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# The role of microalgae-based systems in the dynamics of odorous compounds in the meat processing industry. Part II – olfactometry and sensory relevance

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**ABSTRACT**

This research evaluated the role of microalgae-based systems in deodorizing the meat processing industry by analyzing gas chromatography-olfactometry (GC-O). The olfactometric odorant profile of raw wastewater, the deodorization process along the residence time, and the high-value volatile organic compounds generated by heterotrophic cultures of *Phormidium autumnale* were assessed. The results presented thirty-seven compounds identified by GC-O in the raw wastewater. Indole and skatole were considered the main odor markers with the modified frequency of 91% and 75%, respectively. These compounds did not present sensory perception after 72 h of residence time, suggesting that were completely removed. At the same time, a total of 11 compounds were formed in the microalgae-based process. These compounds were classified as fruity, citrus, green, and resinous by the judges and can be used as a flavoring agent. Finally, the microalgal heterotrophic bioreactor was able to mitigate the most unpleasant odors of the meat processing wastewater, and, in addition, compounds of commercial interest were generated, suggesting the possibility of exploring them for application in the fine chemical or food industry.

*Keywords:* Microalgae/cyanobacteria; Agro-industrial wastes; Olfactometric analysis; Deodorization; Bioproducts

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# The isolated or combined effects of dynamic controlled atmosphere (DCA) and 1-MCP on the chemical composition of cuticular wax and metabolism of 'Maxi Gala' apples after long-term storage

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ABSTRACT

Apples have a continuous hydrophobic layer that covers the surface of the fruit, which is called the cuticle. The effects of 1-methylcyclopropene (1-MCP) on the cuticular wax layer of apples were reported after cold storage, although the interaction between 1-MCP and dynamic controlled atmosphere (DCA) is not yet known. Therefore, this study aimed to analyze the effects of 1-MCP on the wax composition and metabolism of 'Maxi Gala' apples after storage in a controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (DCA-CF) and respiratory quotient (DCA-RQ; RQ = 1.3 and 1.5). The 1-MCP treatment effectively decreased ethylene production for CA and DCA-CF treatments, while in DCA-RQ treatments produced no effect. The average extracted cuticular wax content of 'Maxi Gala' apples was 16.65 g $m^{-2}$ and no differences in storage conditions or 1-MCP application were observed. Alkanes, alcohols, fatty acids, aldehydes, and terpenoids were identified in the chemical composition of the cuticular waxes, being alkanes and fatty acids the predominant ones. Moreover, 1-MCP decreased fatty acid and 10-nonacosanol concentrations in the fruit. Fruit with the 1-MCP application and stored in DCA had lower α-farnesene concentrations. The wax compositions of the DCA-stored apples with and without 1-MCP were similar. However, 1-MCP treatment resulted in a greater mass loss in fruit stored in DCA.

## 1. Introduction

Apples are one of the most important fruit in the world and seasonally harvested, therefore, these fruit must be stored effectively to be available throughout the year (Brackmann et al., 2008). Today, the main method to maintain apple quality during long-term storage is the controlled atmosphere (CA), which employs low temperatures, high relative humidity, high $CO_2$ partial pressure ($pCO_2$), and low $O_2$ partial pressure ($pO_2$) (Brackmann et al., 1993; Brackmann et al., 2005). This technique reduces ethylene biosynthesis and respiration rates, consequently slowing the main biochemical processes during storage (Wright et al., 2015). However, the low $pO_2$ (1.0–1.2 kPa) employed in CA is above the lower oxygen limit (LOL) tolerated by the apples (Bessemans et al., 2016; Weber et al., 2015), which is the minimum $pO_2$ tolerated by the fruit without being damaged by anaerobic metabolism. As a result, apples began to be stored in a dynamic controlled atmosphere (DCA), which was developed to monitor and maintain $pO_2$ as close as possible to LOL during storage.

Four techniques can be used to monitor the LOL during DCA storage (Prange, 2018): one is based on the production of ethanol by the fruit (Veltman et al., 2003), one is based on chlorophyll fluorescence (DCA-CF) (Prange et al., 2007; Wright et al., 2012), the other uses respiratory quotient (DCA-RQ) (Brackmann, 2015; Weber et al., 2015, 2017), and the last is based in the production of $CO_2$ by fruit (DCA-CD) (Thewes

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et al., 2020). By employing these techniques, it is possible to detect the LOL tolerated by the fruit after starting the anaerobic metabolism and adjust the $pO_2$ during storage to avoid excessive anaerobic metabolism (Thewes et al., 2018).

Combined with storage technologies, especially CA, 1-MCP application is widely used as an alternative for fruit quality maintenance. Its application reduces ethylene action/production and respiration rates, preventing loss of flesh firmness and reduced volatile compound production, especially after long storage (DeEll et al., 2007; Lafer, 2008; Lu et al., 2018; Thewes, Brackmann, Anese, et al., 2017; Watkins & Nock, 2012).

Apples have a continuous hydrophobic layer called the cuticle, which covers the surface of the fruit. This layer consists of both cuticular waxes and cutin (Lara et al., 2014). Currently, fruit cuticular wax synthesis is derived from saturated fatty acids with 16 or 18 carbons (C16 or C18). These fatty acids are catalyzed into very long-chain fatty acids (VLCFA, with C20 – C34 chains) (Lara et al., 2015). The VLCFA are then elongated via two biosynthetic pathways to various aliphatic wax components, such as primary and secondary alcohols, wax esters, alkanes, aldehydes, ketones, and triterpenes (Kunst & Samuels, 2003, 2009). This cuticular wax composition is associated with important characteristics related to postharvest quality, including water loss and susceptibility to physical and biological stress (Lara et al., 2014). Studies on apples have shown that the composition of cuticular wax changes continuously during development and postharvest (Belding, Blankenship, Young, & Leidy, 1998; Dong, Rao, Huber, Chang, & Xin, 2012; Ju & Bramlage, 2001). Klein et al. (2020) reported changes in the wax content and composition of 'Cripps Pink' apples during shelf life after CA and DCA storage and described that DCA treatments increased the total wax content. In this work, the authors also found decreased greasiness for DCA-RQ treatments compared to CA, which was attributed to wax composition. Greasiness is a negative sensory quality attribute for consumers and causes some apple cultivars to become unpleasantly greasy on the surface during storage (Curry, 2008; Yang, Zhou, Wang, et al., 2017). Most of these changes in wax constituents are regulated by ethylene (Li et al., 2017; Yang, Zhou, Wang, et al., 2017). Therefore, storage conditions that reduce ethylene production, such as DCA and mainly DCA-RQ (Donadel et al., 2019; Schmidt et al., 2020), and also its association with ethylene action inhibitors, such as 1-MCP, may affect the chemical composition of this cuticular layer of stored apples.

Li et al. (2019) identified four key genes for the synthesis of apple wax (*MdCER4*, *MdCER6*, *MdWSD1,* and *MdMAH1*) and proved their regulation by ethylene. The authors also described that 1-MCP reduced wax density during cold storage of 'Starkrimson' apples (Li et al., 2019). Moreover, Yang, Zhou, Wang, et al. (2017) reported that the accumulation of liquid constituents of the wax and greasiness of the skin of 'Cripps Pink' apples were suppressed in fruit treated with 1-MCP after 70 d of storage at 20 °C. Likewise, Curry (2008) showed that 1-MCP delayed the development of wax constituents considered responsible for greasiness during the cold storage of 'Royal Gala' apples. Nevertheless, there are no results in the literature evaluating the effects of 1-MCP on the cuticular wax composition of apples stored in DCA after long-term storage.

Therefore, determining the composition of fruit cuticular wax from the interaction between DCA methods and 1-MCP may provide important information on postharvest quality in relation to characteristics not previously explored. In this context, the present work aimed to analyze the effects of 1-MCP on the wax composition and overall quality of 'Maxi Gala' apples during long-term CA and DCA storage.

## 2. Materials and methods

### 2.1. Plant material, harvest maturity, and sample preparation

'Maxi Gala' apples were harvested in a commercial orchard in Vacaria, (RS, Brazil) in the 2017 season and immediately transported to the Postharvest Research Center in Santa Maria (RS, Brazil). At a commercial harvest, 140 d after full bloom, the fruit showed an iodine–starch index of 7.20 ± 0.65, soluble solids of 12.43 ± 0.32%, titratable acidity of 0.47 ± 0.03 mg malic acid 100 $g^{-1}$, respiration rate of 0.41 μg $CO_2$ $kg^{-1}$ $s^{-1}$, ethylene production of 5.28 ng $C_2H_4$ $kg^{-1}$ $s^{-1}$, and flesh firmness of 74.6 ± 1.17 N. Damaged and irregular fruit were discarded. Each treatment was applied to three replicates of 25 fruit in a completely randomized experimental design.

Eight storage treatments were applied: conventional CA (with static 1.2 kPa $O_2$ and 2.0 kPa $CO_2$); dynamic controlled atmosphere (DCA) monitored by chlorophyll fluorescence (DCA-CF); DCA monitored by respiratory quotient (DCA-RQ) with two levels (DCA-RQ1.3 and DCA-RQ1.5), with half of the fruit submitted to treatments with 1-MCP and the other half without 1-MCP before storage. The treatments were composed of three replicates of 25 fruit and stored for 9 months. All apples were kept at 5 °C on the first day and the temperature lowered to 2 °C in the course of the following 5 days. Afterwards, the storage conditions were established within individual experimental chambers (233 L), which were all located inside a controlled-temperature storage room. Fruit temperature was monitored daily with mercury thermometers inserted inside the fruit flesh to control pulp temperature. Inside the CA storage chamber, the relative humidity was manually monitored with psychrometers and adjusted with calcium chloride (0.15 kg per treatment), which absorbed the excess humidity and maintained it at an average of 94 ± 2%.

### 2.2. 1-Methylcyclopropene (1-MCP) application

Before storage and after reaching 2 °C, 1-MCP (SmartFresh®, 0.14% of the active ingredient) was applied to half of the fruit. Before treatment, fruit were placed in a 230-L chamber at 2.0 ± 0.1 °C and a solution of 1-MCP was prepared according to the package instructions in order to produce a concentration of 0.625 μL $L^{-1}$ inside the experimental chamber. This solution was placed in Petri dishes inside the chamber. The chamber was hermetically sealed and the air inside circulated with a fan. After 24 h, the fruit were removed from the chamber and stored for 9 months under CA, DCA-CF, DCA-RQ 1.3, and DCA-RQ 1.5.

### 2.3. CA and DCA setup and maintenance

The CA storage rooms were sealed and the storage conditions were established. When the storage temperature reached 2.0 °C, the CA, DCA-CF and DCA-RQ were established by nitrogen flushing to reach 1.2 kPa $O_2$ for CA, and to 0.5 kPa $O_2$ for DCA conditions. Five days were required for this oxygen pull-down. The $pCO_2$ was established by fruit respiration.

During storage, the $pO_2$ was changed according to the fruit metabolism in DCA and maintained statically in CA. To control $pO_2$ in DCA-RQ conditions, the respiratory quotient (RQ) was determined twice a week according to the method proposed by Weber et al. (2015). Thus, the RQ was set to 1.3 and 1.5 and the $pO_2$ changed accordingly to maintain the RQ values (Fig. 1B). The different DCA-RQ levels (1.3 and 1.5) represent the relation of produced $CO_2$/consumed $O_2$, indicating the anaerobic metabolism rate (Weber et al., 2015). The RQ was calculated by considering an interval of 13 h of chamber closure.

Furthermore, DCA-CF was monitored according to Prange et al. (2007). Chlorophyll fluorescence was monitored in six apples during exposure to low $pO_2$. Thus, apples cooled to 2 °C were placed in a perforated plastic container (18 cm width, 27 cm length, 25 cm height) with the fluorescence sensors installed on the top. The container was placed inside an experimental DCA-CF chamber, sealed, and covered with black plastic to protect from the light. The fluorescence monitoring system was activated and the $pO_2$ reduced. Then, the respiration process reduced the $pO_2$ until a change in fluorescence was detected. The lowest $O_2$ set point was determined by identifying the $pO_2$ where an inflection in the fluorescence signal was detected, and then by increasing $pO_2$ by

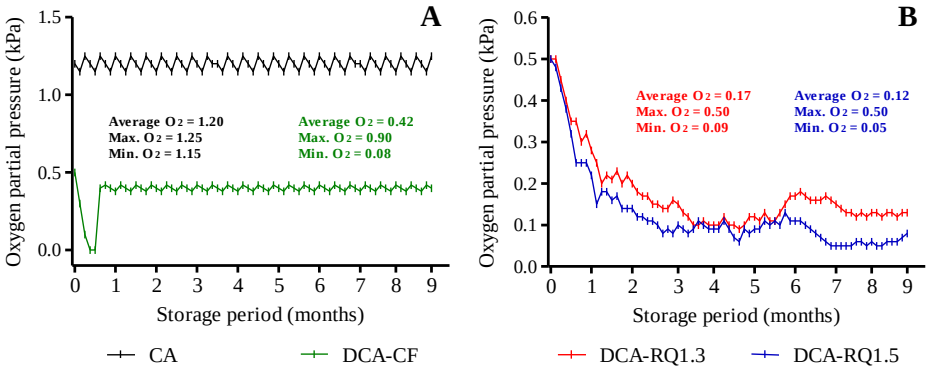



**Fig. 1.** Oxygen variation under controlled atmosphere (CA) and dynamic controlled atmosphere with chlorophyll fluorescence (DCA-CF) (A), dynamic controlled atmosphere with quotient respiratory 1.3 (DCA-RQ1.3) and 1.5 (DCA-RQ1.5) (B) during 9 months of storage.

0.4 kPa as a safety factor (Fig. 1A). Chlorophyll fluorescence was monitored every hour for the entire storage period.

### 2.4. Wax compound extraction, identification, and quantification

Sample preparation and wax compound extraction were carried out according to Klein et al. (2019). The wax was extracted from 10 $cm^2$ of the epidermis homogenized in 5 mL of distilled water, followed by the addition of 2 mL of extraction solvent (chloroform) plus 4 mL of dispersion solvent (acetone). The sample was then shaken for 30 min. The partition procedure was repeated twice with 1 mL of chloroform. The sample was then centrifuged for phase separation and the organic fraction reserved and transferred to a 10-mL volumetric flask, which was added with a 250-μL of a methyl tricosanoate solution (C23:0; 4.006 g $L^{-1}$; Sigma-Aldrich, St. Louis, USA). The extraction yield was gravimetrically obtained from the extracts (5 mL). Results were expressed in g $m^{-2}$ of wax.

Wax compounds were determined from three fractions: fatty acids profile (F1), compounds with high boiling point (F2), and other compounds from the direct analysis of the concentrated extract (F3). The F1 was obtained by transesterification/esterification of 4 mL of the extract after drying under continuous $N_2$ flow at 40 °C. In this extract, the fatty acid methyl esters (FAMEs) were obtained according to Hartman and Lago (1973). The F2 were determined after derivatization with MTBSTFA (N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide; Sigma-Aldrich) according to Oms-Oliu et al. (2011). Finally, F3 compounds were acquired by drying 500 μL of the extract, reconstituting with 50 μL of chloroform, and injecting directly into the chromatographic system.

The three quantitative determinations were carried out by injecting 1 μL of each sample extracts in a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star, 3400 CX, Walnut Creek, USA) and an autosampler (Varian, model 4200, CA, USA) as described by Klein et al. (2019). The FAMEs solution (F1) were injected in splitless mode (splitter off for 1 min; 20:1) and a temperature of 250 °C. The FAMEs separation were carried out using a polar capillary column CP-Wax 52CB (Chrompack, Middelburg, 50 m × 0.32 mm; 0.20 μm of thickness film). The initial column temperature was set at 50 °C, remaining for 1 min, increasing to 180 °C at 10 °C $min^{-1}$. Then, the temperature increase at 2 °C $min^{-1}$ until 200 °C, and then at 10 °C $min^{-1}$ to 230 °C, and maintained in isotherm for 5 min. The detector was maintained at 240 °C. The identification was by comparing their experimental retention times with the ones found in the FAME Mix-37 standard (P/N 47885-U, Sigma-Aldrich, St. Louis, USA).

The fractions F2 and F3 were injected in splitless mode (splitter off for 1 min; 20:1) at 300 °C. Wax compounds were separated using a capillary column of nonpolar phase, BPX-5 (SGE, Australia, 25 m × 0.22 mm; 0.25 μm of thickness film). $H_2$ was used as carrier gas at a constant pressure of 103 kPa. The column initial temperature was 50 °C, where it remained 1 min, increasing to 200 °C at a rate of 10 °C $min^{-1}$ and again increasing to 320 °C at a rate of 3 °C $min^{-1}$, remaining isothermic for 10 min. The detector was maintained at 280 °C. The analytes with no available standard (F2 and F3) were identified by gas chromatography coupled to a mass spectrometer (GC/MS, Shimadzu QP2010 Plus, Kyoto, Japan) based on the comparison of the obtained mass spectra obtained with those available from the National Institute of Standards and Technology (NIST) and the literature. For these analyses, the same chromatographic conditions described above were used and He was used as the carrier gas. The MS operated in the electron impact ionization mode with ionization energy of 70 eV and single quadrupole type analyzer operating in scan mode in a mass range of 35–450 *m/z*.

The concentration of the compounds for all fractions were calculated by internal standardization (C23:0) and the results were expressed as g of compound per kg wax (Klein et al., 2019).

### 2.5. Metabolism and overall quality analysis

#### 2.5.1. Ethylene production and respiration rate

A batch of approximately 1.5 kg fruit was put into a 5-L container and hermetically closed for about 2 h. Thereafter, two samples of 1 mL were taken out of the container and injected into a Varian gas chromatograph model Star CX 3400 (Varian, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and Porapak N80/100 column to determine the ethylene concentration in the container headspace. Injector, column, and detector temperatures were 140, 90, and 200 °C, respectively. Results were expressed in ng $kg^{-1}$ $s^{-1}$. The same air of the container for ethylene measurement was circulated throughout an electronic gas analyzer (Isolcell®, model Oxycarb) to determine fruit respiration by its $CO_2$ production. Respiration rates were expressed in μg $kg^{-1}$ $s^{-1}$. These analyses were performed according to Thewes et al. (2015).

#### 2.5.2. Soluble solids and titratable acidity

Slices of the equatorial region of the fruit were used to produce juice using a juicer (Philips Walita®). Drops of the juice were used to measure the soluble solids by refractometry (Biobrix, Model 103, Curitiba, Brazil), and the results expressed as a percentage. Then, 10 mL from the same juice were taken and diluted in 100 mL of distillated water. This solution was titrated with a 0.1 N NaOH solution until pH 8.1. The results were expressed as mEq 100 $mL^{-1}$.

#### 2.5.3. Juiciness

Using 10 fruit of each replicate, 20 g of the apple pulp was used and pressed for one minute at a pressure of 150 kPa. Juiciness was calculated by weighing the fruit pulp before and after pressing according to the methodology proposed by Lunardi et al. (2004). The results were

expressed as a percentage of the initial fruit pulp mass.

#### 2.5.4. Mass loss

Mass loss was obtained by weighting the fruit before and after the storage and expressed as a percentage of the initial fruit mass (Brackmann et al., 2014).

#### 2.5.5. Pulp cracking

Pulp cracking was determined by counting the fruit that showed any signs of cracking on the pulp compared to the total number of fruit per replicate. Results were presented as a percentage of the total fruits.

### 2.6. Statistical analyses

The experiment was conducted in a completely randomized design with four storage conditions with or without 1-MCP application. Analysis of variance (ANOVA) at 5% of error probability was carried out ($p < 0.05$) and means that showed significant differences were subjected to Tukey's test at 5% error probability. Additionally, data were submitted to multivariate principal component analysis (PCA) using Pirouette 3.11 software (Woodinville, USA, 2003) to show trends in the results. Before PCA, the data matrix was autoscaled in order for each variable to have the same weight during analysis.

## 3. Results and discussion

### 3.1. Apple cuticular wax content

The total apple peel wax of 'Maxi Gala' apple was not affected by storage treatments. The mean amount of cuticular wax isolated from 'Maxi Gala' apple was 16.65 g $m^{-2}$ (Supplementary Material 1). Ju and Bramlage (2001) reported values of 3.1 and 14.1 g $m^{-2}$ of total wax at the start and end of development, respectively, for 'Delicious' apples. Leide et al. (2018) described cuticular wax content of 1.13 and 0.77 g $m^{-2}$, respectively, for 'Florina' and 'Prima' apples shortly after harvesting. On other hand, 'Royal Gala', 'Granny Smith', 'Pink Lady', and 'Red Delicious' apples purchased locally market (RS, Brazil) presented cuticular wax content of 16.12, 15.65, 21.85, and 17.95 g $m^{-2}$, respectively (Klein et al., 2019). Klein et al. (2020) reported mean total wax content for 'Cripps Pink' apples after 8 months of storage in CA, DCA-CF, and DCA-RQ1.3 and RQ1.5 of 21.23 g $m^{-2}$ at 7 d and 26.18 g $m^{-2}$ at 14 d of shelf life at 20 °C.

Studies have shown that ethylene increases the accumulation of cuticular wax of fruit during cold storage, while 1-MCP inhibits this phenomenon (Cajuste et al., 2010; Dong et al., 2012; Ju & Bramlage, 2001; Li et al., 2019). This is because of the regulation of four key genes of the synthesis of apple wax (*MdCER4*, *MdCER6*, *MdWSD1,* and *MdMAH1*), in which ethylene acts directly by increasing the expression levels of *MdCER6* (Li et al., 2019). Following the same authors, *MdCER6* is a key gene to synthesize very long-chain fatty acids (VLCFA), in addition to regulating the pathway of alcohol formation, stimulating *MdCER4* and *MdWSD1*. However, this suppression in the cuticular wax content of 1-MCP-treated apples was not observed here. This may be due to the modulation of the climacteric fruit sensitivity to 1-MCP as a function of the internal ethylene concentration (IEC) (Zhang et al., 2011). It is plausible that endogenous ethylene competes with 1-MCP to bind to receptors and, therefore, despite exogenous ethylene production being suppressed by 1-MCP, the IEC may still be enough for the expression of genes related to wax synthesis. Both et al. (2018) showed that, even with low ethylene production of 1-MCP-treated 'Galaxy' apples, the fruit still had IEC greater than CA after 9 months of storage in DCA-RQ.

### 3.2. Apple cuticular wax composition

Thirty-one compounds including alkanes, alcohols, fatty acids, aldehydes, and terpenoids were identified and quantified in the cuticular waxes of 'Maxi Gala' apples treated or not with 1-MCP after storage (Tables 1 and 2). Alkanes and fatty acids were the predominant constituents of cuticular waxes.

Alkanes constituted an important fraction of the total amount of wax in all treatments, which was mainly influenced by nonacosane. The concentration of this compound did not differ between atmosphere conditions when fruit were treated with 1-MCP. However, it was lower in DCA-RQ1.3 when compared to DCA-CF in fruit without 1-MCP treatment. Similar behavior was found for tetracosane (Table 2). As predominant components of fruit cuticular wax, alkanes play important roles in determining cuticle properties. In *Arabidopsis* leaves, alkanes are associated with better skin barrier properties and are produced in response to water deficits (Bourdenx et al., 2011; Kosma et al., 2009), promoting greater resistance to water movement in the membranes than other cuticular wax components (Grncarevic & Radler, 1967). This indicates that alkanes may be the most effective water-retaining components of wax and therefore essential for preventing weight loss during apple storage, as reported by Chai et al. (2020). The authors showed that the lowest alkane content in the related 'Red Star' apples was related to the highest rate of weight loss during storage at 20 °C for 49 d. Baritelle et al. (2001) related greater weight loss during storage for 'Golden Delicious', 'Red Delicious', 'Fuji', and 'Roma' untreated apples than those treated with 1-MCP. This has always been related to the fact that 1-MCP slows the respiratory rate and also possibly increases alkane content, as observed in this study after CA storage.

Fatty acids were the major constituents of wax in fruit stored in CA, mainly CA without 1-MCP (Table 1). This fraction was lower in DCA treatments, regardless of 1-MCP application. In all storage conditions with 1-MCP, eight fatty acids were not detected, which were currently constituents of the cuticular wax composition in apples without 1-MCP. For treatments without 1-MCP, lauric, myristic, palmitic, palmitoleic, stearic, oleic, and linoleic acids had higher concentrations in the fruit stored in CA. In addition, 1-MCP had lower concentrations of palmitic acid, stearic acid, oleic acid, linoleic acid, α-linolenic acid, and *cis*-11-eicosenoic acid for the same condition (Table 1). Li et al. (2019) reported that 1-MCP decreased the expression level of key genes in the synthesis of VLCFAs and consequently all other wax constituents. Therefore, these lower fatty acids concentrations in fruit treated with 1-MCP found in this study (Table 1), corroborate the previous findings (Curry, 2008; Dong et al., 2012; Li, Min, Song, Shao, & Zhang, 2017). Fatty acid elongation uses C16 and C18 saturated fatty acids and the unsaturated fatty acids C18:1 and C18:2 that are esterified to Co-enzyme A (CoA) before entering the endoplasmic reticulum-bound multi-enzymatic fatty acid elongase (FAE) complexes (Bernard & Joubès, 2013). Low $pO_2$ during storage interferes in the cellular homeostasis of the fruit due to decreased cellular energy, consequently weakening the metabolic processes that consume ATP, including fatty acid synthesis (Geigenberger, 2003). This may explain the lower concentration of palmitic acid, stearic acid, oleic acid, and linoleic acid in DCA-stored apples, where $pO_2$ during storage was lower than CA (Fig. 1).

During the ripening process, the composition of the cuticle is altered due to unsaturated fatty acids accumulation (Yang, Zhou, Zhang, et al., 2017), as observed in both CA groups, with and without 1-MCP. A comparison of both groups points to CA without 1-MCP as being the one with the most accumulation of unsaturated fatty acids. In DCA-RQ1.5, α-linolenic acid, γ-linolenic acid, and *cis*-11-eicosenoic acid had the highest concentrations in the group without 1-MCP (Table 1). Furthermore, 1-MCP application decreased α-linolenic and *cis*-11-eicosenoic acid concentrations in this storage condition.

*Cis*-11,14-eicosadienoic acid and behenic acid had lower concentrations in DCA-RQ1.5 storage without 1-MCP application. Regarding the other treatments with 1-MCP, DCA-RQ1.5 was greater than the same storage condition without 1-MCP (Table 1). Nevertheless, *cis*-13,16-docosadienoic is the acid that most contributed to the fatty acid fraction of the cuticular wax, as its concentration was not influenced by any of

**Table 1**
The fatty acids (F1) of peel wax (g $kg^{-1}$) of 'Maxi Gala' apples, without and with 1-methylcyclopropene (1-MCP) treatment, stored under controlled atmosphere (CA), dynamic controlled atmosphere based on chlorophyll fluorescence (DCA–CF) and dynamic controlled atmosphere by respiratory quotient (DCA–RQ) for 9 months plus 7 d of shelf life.

| Compounds | Without 1-MCP | | | | With 1-MCP | | | |
|---|---|---|---|---|---|---|---|---|
| | CA | DCA-CF | DCA-RQ1.3 | DCA-RQ1.5 | CA | DCA-CF | DCA-RQ1.3 | DCA-RQ1.5 |
| C8:0 (Caprylic acid) | nd | nd | 1.70 ± 0.20 (a) | 1.70 ± 0.31 (a, B) | 7.43 ± 0.68 (a) | 2.43 ± 0.44 (b) | 3.92 ± 1.38 (b) | 3.13 ± 0.50 (b,A) |
| C10:0 (Capric acid) | 0.71 ± 0.07 | nd | nd | nd | nd | nd | nd | nd |
| C11:0 (Undecanoic acid | nd | nd | 0.27 ± 0.04 (a) | 0.32 ± 0.03 (a) | nd | nd | nd | nd |
| C12:0 (Lauric acid) | 1.11 ± 0.20 (a) | 0.35 ± 0.10 (b) | 0.43 ± 0.05 (b) | 0.49 ± 0.07 (b) | nd | nd | nd | nd |
| C14:0 (Myristic acid) | 4.74 ± 0.09 (a) | 0.36 ± 0.10 (b) | 0.55 ± 0.14 (b) | 0.32 ± 0.07 (b) | nd | nd | nd | nd |
| C15:0 (Pentadecanoic acid) | nd | nd | 0.36 ± 0.03 (a) | 0.36 ± 0.08 (a) | nd | nd | nd | nd |
| C15:1 (*cis*-10-Pentadecenoic acid) | nd | nd | 0.52 ± 0.04 (a) | 0.58 ± 0.07 (a) | nd | nd | nd | nd |
| C16:0 (Palmitic acid) | 87.84 ± 10.4 (a, A) | 18.65 ± 4.39 (b) | 17.03 ± 4.38 (b) | 21.09 ± 4.29 (b) | 26.75 ± 3.45 (a,B) | 16.69 ± 3.59 (b) | 19.79 ± 4.04 (ab) | 16.41 ± 0.93 (b) |
| C16:1 (Palmitoleic acid) | 5.37 ± 1.63 (a) | nd | 0.27 ± 0.04 (b) | nd | nd | nd | nd | nd |
| C18:0 (Stearic acid) | 42.64 ± 9.17 (a, A) | 10.99 ± 2.12 (b) | 10.91 ± 3.20 (b) | 12.72 ± 4.24 (b) | 13.10 ± 0.78 (B) | 9.15 ± 2.83 | 10.14 ± 1.28 | 8.67 ± 1.42 |
| C18:1n9 (Oleic acid) | 126.9 ± 26.66 (a,A) | 9.53 ± 1.70 (b) | 11.65 ± 2.32 (b) | 11.01 ± 1.36 (b) | 14.9 ± 0.92 (a, B) | 9.57 ± 1.29 (b) | 12.25 ± 2.02 (ab) | 10.24 ± 0.81 (b) |
| C18:2n6 (Linoleic acid) | 47.28 ± 1.38 (a, A) | 7.79 ± 1.21 (b) | 8.41 ± 1.95 (b) | 8.40 ± 0.86 (b) | 11.28 ± 1.39 (a,B) | 7.98 ± 1.11 (b) | 9.03 ± 0.31 (ab) | 8.36 ± 0.79 (b) |
| C18:3n6 (γ-Linolenic acid) | 0.25 ± 0.05 (b) | nd | 0.62 ± 0.13 (a) | 0.84 ± 0.11 (a) | nd | nd | nd | nd |
| C18:3n3 (α-Linolenic acid) | 3.40 ± 0.40 (b, A) | 0.83 ± 0.17 (c) | nd | 5.61 ± 0.10 (a, A) | 0.64 ± 0.23 (B) | 0.65 ± 0.06 | 0.82 ± 0.05 | 0.74 ± 0.06 (B) |
| C20:0 (Arachidic acid) | 7.05 ± 0.91 (a) | 5.22 ± 0.72 (a) | 5.67 ± 0.96 (a) | 0.81 ± 0.06 (b, B) | 5.86 ± 0.35 | 5.22 ± 0.62 | 5.76 ± 0.45 | 5.83 ± 1.10 (A) |
| C20:1n9 (*cis*-11-Eicosenoic acid) | 7.39 ± 1.06 (b, A) | 0.71 ± 0.20 (c) | 0.71 ± 0.07 (c) | 12.67 ± 0.46 (a,A) | 0.72 ± 0.27 (B) | 0.70 ± 0.11 | 0.84 ± 0.05 | 0.82 ± 0.04 (B) |
| C20:2 (*cis*-11,14-Eicosadienoic acid) | 11.92 ± 2.14 (a) | 11.18 ± 1.69 (a) | 10.94 ± 1.29 (a) | 6.97 ± 0.23 (b, B) | 11.99 ± 1.57 | 12.31 ± 1.77 | 11.69 ± 1.47 | 12.47 ± 0.83 (A) |
| C22:0 (Behenic acid) | 8.11 ± 1.70 (a) | 6.03 ± 1.21 (a) | 6.88 ± 0.89 (a) | 0.69 ± 0.16 (b, B) | 7.42 ± 0.64 | 6.14 ± 0.85 | 6.67 ± 0.51 | 7.14 ± 0.95 (A) |
| C22:2 (*cis*-13,16-Docosadienoic acid) | 127.04 ± 0.96 | 132.96 ± 7.78 | 124.59 ± 2.19 | 133.42 ± 3.85 | 135.66 ± 14.32 | 135.50 ± 6.70 | 132.28 ± 12.89 | 138.87 ± 8.97 |
| C24:0 (Lignoceric acid) | nd | nd | nd | nd | 23.87 ± 0.74 (a) | 3.19 ± 0.33 (b) | 3.56 ± 0.49 (b) | 3.61 ± 0.17 (b) |

Nd: Not detected. Means followed by equal uppercase letter do not differ between with and without 1-MCP application, in each storage condition. Means followed by equal lowercase letter do not differ between storage conditions in each 1-MCP strategy, by Tukey test at 5% error probability.

**Table 2**
Other compounds (F2 and F3) of peel wax (g $kg^{-1}$) of 'Maxi Gala' apples, without and with 1-methylcyclopropene (1-MCP) treatment, stored under controlled atmosphere (CA), dynamic controlled atmosphere based on chlorophyll fluorescence (DCA–CF) and dynamic controlled atmosphere by respiratory quotient (DCA–RQ) for 9 months plus 7 d of shelf life.

| Compounds | Without 1-MCP | | | | With 1-MCP | | | |
|---|---|---|---|---|---|---|---|---|
| | CA | DCA-CF | DCA-RQ1.3 | DCA-RQ1.5 | CA | DCA-CF | DCA-RQ1.3 | DCA-RQ1.5 |
| α-farnesene | 2.14 ± 0.30 (b) | 4.62 ± 0.36 (a,A) | 4.79 ± 0.03 (a, A) | 1.67 ± 0.41 (b,A) | 1.98 ± 0.17(a) | 2.09 ± 0.16 (a, B) | 0.50 ± 0.09 (b, B) | 0.61 ± 0.20 (b, B) |
| 10-nonacosanol | 4.83 ± 1.62 (a,A) | 1.66 ± 0.79 (b,A) | 0.49 ± 0.03 (b, A) | 0.54 ± 0.14 (b,A) | 0.32 ± 0.03 (B) | 0.40 ± 0.09 (B) | 0.30 ± 0.03 (B) | 0.26 ± 0.08 (B) |
| Tetracosane | 9.98 ± 0.05 (a,B) | 10.06 ± 0.54 (a) | 8.34 ± 0.01 (b) | 10.67 ± 0.15 (a) | 10.61 ± 0.10 (A) | 9.99 ± 1.03 | 9.84 ± 1.08 | 10.23 ± 0.68 |
| Hexacosane | 2.53 ± 1.01 | 3.40 ± 2.39 | 1.67 ± 0.12 (B) | 4.60 ± 2.57 | 2.76 ± 0.11 | 3.99 ± 2.29 | 2.42 ± 0.30 (A) | 2.45 ± 1.16 |
| Nonacosane | 104.64 ± 3.96 (ab, B) | 113.85 ± 11.86 (a) | 92.67 ± 4.44 (b) | 108.44 ± 6.08 (ab) | 112.07 ± 0.79 (A) | 116.02 ± 2.51 | 102.97 ± 9.41 | 113.59 ± 11.18 |
| Hexacosanal | 5.55 ± 0.39 (B) | 6.67 ± 0.97 | 4.74 ± 1.26 | 5.22 ± 2.42 | 7.11 ± 0.65 (A) | 8.05 ± 0.41 | 7.01 ± 0.85 | 8.23 ± 1.45 |
| Heptacosanal | 5.09 ± 0.59 (B) | 6.51 ± 0.95 | 3.85 ± 1.61 (B) | 4.20 ± 2.10 | 6.42 ± 0.44 (A) | 8.15 ± 1.41 | 6.79 ± 0.62 (A) | 7.62 ± 1.36 |
| Henicosanal | 2.36 ± 0.10 | 2.72 ± 0.24 | 2.00 ± 0.54 | 2.25 ± 0.80 | 2.83 ± 0.36 | 3.07 ± 0.39 | 2.94 ± 0.27 | 3.45 ± 0.41 |
| Squalene | 2.93 ± 0.66 | 2.85 ± 0.94 | 2.72 ± 0.42 | 2.81 ± 0.58 | 3.65 ± 0.16 (a) | 2.94 ± 0.35 (b) | 2.62 ± 0.07 (bc) | 2.20 ± 0.17 (c) |
| Oleanolic acid | 0.61 ± 0.10 | 0.53 ± 0.11 | 0.73 ± 0.34 | 0.41 ± 0.03 (A) | 0.62 ± 0.43 | 0.31 ± 0.20 | 0.33 ± 0.06 | 0.27 ± 0.10 (B) |
| Ursolic acid | 5.45 ± 0.68 (B) | 6.01 ± 0.44 (B) | 6.25 ± 0.17 | 6.09 ± 0.46 | 8.92 ± 0.58 (A) | 12.57 ± 1.48 (A) | 7.09 ± 0.80 | 6.60 ± 0.60 |

Means followed by equal uppercase letter do not differ between with and without 1-MCP application, in each storage condition. Means followed by equal lowercase letter do not differ between storage conditions in each 1-MCP strategy, by Tukey test at 5% error probability.

the tested variables, in addition to having a mean wax of 133 g $kg^{-1}$ in all treatments. In 'Cripps Pink' apples stored under the same conditions of this study (after 14 d of shelf life), *cis*-11,14-eicosadienoic was the most abundant acid in this fraction of the wax (Klein et al., 2020). Lignoceric acid was only present in fruit treated with 1-MCP and the highest concentration was in CA-stored fruit.

10-nonacosanol was identified in all treatments, although 1-MCP-treated fruit had lower concentrations than untreated ones (Table 2). No influences between storage conditions was observed in 1-MCP-treated fruit, although higher 10-nonacosanol was observed in fruit without 1-MCP in CA (Table 2). Similar behavior was observed in ethylene production (Table 1), corroborating Li et al. (2017, 2019), who reported that 1-MCP decreased the expression of the *MdCER4* gene, which is the main regulator of the alcohol formation pathway in 'Starkrimson' apple in cold storage.

α-Farnesene oxidation products are involved in superficial scald development in apples (Delong & Prange, 2003). Thus, the pre-storage treatment of susceptible fruit using antioxidants, including DPA (diphenylamine), may delay α-farnesene autoxidation and reduce respiration rates and ethylene evolution (Whitaker et al., 2000). Fruit without 1-MCP application and stored in DCA had higher α-farnesene concentrations compared to those with the 1-MCP application. Among the storage conditions in fruit without 1-MCP, DCA-CF and DCA-RQ1.3 had the highest concentrations (Table 2). This influence of 1-MCP was also observed by Ding et al. (2020), who showed that 1-MCP effectively inhibited α-farnesene accumulation compared to the control group without 1-MCP during 113 d of storage at 0 °C in 'White Winter Pear-main' apples. On the other hand, Yang et al. (2016) described that 1-MCP to significantly delayed α-farnesene production in 'Golden Delicious' apples during shelf life at 20 °C. Nevertheless, fruits treated with 1-MCP had α-farnesene production similar to untreated fruits, but this accumulation was only observed at 40 d. Therefore, this lower α-farnesene concentration in 1-MCP-treated fruit and stored in DCA from our study may only be a delay in the production. In the 1-MCP-treated group, the storage conditions that contributed to higher α-farnesene concentrations were CA and DCA-CF, which is due to the higher $pO_2$ levels (Fig. 1) during storage. Thus, there may be a favorable interaction between 1-MCP and DCA-RQ capable of suppressing α-farnesene production. Notably, DCA-CF was identified as a potential method of controlling superficial scald (DeLong et al., 2007; Zanella et al., 2005), which is mainly associated with low $pO_2$ during storage. Therefore, the present study has shown that DCA-RQ decreases $pO_2$ even lower (Fig. 1) and possibly improves superficial scald control.

Regarding the identified aldehydes, the storage condition did not influence the concentration of these compounds, although this was not the case for hexacosanal and heptacosanal, as 1-MCP promoted higher concentrations in fruit stored in CA and DCA-RQ1.3 (Table 2).

Ursolic acid (UA) concentrations did not differ between the storage conditions, despite 1-MCP contributing to higher UA concentrations in CA and DCA-CF (Table 2). Oleanolic acid (OA) was not influenced by any of the tested variables. Fruit without 1-MCP had no changes in squalene concentrations in relation to storage conditions, but for 1-MCP-treated fruit, CA storage promoted higher concentration. Squalene is another terpenoid with important biological functions, including protecting against bacterial and fungal infections (Becker et al., 2019). Chai et al. (2020) analyzed 10 apple cultivars and found no terpenoids before cold storage, although these compounds were present in all cultivars after storage except for 'Red Star', which may partially explain why this cultivar had a lower shelf life than the others.

In general, it was possible to observe the important effects of 1-MCP on the wax composition of 'Maxi Gala' apples in CA compared to non-treated fruit. However, the same comparison employing DCA storage technologies had little to no effects. This may have occurred due to the influence of ethylene concentration on the cuticular wax synthesis, which was higher in CA-stored fruit. Hence, CA was the storage atmosphere that had the highest differences in wax composition compared to the other storage conditions treated with 1-MCP. Therefore, regarding the cuticle wax composition of 'Maxi Gala' apples, 1-MCP had little effect when using storage technologies that are effective in suppressing ethylene production, such as the DCA methods used in this study.

### 3.3. Metabolism and overall quality of fruit

The 1-MCP treatment was effective in decreasing ethylene production for CA and DCA-CF treatments, while 1-MCP had no effect on ethylene production for DCA-RQ1.3 and RQ1.5 treatments (Table 3). This is important because it shows the effectiveness of DCA-RQ storage in reducing ethylene production. This, in addition to the reduced financial cost, other studies have already shown a better volatile profile of apples stored in the DCA-RQ without 1-MCP (Thewes, Brackmann, Both, et al., 2017). Without 1-MCP, DCA-CF and DCA-RQ reduced more effectively ethylene production than CA storage. This may be an effect of the higher ethanol, acetaldehyde and ethyl acetate production by fruit submitted to DCA-RQ, which affect ethylene suppression (Pesis, 2005; Weber et al., 2016) by the ACC synthase and reduced ACC oxidase enzyme activity (Asoda, Terai, Kato, & Suzuki, 2009; Weber et al., 2016), as this storage condition maintained $O_2$ levels low (Fig. 1B) and induced anaerobic metabolism. With the 1-MCP application, there were no differences between treatments for ethylene production.

Respiration rate was affected by storage conditions, being higher in CA compared to DCA treatments for fruit with and without 1-MCP. The 1-MCP application did not affect the respiration rate, regardless of the storage condition (Table 3). In climacteric fruit, the increased ethylene concentration stimulated the respiration rate of the fruit (Both et al., 2017; Thewes et al., 2015; Weber et al., 2015), as evidenced in the present study for 'Maxi Gala' apples stored in CA. However, the 1-MCP application generally reduced respiration rate compared to fruit without its application, which was not observed here.

Neither CA nor DCA conditions, with or without 1-MCP, affected soluble solid content or acidity in 'Maxi Gala' after storage (Table 3). DCA-RQ1.5 with 1-MCP had less juiciness when compared to the other storage conditions and also in the same condition with the 1-MCP application. In fruit without 1-MCP, the juiciness was not affected by the atmospheric conditions. Fruit with 1-MCP application had greater weight loss during DCA storage, in particular, DCA-RQ1.5 greater mass loss coincided with less juiciness. For DCA-RQ1.5 without 1-MCP, the juiciness was greater and had less mass loss. Only fruit stored in DCA-CF without 1-MCP showed pulp cracking, which is probably correlated to the less weight loss.

Postharvest weight loss of fruit results from water loss and respiration ($CO_2$ loss) due to its diffusion through the fruit cuticle (Lara et al., 2014). Excessive water loss, which is the main cause of weight loss in fruit storage, promotes browning and loss of fruit texture and flavor (Lufu et al., 2020). Chai et al. (2020) evaluated the weight loss of 10 apple cultivars after 49 days of storage (25 °C, 90% relative humidity) and reported that the highest weight loss rate of 8.37% was for 'Red Star' apples, while the lowest rate of weight loss of 3.64% was observed for the 'Mutsu' apples. The authors also correlated that the alkanes of the cuticular wax are essential to maintain the weight of the apple fruit during storage. This group stands out compared to other wax components for providing greater resistance to the movement of water in artificial membranes and experimental models with Arabidopsis (Grncarevic & Radler, 1967; Bourdenx et al., 2011). In our study, weight loss was related to fruit treated with 1-MCP (Fig. 2), especially those stored in DCA (Table 3). Although alkanes did not differ significantly in fruit stored in DCA with or without 1-MCP (Table 2). However, the principal component analysis shows for treatments without 1-MCP (Fig. 3) that alkanes are inversely correlated with weight loss and juiciness, which is maintained when fruits lose less water. Further evidence that corroborates previous studies is that in fruit without 1-MCP, the condition of DCA-CF had a higher concentration of nonacosane and lower mass loss (Table 2 and 3).

**Table 3**
Metabolism and overall quality analysis of 'Maxi Gala' apples, without and with 1-methylcyclopropene (1-MCP) treatment, stored under controlled atmosphere (CA) and different dynamic controlled atmosphere (DCA-CF and RQ) for 9 months plus 7 d of shelf life.

| | Without 1-MCP | | | | With 1-MCP | | | |
|---|---|---|---|---|---|---|---|---|
| | CA | DCA-CF | DCA-RQ1.3 | DCA-RQ1.5 | CA | DCA-CF | DCA-RQ1.3 | DCA-RQ1.5 |
| Ethylene | 14.82 ± 3.06* (a, A) | 2.68 ± 1.61 (b, A) | 0.52 ± 0.54 (b, A) | 0.32 ± 0.47 (b, A) | 0.05 ± 0.00 (a, B) | 0.07 ± 0.08 (a,B) | 0.02 ± 0.01 (a, A) | 0.03 ± 0.01 (a, A) |
| Respiration rate | 2.47 ± 0.42 (a,A) | 0.92 ± 0.06 (b, A) | 1.14 ± 0.07 (b, A) | 1.10 ± 0.08 (b, A) | 2.87 ± 0.64 (a, A) | 1.48 ± 0.72 (b, A) | 0.94 ± 0.16 (b, A) | 1.05 ± 0.10 (b, A) |
| Soluble solids | 13.25 ± 0.30 | 13.50 ± 0.62 | 13.45 ± 0.38 | 13.60 ± 0.37 | 13.30 ± 0.26 | 13.50 ± 0.50 | 13.80 ± 0.16 | 13.90 ± 0.26 |
| Acidity | 4.50 ± 0.28 | 4.51 ± 0.54 | 4.65 ± 0.20 | 4.73 ± 0.25 | 4.66 ± 0.15 | 4.71 ± 0.28 | 4.64 ± 0.19 | 4.70 ± 0.08 |
| Juiciness | 65.33 ± 0.80 | 66.02 ± 1.52 | 66.18 ± 1.53 | 68.96 ± 2.62 (A) | 66.24 ± 1.87 (a) | 67.82 ± 1.26 (a) | 65.62 ± 0.91 (ab) | 63.02 ± 1.64 (b, B) |
| Mass loss | 2.95 ± 0.25 (a) | 1.84 ± 0.42 (b, B) | 2.88 ± 0.61 (a, B) | 2.90 ± 0.40 (a, B) | 2.60 ± 0.40 (c) | 3.17 ± 0.39 (bc, A) | 3.73 ± 0.34 (ab, A) | 4.22 ± 0.42 (a, A) |
| Pulp cracking | 0 | 4.00 ± 1.63 | 0 | 0 | 0 | 0 | 0 | 0 |

*Means ± SD (standard deviation) values followed by equal uppercase letter do not differ between with and without 1-MCP application, in each storage condition. Means followed by equal lowercase letter do not differ between storage conditions in each 1-MCP strategy group, by Tukey test at 5% error probability. Controlled atmosphere (CA), dynamic controlled atmosphere based on chlorophyll fluorescence (DCA–CF) and dynamic controlled atmosphere by respiratory quotient (DCA–RQ). Results expressed in: Ethylene (ng $kg^{-1}$ $s^{-1}$); Respiration rate (μg $kg^{-1}$ $s^{-1}$); Soluble solids (%); Acidity (mEq 100 $mL^{-1}$); Juiciness, mass loss and pulp cracking (%).

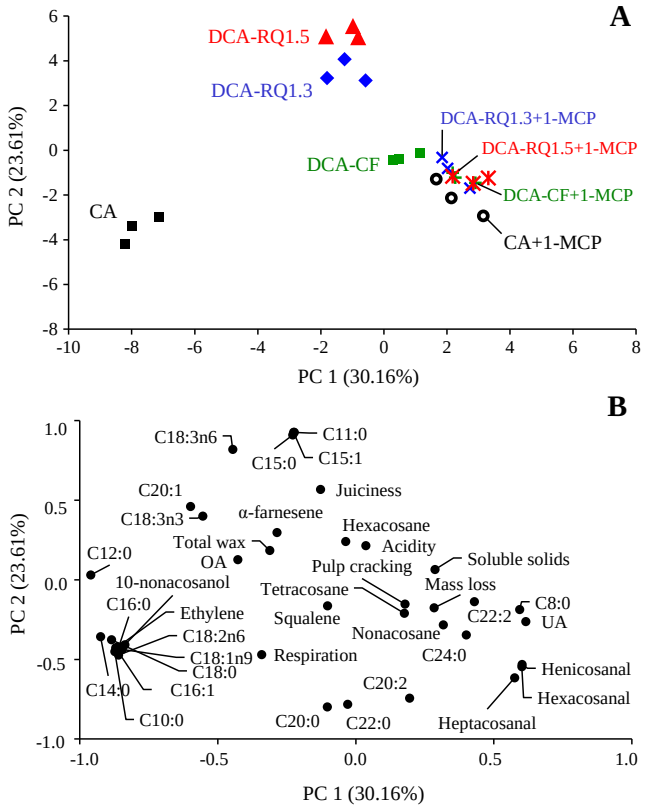



**Fig. 2.** Scores (treatments) (A) and loadings (variables) (B) plots of the first two principal components of the identified wax compounds, total wax, ethylene production, and respiration rate of 'Maxi Gala' apples, without and with 1-methylcyclopropene (1-MCP) treatment, stored under controlled atmosphere (CA), dynamic controlled atmosphere based on chlorophyll fluorescence (DCA–CF) and dynamic controlled atmosphere by respiratory quotient (DCA–RQ) for 9 months plus 7 d of shelf life. UA: ursolic acid; OA: oleanolic acid.

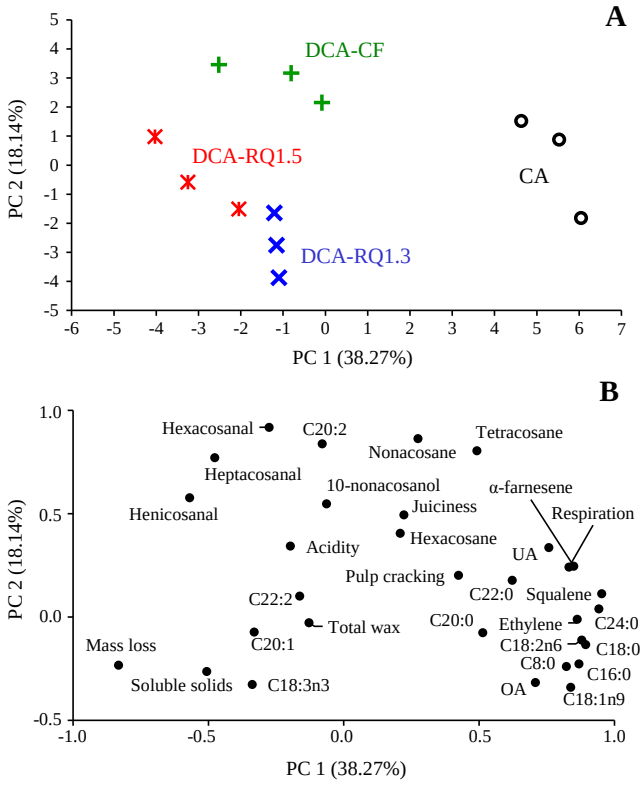



**Fig. 3.** Scores (treatments) (A) and loadings (variables) (B) plots of the first two principal components of the identified wax compounds, total wax, ethylene production, and respiration rate of 'Maxi Gala' apples, with 1-methylcyclopropene (1-MCP) treatment, stored under controlled atmosphere (CA), dynamic controlled atmosphere based on chlorophyll fluorescence (DCA–CF) and dynamic controlled atmosphere by respiratory quotient (DCA–RQ) for 9 months plus 7 d of shelf life. UA: ursolic acid; OA: oleanolic acid.

### 3.4. Multivariate analysis (PCA)

Principal component analysis (PCA) was performed to analyze the 1-MCP treatment, storage conditions, and variables determined in this experiment. All experimental data are shown in Fig. 2. The first and second principal component (PC1 and PC2) defined 53.77% of the overall variation and, throughout the PC1, treatments with and without 1-MCP were discriminated. However, there was a greater difference between CA and DCA without 1-MCP than with 1-MCP-treated fruit. This occurred as a result of the higher abundance of several fatty acids, mainly important fatty acids for wax biosynthesis, such as C16:0 and C18:0, 10-nonacosanol, and ethylene production that were positively correlated with CA without 1-MCP. In addition, the fruit stored in DCA-RQ without 1-MCP were discriminated from the other treatments as

observed throughout the PC2, when they were positively correlated to the presence of α-farnesene and fatty acids C15:0, C11:0, C15:1, and C18:3n6.

Regarding apples without 1-MCP, all treatments were well discriminated. Nevertheless, treatments with the 1-MCP application were grouped due to the lower difference in concentration among compounds than fruit without 1-MCP. Because of this, another PCA was performed only for the group of samples treated with 1-MCP (Fig. 3). PC1 and PC2 defined 56.41% of the overall variation. It was possible to observe the discrimination of CA from DCA in PC1 (38.27%) due to its correlation with the fatty acids C16:0, C18:0, C18:2n6, C18:1n9, C24:0, C8:0, and C22:0, squalene, UA, and OA in addition to higher ethylene production and respiration rate. On the other hand, DCA-stored apples discriminated DCA-CF storage, which was associated mainly with compounds such as heptacosanal, hexacosanal, henicosanal, and C20:2 as observed in the PC2. The samples stored in DCA-RQ1.3 and DCA-RQ1.5 were not discriminated, but they correlated with weight loss.

## 4. Conclusion

The isolated or combined effects of 1-MCP and the dynamic controlled atmosphere on the cuticle wax composition of 'Maxi Gala' apples were studied. 1-MCP application was effective in reducing ethylene production in CA, although DCA, especially DCA-RQ, has also been shown to be efficient in reducing the overall metabolism of the fruit similarly as 1-MCP treatment in CA. The storage atmospheres and treatment with 1-MCP did not influence the total wax content. The wax compositions of the apples stored in DCA were similar when 1-MCP was applied. However, 1-MCP treatment resulted in a greater mass loss in fruit stored in DCA.

## CRediT authorship contribution statement

**Bruna Klein:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Quellen Machado Ribeiro:** Investigation, Data curation. **Fabio Rodrigo Thewes:** Investigation, Data curation, Writing - review & editing. **Rogerio de Oliveira Anese:** Investigation, Data curation. **Fernanda de Candido de Oliveira:** Investigation, Formal analysis, Data curation. **Ingrid Duarte dos Santos:** Investigation, Data curation, Writing - review & editing. **Stephanie Reis Ribeiro:** Investigation, Data curation. **Jossiê Zamperetti Donadel:** Resources, Conceptualization. **Auri Brackmann:** Conceptualization, Resources. **Juliano Smanioto Barin:** Resources, Writing - review & editing. **Alexandre José Cichoski:** Investigation, Data curation. **Roger Wagner:** Conceptualization, Methodology, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2020.109900.

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# Safety profile, antimicrobial and antibiofilm activities of a nanostructured lipid carrier containing oil and butter from *Astrocaryum vulgare: in vitro* studies

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## Abstract

***Ethnopharmacological relevance:*** *Tucumã (Astrocaryum vulgare)is a fruit native to the Amazon region. Extracts from the peel and pulp are thought of as promising treatments for bacterial infections. The primary constituents of Tucumã oil and butter possess unsaturated carbon chains that are susceptible to oxidation by light or heat. The oils have high volatility and low aqueous solubility that limits their use without a vehicle. Nanotechnology refers to techniques to solve these problems. Nanostructured lipid carriers (NLC), for example, protect fixed oils degradation by heat or light, as well as from oxidation and evaporation, ensuring greater stability and function, thereby prolonging the useful life of the final product.* ***Study objectives:*** *The objective of this study was to evaluate the hemolytic, cytotoxic, antimicrobial and antibiofilm properties of an NLC containing Tucumã butter and oil soasto improve the solubility and photosensitivity of the compounds, generating better pharmacological efficacy.* ***Materials and methods:*** *The NLC was assessed for stability for 60 days. The cytotoxicity of nanoparticles in peripheral blood mononucleated cells was determined in culture using assays for cell viability, DNA damage, oxidative metabolism and damage to human erythrocytes. Antimicrobial activity was determined using the broth microdilution technique and antibiofilm activity according to standardized protocols.* ***Results:*** *The Tucumã NLC remained stable throughout the evaluated period, with pH between 5.22–5.35, monodisperse distribution (PDI<0.3) and average particle size of 170.7 ± 3nm. Cytotoxicity studies revealed that the NLC is safe and modulates inflammatory processes, demonstrated by increased cell viability and nitric oxide levels. There was low hemolytic activity of the NLC against human erythrocytes atmost concentrations*

*tested.* ***Conclusion:*** *Taken together, the data suggest that NLC containing Tucumã oil and butter showed antimicrobial and antibiofilm activity against organisms that cause morbidity and mortality in humans. They may be alternative solutions to public health problems related to bacterial infections.*

***Keywords:*** Microorganisms; Infections; Nanoparticles; Tucumã.

## 1. Introduction

Bacterial infections are one of the main causes of chronic diseases and mortality, causing economic losses for patients and public health systems. Each year in the United States, at least 2.8 million people are infected with antibiotic-resistant bacteria, with at least 35,000 deaths (CDC, 2019). Based on the incidence of antibiotic-resistant infections in 2014, each infection would cost $ 1,383 to be properly treated, resulting in national health care costs for the adult population of approximately 2.8 billion per year (CDC, 2019). Antibiotics have been the preferred treatment method because antimicrobial agents kill microorganisms or stop their growth. Nevertheless, several studies have provided direct evidence that the widespread use of antibiotics leads to the emergence of multidrug-resistant bacterial strains (KNETSCH and KOOLE, 2011; PORSE et al., 2020), now considered a significant health problem worldwide (TARSILLO and PRIEFER, 2020).

Antibacterial agents based on herbal products have aroused growing interest because of their technological, economic, nutritional, and bactericidal activities(SUTILLI et al., 2018; JOSHI, 2018).These herbs include Tucumã (*Astrocaryum vulgare*) (HOVORKOVÁ et al., 2018),a fruit native to the Amazon region that is widely used as an anti-inflammatory and antioxidant (BONY et al., 2012).Recent studies have revealed its potential use as a hypoglycemic and antioxidant in a model of diabetes induced by alloxan (BALDISSERA et al., 2017). In the particular interest of the present study, Hovorková et al. (2018) studied the antibacterial activity of Tucumã against gram-positive and commensal pathogenic bacteria in the intestine, revealing that Tucumã is a promising herbal product for use as an antibacterial agent. Jobim et al. (2014) tested extracts of pulp and bark from Tucumã (*Astrocaryum aculeatum*) against 37 microorganisms and found significant antibacterial activity against three important gram-positive bacteria and antifungal activity against *C. albicans*. The antimicrobial mechanism of action of Tucumã appeared to involve redox imbalance that interrupts the growth of microorganisms and/or causes increased mortality. This effect has some specificity for each microorganism involving the role of different chemical compounds found in Tucumã extracts.

Even with good biological activity, oils in generation can undergo oxidation, with consequent loss of biological function. In this context, nanotechnology may provide an important alternative, because substances on the nanometer scale have different properties from those on the macrometric scale (FREIRE et al., 2018). In addition, nanoparticles (NPs) increase the retention of a drug in the particle, increasing the release time and controlling the release of the molecule. Nanostructured lipid carriers (NLC) protect fixed oils against degradation by heat and light, oxidation and evaporation, ensuring greater stability and function, consequently prolonging the useful life of the final product (PIRAN et al., 2017). We believe that nanotechnology can improve the antimicrobial activity of Tucumã oil and prevent its activity from being

reduced.

Most antimicrobial resistance mechanisms are irrelevant to NPs, because NPs are in direct contact with the bacterial cell wall, without the need to penetrate the cell. This shows that NPs are less likely to promote resistance in bacteria than are antibiotics. In addition, in view of the serious health concern associated with antimicrobial resistance, new approaches to inhibit microbial growth and the biofilms formed by them have been studied. The usefulness of nanomaterials for the efficient administration of antibacterials and the development of anti-biofilm agents is a promising strategy to overcome the resistance of microorganisms to antimicrobials (LOPES et al., 2019; WANG et al., 2017).

## 2. Materials and methods

### *2.1 Acquisition of Tucumã oil and butter*

Tucumã oil and butter were purchased commercially from Amazon oil Industry - (Pará, Brazil).

2.1.1 Characterization of Tucumã oil and butter

The oil was previously characterized by Baldissera et al. (2017) using the gas chromatography method. The major components were oleic (368.7 mg/g) and palmitic acid (198.23 mg/g).

Tucumã butter was characterized using the method of Hartman and Lago (1973). A total of 20 mg of lipids from 1 mL of 0.4 M KOH methanolic solution were added in a test tube and vortexed for 1 min. The samples were kept in a water bath for 10 minutes at the boiling point and subsequently cooled to room temperature. Then, 3 mL of1 M $H_2SO_4$ methanolic solution were added and vortexed and kept in a water bath for 10 min. After cooling, 2 ml of hexane was added and centrifuged at 1050*g* for 5 min. Finally, the hexane with the fatty acid methyl esters (FAME) was subjected to chromatographic analysis. For FAME determination, a gas chromatograph model 3400CX was equipped with a flame ionization detector (Varian, Palo Alto, CA). A microliter of samples was injected into an injector without division, operated in split mode (20:1) at 250 °C. Hydrogen was used as a carrier gas at a constant pressure of 30 psi. The FAMEs were separated using an HP-88 chromatography column (100 m × 0.25 mm × 0.20 μm thick film, Agilent, J & W, Folson, CA, USA). The initial oven temperature was programmed at 50 °C for 1 min and increased to 185 °C, at a rate of 15 °C/min. Then, increasing to 195 °C, at a rate of 0.5 °C/min, and finally up to 230 °C, increasing by 15 °C/min, and maintained for 5 min at isothermal. The detector temperature was kept constant at 250 °C. FAME compounds were identified by comparing the experimental retention time with those of the authentic standard (FAME Mix-37, Sigma Aldrich, St. Louis, MO). The results were presented as a percentage of each FA identified in the lipid fraction, considering the equivalent factor of the chain size from FAME to FID and the conversion factor of the ester in the respective acid, according to Visentainer and Franco (2006).

### *2.2 Preparation and characterization of the Nanostructured Lipid Carrier*

The NLC containing the Tucumã oil and butter was prepared using a method developed by pre-formulation tests using Ultra Turrax® equipment and characterized according to pH, polydispersion index (PDI), size and zeta potential. The diameter and PDI determinations of the nanoparticles were carried out through

dynamic light scattering; the zeta potential was measured using electrophoresis (Zetasizer® nano-Zs model ZEN 3600, Malvern), the determination of the pH of the nanoparticles was performed in pH-meter. The formulation was prepared at n = 3 and maintained under various temperature and storage conditions (refrigerator at 28ºC, oven at 40ºC, room temperature with exposure to light and room temperature in a dark place) over60 days.

### *2.3 Biocompatibility Parameters*

#### 2.3.1 Hemolytic activity

The hemolytic assay was determined according to Souza Filho et al. (2019) with modifications. Blood was added with 1x PBS solution (1:1 v/v) and centrifuged for 15 minutes at 168*g*. The supernatants were discarded, and this procedure was repeated three times. Subsequently, in microtubes containing 1mL of 1x PBS at different pHs (pH 7.2 simulating cases of sepsis due to metabolic acidosis; pH 7.4 simulating the normal pH of the organism and pH 7.5 simulating alkalemia), 50µL of red blood cells were added washed and 10µL were taken. As controls, we used the following: negative control (NC) (erythrocytes + 0.9% sodium chloride); positive control (PC) (erythrocytes + distilled water); surfactants (TS) (erythrocytes + mixture of surfactants, Tween® 60 + Spam 60®, in the same concentrations as treatments with NLC). The tubes were incubated at 37 °C at room temperature, under rotation, for 1 hour. After the microtubes were centrifuged for 15 minutes at 168*g*, 200µL of the supernatants were transferred to 96-well plates and were read in the ELISA reader at 540nm. The results were expressed as a percentage of the positive control.

#### 2.3.2 Coagulation test

This assay was performed according to Souza Filho et al. (2019). Whole blood was collected in citrate tubes and centrifuged for 10 minutes at 1050*g*. Then, 225 µL of plasma were separated into wells along with 25 µL of NLC treatments and incubated at 37 °C for 30 minutes. Two independent experiments were carried out in duplicate with different donors. Subsequently, a properly calibrated Quick Timer II (Drake) coagulometer was read, according to the manufacturer's recommendations for tests for TP hemostasis (Labtest - lot: 4008) and TTPa hemostasis (Labtest - lot: 4006). As a reference we used the interval between 25 and 35 seconds for the normal value of TTPa, while the baseline values of TP used the interval between 11 and 15 seconds, according to authors (SALVADOR-MORALES et al., 2009; ADAMSON et al., 1993).

### *2.4 Cytotoxicity evaluation*

#### 2.4.1 Cell culture and treatments

Peripheral blood mononuclear cells (PBMCs) derived from whole blood samples discarded from healthy adults were obtained from the Clinical Analysis Laboratory of the Franciscan University (LEAC-UFN) (experimental protocol approved by the UFN Ethics Committee on Human Beings) (CAAE número: 31211214.4.0000.5306) with absence of identification data.

Blood samples were processed for PBMC separation using procedure based on the difference in density gradient using the Ficoll Histopaque-1077VR reagent (Sigma-Aldrich). After the blood was disposed of in the reagent (1:1 v/v), the samples were centrifuged for 30 minutes at room temperature. PBMCs were distributed in 96-well plates containing RPMI 1640 cell medium (Sigma-Aldrich) containing 10% fetal

bovine serum and supplemented with 1% antibiotics. The cells were grown at $2x10^5$cells $ml^{-1}$per well (BOTTON et al. 2015). Then, the cells were exposed to each compound tested for the efficacy protocol described in this research, in the same concentrations for 24 h, to evaluate its effect on cell modulation through different colorimetric and fluorometric assays. All treatments and trials were carried out in at least triplicate to ensure coherent statistical analysis; hydrogen peroxide ($H_2O_2$) at 200 µM was used as a positive control for all tests.

**2.4.2 Cell viability measurements**

After the treatment period, cell viability was assessed. The first assay performed was MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide) using the protocol according to Mossman (1983), when completing the incubation time of the cells with the treatment were 20µL of the MTT solution (0.01M and pH 7.4) was added at a concentration of 5mg/mL diluted in PBS (1X phosphate buffer). The plates that received the MTT solution were protected from light and kept at 37 °C, in an oven with 5% $CO_2$, which was homogenized and incubated for 2 h. After incubation, the supernatants were removed from the wells and the cells resuspended in 150µL of dimethyl sulfoxide (DMSO). Absorbance was determined in an ELISA reader at 560nm.

The determination of cell viability was complemented by the quantification of free DNA in the medium using the DNA-PicoGreen® reagent (Invitrogen, Life Technologies). DNA-PicoGreen® reagent was incubated for 5 min together with the sample dark 96-well plates, and fluorescence reading was performed on the spectrofluorometer at 480nm (excitation) and 520nm (emission) (SAGRILLO et al., 2015).

**2.4.3 Quantification of total levels of reactive oxygen species**

The total ROS levels were measured by fluorescence according to the technique described by Esposti (2002) using wavelengths of 488 nm (excitation) and 525 nm (emission). The results were expressed as% of the negative control.

**2.4.4 Determination of nitric oxide (NO) levels**

NO levels were measured according to the technique described by Choi et al. (2012). For this, 50µL of cell culture supernatant and 50µL of Griess reagent were added to96-well plates. The plates were maintained at room temperature for 15 min and the reading was performed spectrophotometrically at 540nm. The results were expressed in percentages in relation to the negative control.

***2.5 Evaluation of the antimicrobial activity of the Nanostructured Lipid Carrier***

The sensitivity tests for determining the minimum inhibitory concentration (MIC) of the different microorganisms tested were determined using the broth microdilution method according to the protocol M07-A8 approved by the *Clinical and Laboratory Standards Institute* (CLSI).

The bacterial strains of *Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus mutans, Enterococcus faecalis* and methicillin-resistant *Staphylococcus aureus* (MRSA) used in this study are clinical isolates from inmates at the Santa Maria University Hospital who were previously identified by gender-specific phenotypic

methods. These microorganisms are part of the collection of strains of the Microbiology Laboratory of the Franciscan University, Santa Maria - RS. The strains of *Aeromonas hydrophila* and *Pseudomonas aeruginosa* were provided by the Microbiology and Pathology Laboratory of the Federal University of Santa Maria - UFSM, Santa Maria - RS, Brazil.

MIC was determined to assess the NLC's antimicrobial activity against the microorganisms tested. In this sense, the bacterial samples were cultured on Mueller–Hinton agar (MH), and the colonies were inoculated in 5 mL of sterile saline, the absorbance was controlled until a transmittance of 0.5 was obtained on the MacFarland scale (1 x $10^6$to 5 x $10^6$cellsper mL), then a 1:10 dilution in MH broth was performed resulting in a concentration of $10^4$cells per mL.

After inoculum preparation, MIC was performed in 96-well polystyrene plates. The tests were performed in triplicate where, first, 100µL of MH broth were distributed in each well. A 100µL aliquot of the NLC solution containing Tucumã butter and oil was added to the first well and, after homogenization, transferred to the second, and so on until the twelfth well. Once the serial dilutions were made, the following dilutions were obtained: 25000, 12500, 6250, 3125, 1562.5, 781.25, 390.6, 195.3, 97.65, 48.8,24.41, and 12.2µg/mL.Soon after, the microorganisms were inoculated, where 10 µL of the standardized inoculum was then added to each well of the microdilution plate. After pipetting, the plates were incubated at 37 ºC for 48 h. The plates were read by adding 20µL of a 1% solution of the 2,3,5-triphenyl tetrazolium chloride dye (Vetec®), in order to assist in the MIC development. As a negative control (-) three wells were used with the MH broth and, as a positive control (+), three wells were used with the MH broth and the bacterial inoculum and as a surfactant control (TS) three wells with a mixture of surfactants, Tween® 60 + Spam 60®, in the same concentrations as NLC treatments.

### *2.6 Interference of the Nanostructured Lipid Carrier in the formation of biofilm*

To assess the interference capacity of the NLC on the biofilm in formation of *K. pneumoniae, E.coli, P. aeruginosa, S.aureus, S. epidermidis*, *S.agalactiae*, methicillin-resistant *S.aureus* (MRSA) and *A.hydrophila*, one amount of each bacterial isolate was cultured in sterile petri dishes with MH Agar and incubated at 37ºC for 24 h. Then the colonies were suspended in sterile 0.85% saline to prepare the inoculum. Cell density was adjusted in a spectrophotometer in order to obtain a transmittance equivalent to the 0.5 tube on the McFarland scale(1 x $10^6$ a 5 x $10^6$ cells/mL) at a wavelength of 630nm.

In 96-well plates of sterile polystyrene, 90µL of brain–heart infusion (BHI) broth supplemented with 1% glucose was added and then 20µL of the inoculum of each microorganism in each well and 90µL of the NLC solution were dispensed to test the antibiofilm activity of the nanoparticles. For the negative control, only broth was added, and for the positive control, the culture medium plus the solution containing the microorganisms was added. For the surfactant control (TS), the medium and the mixture of surfactants, Tween® 60 + Spam, was added. 60®, in the same concentrations as NLC treatments. After pipetting, the plates were incubated at 37ºC for 48 h. After this period, the samples were washed with 200µL of distilled water, three times. Then, the biofilm was fixed with 150µL of methane for 20min, after the plates were emptied and the wells were stained with 150µL of gentian violet for 15min and again washed with 200µL of distilled water. Subsequently, 150µL of 95% ethanol was added to dilute the crystals. Finally, the OD (optical density) was determined in a microplate reader, measured at 570nm (STEPANOVI et al. 2007).

### *2.7 Statistical analysis*

The results of hemolysis and cytotoxicity were presented as a percentage of the untreated control group (negative control). The analyses were performed using one-way bilateral analysis of variance (ANOVA) followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. The data were expressed as mean ± standard deviation. The graphs were prepared using GraphPadPrism version 5.01 (GraphPad Software, La Jolla, CA, USA).

The OD readings obtained in the biofilm formation assay were recorded as mean ± standard deviation (SD) and bilateral one-way ANOVA followed by Dunnett's post hoc test. Values with $p < 0.05$ were considered statistically significant. The graphs were prepared using GraphPadPrism version 5.01 (GraphPad Software, La Jolla, CA, USA).

## 3. Results

### *3.1 Characterization of Tucumã butter*

The determination of the concentration of fatty acids is shown in Table 1. The analysis of Tucumã butter by gas chromatography identified 12 fatty acids, the majority of which was lauric acid (41.95 mg/g), myristic acid (28.08 mg/g) and oleic acid (10.07 mg/g).

Table 1. Determination of fatty acid composition in tucumã butter (*Astrocaryum vulgare*) by gas chromatography.

| Fattyacid | mg of fatty acid/g of tucumã butter |
|---|---|
| Ácido capróico (C6:0) | 0,07 |
| Ácido caprílico (C8:0) | 1,77 |
| *Ácido cáprico (C10:0)* | 1,89 |
| *Ácido undecanóico (C11:0)* | 0,07 |
| *Ácido láurico (C12:0)* | 41,94 |
| *Ácido isomerístico (C13:0)* | 0,10 |
| *Ácido mirístico (C14:0)* | 28,3 |
| *Ácido palmítico (C16:0)* | 9,03 |
| *Ácido esteárico (C18:0)* | 2,55 |
| *Ácido oléico (C18:1cis)* | 10,07 |
| *Ácido linoléico (C18:2cis)* | 2,64 |
| *Ácido γ-linolênico (C18:3n6)* | 0,03 |

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### *3.2 Preparation and characterization of the Nanostructured Lipid Carrier*

The prepared formulation had a monodispersed distribution of particles showing PDI value of 0.256 ± 2, average size of 170.7 ± 3nm, zeta potential of -18.2 ± 2 and pH of 5.22–5.35. When evaluating the NLC formulations under various conditions of temperature and storage, there were no significant changes in the stability of the formulations over 60 days in any of the conditions of storage and temperature that they were

exposed to. The formulations stored in the refrigerator at -4ºC, showed a PDI value of 0.252 ± 2, average size of 168.9 ± 3nm, zeta potential of -18.8 ± 2 and pH of 5.32–5.55. The formulations stored in an oven at 40ºC showed a PDI value of 0.255 ± 2, an average size of 165.9 ± 3nm, zeta potential of -18.6 ± 2 and pH of 5.42–5.53. When stored at room temperature in a dark environment, the formulations showed a PDI value of 0.257 ± 2, average size of 171.3 ± 3nm, zeta potential of -18.4 ± 2 and pH of 5.37–5.43.In a clear environment, PDI value was0.254 ± 2, average size was171.8 ± 3nm, zeta potential was-18.0 ± 2 and pH was5.39–5.45.The formulations maintained in a clear environment and at room temperature underwent a color change.

### *3.3 Biocompatibility Parameters*

#### 3.3.1 Hemolytic activity

Hemolytic activity was significantly higher in the treatment with 12500 µg/mL NLC with pH 7.2 (acidosis) compared to the NC group, while it was significantly higher in the treatment with 25000 µg/mL NLC with pH 7.4 (physiological) compared to the NC group. There was no significant difference between groups treated with NLC in relation to hemolytic activity at pH 7.5 (alkalosis) (Figure 1).

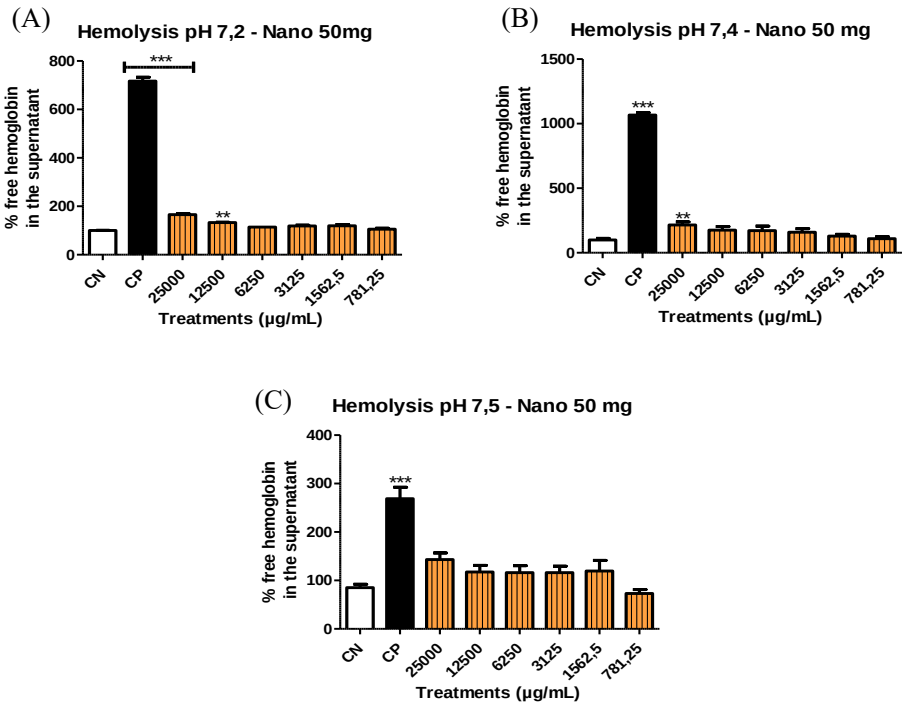



Figure 1. Results of hemolysis at different concentrations of the Nanostructured Lipid Carrier (NLC) at different pHs. (A) Results of hemolysis at pH 7.2 simulating cases of metabolic acidosis. (B) Hemolysis results at pH 7.4 simulating cases of physiological pH. (C) Hemolysis results at pH 7.5 simulating cases of metabolic alkalosis. Note: Results expressed as a percentage of positive control (100%). Values with p <0.05 were considered statistically significant.

#### 3.3.2 Coagulation test

The results of the coagulation tests are related to the type and concentration of treatments, as can be seen in Figure 2A (TP) and 2B (TTPa). Where for the TP test there were no results outside the biological range.

In the TTPa test at treatment concentrations of 781.25 µg/mL and 12.2 µg/mL. The results were below the biological range.

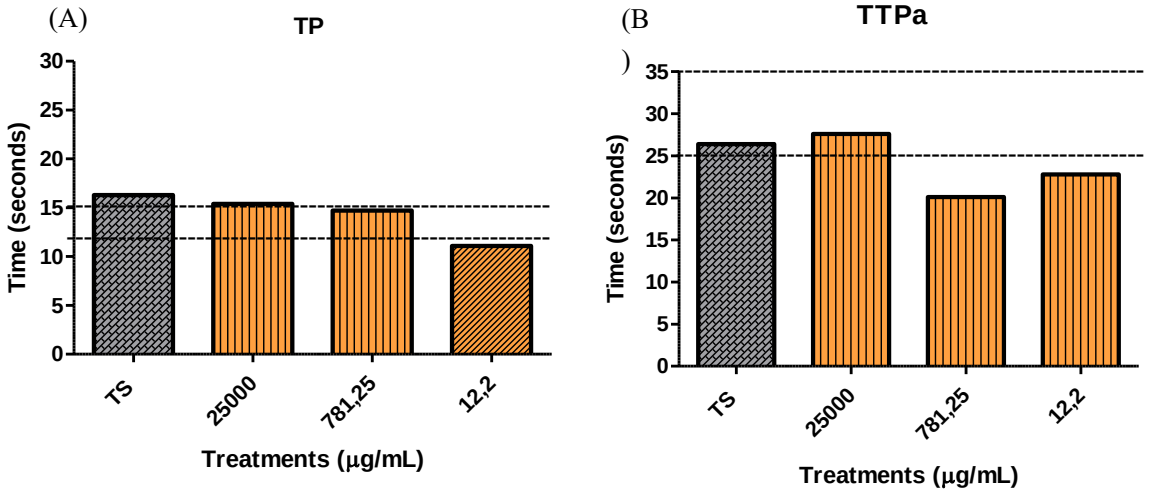



Figure 2. Results of coagulation tests at different concentrations of the Nanostructured Lipid Carrier (NLC). (A) prothrombin time (TP) results. (B) results from partially activated thromboplastin time (TTPa). The dotted limits correspond to the expected physiological times for healthy donors (between 12 and 15 seconds for PT and 25 and 35 seconds for TTPa). Values expressed as mean ± standard deviation (SD).

### *3.4 Cytotoxicity evaluation*

#### 3.4.1 Cell viability measurements

Cell viability was significantly higher in the treatment with 25000 µg/mL NLC than in the NC group (Figure 3A). Figure 3B shows the results for detecting double-stranded DNA damage in the cell culture supernatant, where no DNA damage was observed in any of the tested concentrations.

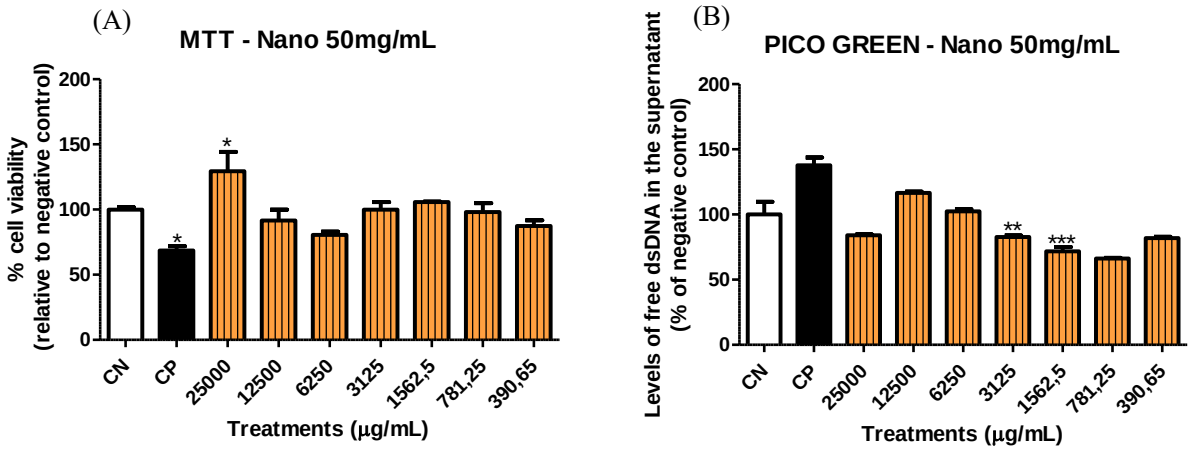



Figure 3. (A) MTT assay (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazoline bromide) after 24h incubation. (B) Quantification quantity of free dsDNA after 24h of treatment. The data are presented as% of the untreated control group (CN). Values with $p < 0.05$ were considered statistically significant.

### 3.4.2 Quantification of total levels of reactive oxygen species

Treatments with 6250 µg/mL and 3125 µg/mL NLC showed a significant increase in DCF production compared to the NC group (Figure 4).

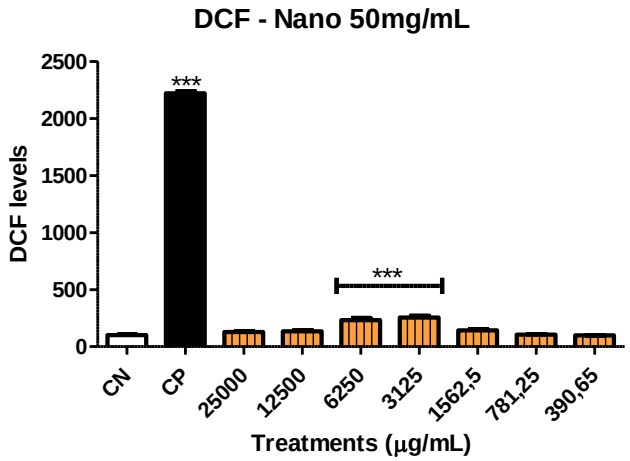



Figure 4. 2 ', 7'-dichlorofluorescein diacetate (DCF) assay with 24h incubation. The data are presented as% of the untreated control group (CN). Values with p <0.05 were considered statistically significant.

### 3.4.3 Determination of nitric oxide levels

ON levels were significantly higher in the treatment with 25000 µg/mL NLC than in the NC group (Figure 5).

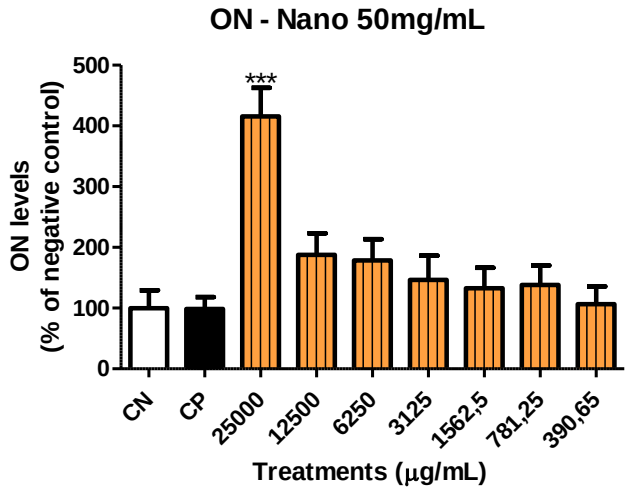



Figure 5. Nitric oxide (NO) assay with 24h incubation. The data are presented as% of the untreated control group (CN). Values with p <0.05 were considered statistically significant.

### *3.5 In vitro evaluation of the antimicrobial activity of the Nanostructured Lipid Carrier*

The NLC containing Tucumã oil and butter showed antimicrobial activity in vitro against ten microorganisms of broad clinical importance to humans. The results also indicate that there was a greater antibacterial efficiency for gram-negative bacteria than for gram-positive bacteria (Table 2).

Table 2. Minimum Inhibitory Concentration (MIC) of the Nanostructured Lipid Carrier (CLN) containing Tucumã oil and butter *Astrocaryum vulgare*.

| MICROORGANISMS | MIC(µg/mL) |
|---|---|
| *Staphylococcus aureus* | 12500 |
| *Staphylococcus aureus resistente a Meticilina (MRSA)* | 12500 |
| *Staphylococcusepidermidis* | 3125 |
| *Streptococcus mutans* | --- |
| *Streptococcus agalactiae* | 6250 |
| *Enterococcusfaecalis* | 6250 |
| *Klebsiellapneumoniae* | 12500 |
| *Escherichia coli* | 12500 |
| *Pseudomonasaeruginosa* | 25000 |
| *Aeromonashydrophila* | 25000 |

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### *3.6 Interference of the Nanostructured Lipid Carrier in the formation of biofilm*

The biofilm formation for *A.hydrophila*, *E.coli*, *K.pneumoniae*, *S.agalactiae* and were significantly lower at concentrations of 25,000, 12500 and 6250 µg/mL NLC compared to that of PC. By contrast, biofilm formation for *S. epidermidis* and *S.aureus* was significantly lower at concentrations of 25000 and 12500µg/mL NLC compared to PC (Figure 6).

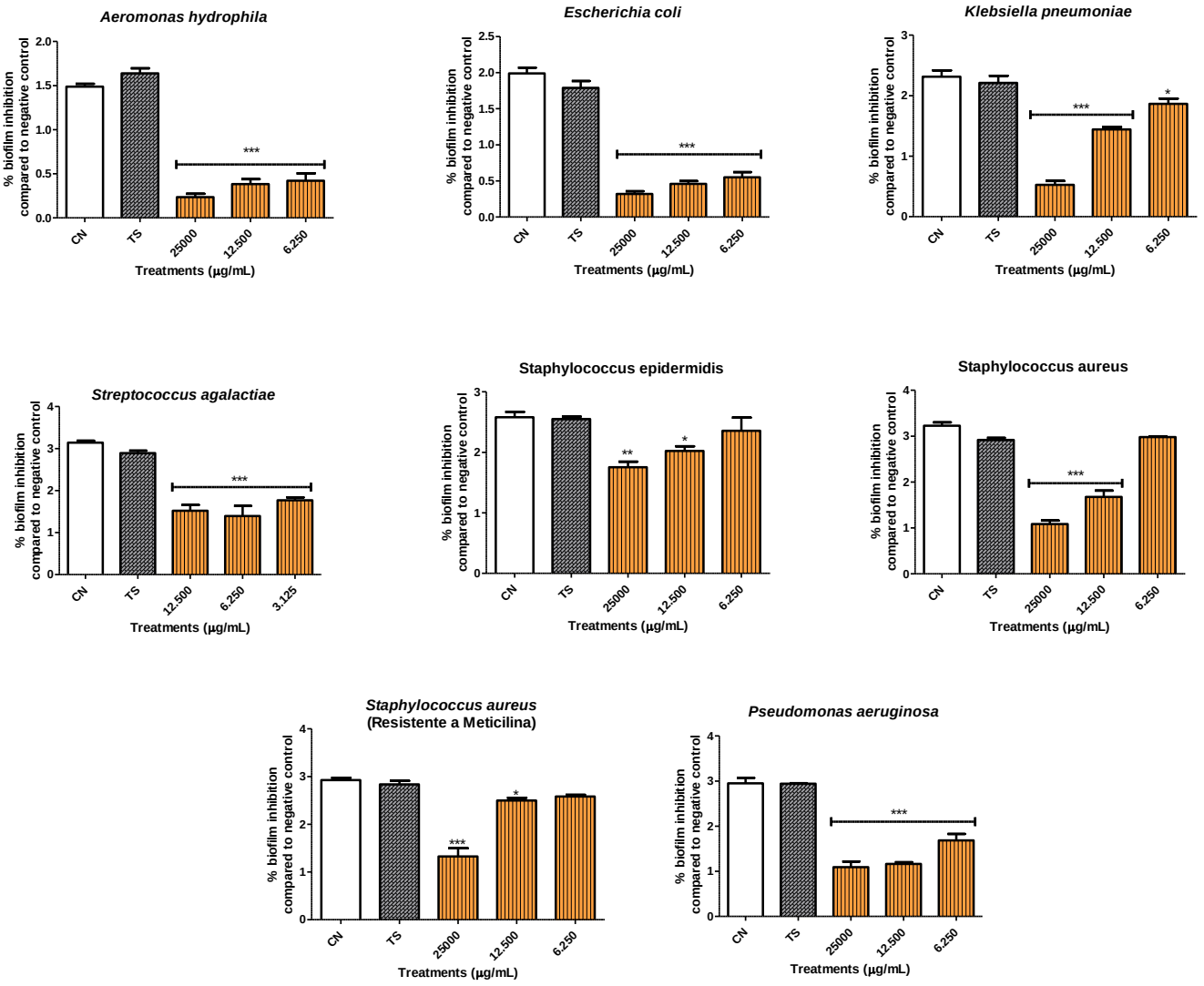



Figure 6. Effect of different concentrations of the Nanostructured Lipid Carrier (NLC) on the biofilm inhibition of different microorganisms. Significant difference between the tested concentration and the positive control (CP). Values with $p < 0.05$ were considered statistically significant.

## 4. Discussion

We observed for the first time that a nanoformulation of Tucumã oil in the form of NLC showed potent antimicrobial and anti-biofilm activities against various gram-positive and gram-negative bacteria that are human and animal pathogens. Our findings reveal that this formulation has morphological characteristics and an adequate safety profile, which makes this formulation an alternative to combat these pathogens.

A complete characterization of NLC is important, considering the influence of such parameters in terms of biological effects, because the physical-chemical analysis provides important information about the stability of these types of nanoformulation over time (GIORDANI et al., 2014). Particle size is an important attribute of lipid nanocarriers, affecting stability, encapsulation efficiency, drug release profile, biodistribution, mucus adhesion, and cell uptake. A rapid increase in particle size, for example, indicates low stability of the colloidal system (BAHARI and HAMISHEHK, 2016). For a particle to be considered

nanometric, it is necessary that at least one of its dimensions is less than 1000 nm and that this changes its nature properties. Thus, the results found for the sizes of the produced nanoparticles agree with the criteria established in the literature, suggesting adequate stability and better therapeutic efficacy. The results of the characterization of the nanoparticles suggestadequate homogeneity: all formulations must be monodispersed (PDI <0.3) and diameter less than 300 nm. The numerical value of the PDI varies from 0.0 (for a perfectly uniform sample in relation to the particle size) to 1.0 (for a highly polydispersed sample with various particle size populations). Values of 0.2 and below are generally considered acceptable in practice for nanoparticles based on material polymers. In drug delivery applications using lipid-based carriers, such as liposomes and nanoliposomes formulations, a PDI of 0.3 and below is considered acceptable and indicates a homogeneous population of phospholipid vesicles (CHEN et al. 2011). In this study, the polydispersity index was low (<0.3) for all dispersions obtained, indicating the formation of monodispersed systems.

The stability studies of the Nanostructured Lipid Carrier (NLC) showed that it remained stable for the period of 60 days in all established temperature and storage conditions. There were no visible trends in sedimentation, phase separation or aggregation over time. The average particle size, PDI, zeta potential and pH are considered good indicators of stability of suspended nanoparticles (BERNARDI et al., 2011).Considering that the zeta potential reflects the electrostatic repulsion between the particles, and that the standard value of the zeta potential is equal to or greater than +/- 30mV, it is associated with stable solutions, it can be said that the prepared formulation was stable. In addition, high values of polydispersity index indicate heterogeneity in the diameter of the suspended particles; variations in polydispersity values as a function of time indicate the formation of particle populations with diameters that did not initially exist, which may be due to particle aggregation or breaking/degradation (MACHADO et al., 2019). The characterization and stability results obtained demonstrate that the NLC was successfully developed, remaining stable for a substantial period of time in all temperature and storage conditions to which it was exposed. In order for nanoformulations to be used efficiently in technological and biomedical applications, they must be able to remain stable for a long period of time, without losing their properties or structural changes.

The hemolysis assay is useful to determine cytotoxic activity and may be related to direct damage to the erythrocyte cell membrane (ALENCAR et al., 2015). For this reason, we performed this test in three conditions (physiological, acidosis, and alkalosis) to see if NLC were capable of causing damage to erythrocytes. We found that the highest concentrations of NLC (12500 µg/mL in the condition of acidosis; 25000 µg/mL in physiological conditions) caused hemolysis in the erythrocytes, suggesting a possible cytotoxic effect when applied in high concentrations. To the best of our knowledge, there are no reports on the hemolytic effects of NLC; however, that effect can be explained by one of its major compounds, i.e., oleic acid. Hoque et al. (2013) found that high concentrations of oleic acid caused hemolysis in human and goat erythrocytes. According to what was observed in the present study, where only the two highest concentrations of NLC have hemolytic effect, we believe that the high amount of oleic acid present in Tucumã oil and butter may be responsible for the hemolytic action of NLC. Ashokraja et al. (2017) suggested that the observed hemolysis properties of synthesized NPs can be essentially attributed to their size, surface chemistry, and physicochemical properties. Nevertheless, according to these authors, the

hemolytic process involves the denaturation of cells through the physical-chemical interaction between NPs and the cell surface. Red blood cells do not have cellular organelles and the structure of red blood cells is maintained by few membrane proteins, phospholipids and carbohydrates in their cell membrane. In such physiological conditions, red blood cell senescence occurs due to physical-chemical changes in its membrane. More molecules of the NLC are being released at pH 7.2 and 7.4 than the NLC at pH 7.5, as blood pH variation can also lead to hypertonic condition causing hemolysis. Likewise, the main cause of the higher hemolysis properties of the NLC may be due to the interactions of reactive species with the cell surface or due to changes in pH (ASHOKRAJA et al., 2017).

Nevertheless, nanoparticles can have longer systemic circulation times. This prolonged time of circulation in the blood stream increases the duration of contact with these blood components, including the coagulation system, potentially amplifying the activation of the coagulation cascade. The results of the coagulation tests showed normal TP activities, in all concentrations tested with TP values within the biological range, and abnormal TPPa values, below the biological range, in the two lowest concentrations tested, but without statistical significance. According to Zare-Zardini et al. (2018), one of the most important things about coagulation is the idea of the intrinsic and extrinsic arms of the coagulation cascade and the way they interact during coagulation in the body. For a potent application of any compound, an investigation of its effect on blood clotting is necessary. The results demonstrated that the NLC does not interfere in the extrinsic and intrinsic clotting pathways. However, as changes in the TTPa test were observed, we did not rule out interactions with intrinsic coagulation factors such as factors VIII, IX, XI, XII, and pre-kallikrein protein. For these reasons, different methods of analysis must be performed to confirm and better understand our findings.

To investigate the safety profile of this formulation, we performed MTT assays and measured changes in double-stranded DNA. In the MTT assay, no concentration was able to reduce the cellular viability of PBMCs after 24 h of culture, suggesting that this treatment does not have cytotoxic effects for mononuclear cells such as lymphocytes and monocytes. These results agree with those of Sagrillo et al. (2015), who reported that Tucumã fruit extracts did not have cytotoxic effects and reduced the toxic effects of human lymphocytes exposed to hydrogen peroxide. Furthermore, we did not detect the presence of double-stranded DNA damage in any of the tested concentrations.

To identify possible mechanisms of action associated with these few cytotoxic effects of the highest concentrations of NLC, we evaluated the production of ROS and nitric oxide, two important mediators of oxidative and inflammatory damage related to cytotoxic effects (Baldissera et al., 2017). According to these authors, the production of reactive species are among the main causes of DNA damage. These species react with various DNA components to produce DNA damage, modifying bases, inducing interconnection between chains and intra-chains, and promoting chromosomal abnormalities. In the present study, a significant increase in the production of ROS (6250 and 3125 μg/mL) and NO (25000μg/mL) was observed compared to the negative control, suggesting that the production of ROS and NO may be involved in these cytotoxic effects found in red blood cells and PBMC exposed to NLC for 24 h. Although it has caused some negative effects, a recent study by Baldissera et al. (2017) found that Tucumã oil reduced oxidative damage in the brain of diabetic mice, which reveals its potent antioxidant action. On the other hand, β-carotene, another main constituent of Tucumã oil and butter prevented cell damage and decreased levels of

DCF (BESTWICK and MILNE, 1999). Another important constituent of both oil and Tucumã butter is tocopherol, an excellent inhibitor of lipid peroxidation, which acts as a hydrogen donor for the peroxyl radical, interrupting the radical chain reaction (BARREIROS and DAVDI, 2006). It is important to note that the cytotoxic effects of NLC may be due to its constituents, because the literature reports the absence of toxic effects of Tucumã oil.

The results related to antimicrobial activity are important, because these microorganisms isolated in hospital environments can colonize and adhere to the surfaces of medical instruments and implants. Because they are already resistant to various drugs, the ability to adhere can effectively reduce antimicrobial options and can further worsen infections. The main reason why NPs are being considered as an alternative to antimicrobials is that they can effectively prevent microbial resistance to drugs in certain cases. The rampant use of antibiotics has led to the emergence of several risks to public health, including the emergence of superbugs that do not respond to any antibioticand an existing epidemic against which the medication has no defense. The search for new effective antibacterial materials is significant for combating drug resistance, and NPs are being established as a promising approach to solving this problem (WANG et al., 2017).Rossato et al. (2019) showed that Tucumã-free oil showed antimicrobial activity against 5 microorganisms. *A.vulgare* oil is rich in lipids, carotenes, fibers and tocopherol, providing the fruit with high energy and nutritive content. Probably, the antimicrobial effect of Tucumã oil may be associated with its chemical composition, which includes several types of polyphenol molecules and fatty acids. Still, according to Rossato et al. (2019) polyphenols are secondary metabolites produced by higher plants, which play several essential roles in the physiology of plants that have potential healthy properties in the human body, mainly as antioxidants, anti-inflammatories, anti-allergens, and antimicrobials. This suggests that low uptake of free oil may limit its effectiveness, and that lipid encapsulation can help the active ingredients achieve their goals. Piran et al. (2017) demonstrated that NLC's increase the retention of a drug in the particle increased the release time and reduced the amount of drug needed to produce therapeutic action. According to these authors, this proficiency can be used to control the release of essential oils to improve the efficiency and reduce the quantity and toxicity of the essential oils used. By controlling the release of the drug molecule, the nanocarriers also protect essential oils against possible thermal or photoelectric degradation, oxidation or evaporation, which guarantees greater stability, flavor and function, consequently prolonging the useful life of the final product. According to Hu and Kwon (2011) most types of NP can overcome at least one of the common resistance mechanisms of microorganisms (including the interruption of bacterial membranes and the prevention of biofilm formation). These effects are the result of the bactericidal mode of NPs, which is based on their specific physicochemical properties. Unlike traditional antibiotics, NPs have dimensions <100 nm. The irexceptionally small size results in properties such as greater interaction with the cells due to greater area-to-surface ratiosand versatile and controllable application. According to Mühling et al. (2009),NPs cross the bacterial membrane and gather along the metabolic pathway, influencing the shape and function of the cell membrane. Then, NPs interact with the basic components of the bacterial cell, including DNA, lysosomes, ribosomes, and enzymes, leading to oxidative stress, disturbances of the electrolyte balance, inhibition of enzymes, heterogeneous changes, changes in the permeability of the cell membrane, deactivation of proteins, and changes in expression genes, all of which can be considered possible

mechanisms of action of nanoparticles against bacteria. According to Karimi et al. (2018) NLC systems allow the transfer of antibacterial extracts through the cell membrane layer. They acts carrier agents, bringing concentrations of compounds within the aqueous phase to microorganisms. According to these authors, there are “holes” in the cell membranes of the bacteria that act as component transfer areas, and smaller nanoparticles can be more easily inserted into the cavities and release their bioactive compounds, also contributing to the improvement of the antimicrobial activity of the nanoformulations. Finally, Mokarizadeh et al. (2017) found that lipid-based nanocarriers can protect essential oils against thermal or photoelectric degradation, increasing product stability and consequently, prolonging the life of the final product, in addition to not affecting the appearance, texture, or the taste of the product. The use of such delivery systems can increase the concentration of antimicrobials in areas in which microorganisms are preferentially located and can potentially increase the passive mechanisms of cell absorption that affect the stability of the lipid membrane and, as a result, increase antibacterial activity.

Comparing the MICs and the concentrations capable of inhibiting bacterial biofilm, we observed that the NLCs containing Tucumã oil and butter inhibited the formation of biofilm in concentrations lower than the MICs in most of the tested microorganisms. This suggests inhibition of biofilm formation of the microorganism in the sessile form (adhered to solid surfaces), not only in the inhibition of bacterial growth in the planktonic form (microorganisms in suspension and dispersed in the aqueous medium). Size of a NP can greatly affect its antibacterial activity. In addition to the rupture of bacterial membranes, the prevention of biofilm formation is an important mechanism becausebiofilms play fundamental roles in the development of bacterial resistance. The unique structure and composition of bacterial biofilms provides shelter or protection for embedded microorganisms, helping them to protect themselves from most antibiotics. In addition, bacterial biofilms are "a breeding ground" for frequent resistance mutations and the exchange and alteration of these mutations between bacterial cells. Studies have shown that many NPs can prevent or overcome biofilm formation (PEULEN and WILKINSON, 2011).Most bacteria exist in the form of biofilms, usually containing several species that interact with each other and with the environment. Biofilms are specifically microbial aggregates that depend on a solid surface and extracellular products, including extracellular polymeric substances (EPSs) (WANG et al., 2017). Astudy by Alalaiwe et al. (2019) using the biofilm, demonstrated that oxacillin-loaded nanoparticles penetrated the EPS, eradicating the MRSA biofilm more effectively than the individual treatment. According to these authors, extracellular DNA plays a fundamental role in the production of biofilm, acting as a chelator of cationic molecules. The interaction between EPS and nanoparticles with lipids can cause strong affinity and disintegration of the biofilm.

## 5. Conclusion

The Tucumã nanostructured lipid carrier was successfully developed by the homogenization method using Ultra Turrax. It remained stable for a considerable period of time. Cytotoxicity studies demonstrated that the nanoparticle suspension is safe because no concentration reduced cellular viability of PBMCs or caused double-stranded DNA damage, in addition to having low NLC hemolytic activity against human erythrocytes in most concentrations tested and few toxic effects at high concentrations. The NLC-

containing Tucumã oil and butter showed antimicrobial and antibiofilm activity against important agents that cause human morbidity and mortality Our findings suggest that this treatment, in nanostructured form, may be a potential alternative therapeutic agent for the elimination of bacteria and biofilms.

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# Respiratory quotient level variation during storage: Critical period for low oxygen tolerance, metabolism, and quality of 'Galaxy' apples

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ABSTRACT

New technologies for apple preservation include storage under dynamic controlled atmosphere (DCA) with respiratory quotient (RQ) monitoring or chlorophyll fluorescence (CF). Therefore, this study aimed to evaluate the effects of RQ variation on the metabolism, overall quality, and volatile organic compound concentration of 'Galaxy' apples after long-term storage and compare them to fixed RQ levels, controlled atmosphere (CA), and DCA-CF. We also aimed to identify the period during storage in which apples are more susceptible to low oxygen damage. Fruit were evaluated after nine months of storage plus 7 d of shelf life at 20 °C. Higher RQ (1.5 and 2.0) levels throughout the storage period or the first three months resulted in higher anaerobic metabolism, increasing the internal disorders and reducing overall fruit quality, healthy fruit and flesh firmness. The results evidenced that the critical period for low oxygen damage is the first three months of storage. Overall fruit quality is better maintained when fruit were stored under DCA-CF, DCA-RQ 1.1, DCA-RQ 1.3, and changing RQ from 1.1; 1.3; 1.5, being three months at each RQ level. Moreover, more healthy fruit, higher flesh firmness, and lower physiological disorders were observed under these storage conditions, although the main ester accumulation was reduced. In general, higher 2-methylbutyl acetate and butyl acetate were observed in fruit stored under CA and high RQ levels (1.5 and 2.0). This demonstrates that even with RQ level variations during storage, it was not possible to obtain apples with high overall quality and ester concentrations under the same storage condition.

## 1. Introduction

Apples (*Malus domestica*) are seasonal fruit with a harvest window concentrated over two or three months, making storage necessary to provide fruit throughout the off-season. The main storage method is the controlled atmosphere (CA), in which low storage temperatures are combined with low oxygen and increased carbon dioxide partial pressures (Argenta et al., 2000; Brackmann et al., 2008; Kittemann et al., 2015; Weber et al., 2011). However, the oxygen partial pressure ($pO_2$) is maintained far above the lower oxygen limit (LOL) because the fruit metabolism cannot be monitored periodically during storage.

Adopting $pO_2$ levels above the LOL does not reduce the fruit metabolism to a minimum during storage. Contrarily, the fruit metabolism will be reduced to a minimum with levels closer to the LOL. In practical terms, the LOL can be estimated by several methods, such as ethanol production (Schouten et al., 1998; Veltman et al., 2003), chlorophyll fluorescence (Prange, 2018; Prange et al., 2003; Wright et al., 2012), respiratory quotient (Bessemans et al., 2016; Gasser et al., 2008; Weber et al., 2015), and carbon dioxide production (Thewes et al., 2020b). Among these methods, storage under a dynamic controlled atmosphere based on chlorophyll fluorescence (DCA-CF) is the most widely studied and adopted method in commercial apple storage. Some studies have reported that DCA-CF maintained higher overall fruit quality (Eren et al., 2015; Prange, 2018; Raffo et al., 2009; Thewes et al., 2015; Tran et al., 2015), albeit reducing volatile organic compound (VOCs) emission, especially esters, compared to CA (Aubert et al., 2015; Both et al., 2017; Donadel et al., 2019; Thewes et al., 2017b). Nonetheless, apple fruit storage under a dynamic controlled atmosphere based on

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respiratory quotient (DCA-RQ) maintained higher VOC emission than DCA-CF and comparable overall quality (Both et al., 2017; Thewes et al., 2020a).

Some studies have attributed better quality maintenance under DCA-RQ to the induction of anaerobic metabolism in safe levels by storage under RQ > 1.0 (Bessemans et al., 2016; Both et al., 2017; Thewes et al., 2017c; Weber et al., 2015). According to the literature, apple storage under DCA-RQ 1.3 maintains the physical and chemical quality better than storage under CA (Anese et al., 2020; Thewes et al., 2017a; Weber et al., 2015). However, higher VOCs are maintained by storage using higher RQ levels, such as RQ 1.5 and 2.0 (Both et al., 2017; Donadel et al., 2019; Thewes et al., 2017c, 2017b). These studies were performed using a fixed RQ level throughout the storage period (i.e., a fixed anaerobic metabolism level), changing the $pO_2$ to obtain the pre-established RQ. Thus, the RQ level that maintains the highest overall quality does not allow high VOC emission. Taking this into account is necessary to develop storage techniques that allow maintaining the overall quality of apples and high VOC concentration.

It is well known that fruit tolerance to low oxygen atmospheres and, consequently, anaerobic metabolism, changes over the storage period because the whole metabolism is affected by the atmosphere, temperature, relative humidity, storage time, among other factors (Prange, 2018; Weber et al., 2015; Wright et al., 2012). Therefore, it is probable that fruit will tolerate different RQ levels during storage. Nevertheless, at least to our knowledge, there is still no research evaluating RQ level variations throughout the storage period on the metabolism, overall fruit quality, and VOC emission of apples. Additionally, the study of RQ variations during storage will allow to detect the period in which fruit are more susceptible to low oxygen damage and the moments when oxygen should be more accurately analyzed and supplied.

Given the above, this study sought to evaluate the effects of RQ level variation during the storage on the metabolism, overall quality, and VOCs of 'Galaxy' apples after long-term storage and compare them to fixed RQ levels, CA, and DCA-CF. We also aimed to identify the period during storage that apples were more susceptible to low $pO_2$.

## 2. Materials and methods

### 2.1. Experimental material

'Galaxy' apples, a 'Royal Gala' strain, were harvested in a commercial orchard in Vacaria (RS, Brazil). Fruit were harvested in a commercial orchard at the harvest peak (optimal harvest date) to represent the main commercial storage rooms of this season. We used only one harvest maturity stage and orchard because we want a homogenous patch of fruit in order to isolate the effects of CA, DCA – CF and DCA – RQ (fixed or variation) on fruit metabolism/quality and identify the critical period for low oxygen damages. The fruit were harvested in the morning and immediately transported to the postharvest research center of the Federal University of Santa Maria (Brazil). At the postharvest research center, the fruit were submitted to a selection process to homogenize the fruit size in the samples and eliminate those with defects.

### 2.2. Sample preparation

After the selection process and discarding fruit with defects, the apples were randomly selected to make 25-fruit samples. Each treatment was composed of four samples. The four samples of each treatment were put into an experimental chamber (230 L) that could be hermetically sealed. Ten chambers were used (one for each atmospheric condition) and put into a cold room, which allowed to control the storage temperatures of all chambers equally.

### 2.3. Storage temperature and relative humidity

Once the fruit were inside the storage chambers, the temperature was reduced to 5 °C and maintained for one day. The temperature was then gradually reduced to 2 °C during an additional five-day period. On the day the temperature reached 2 °C, the chamber was hermetically sealed to establish the atmospheric conditions. The relative humidity was maintained at 94 ± 2% throughout the nine-month storage period by allocating calcium chloride, which absorbed the excess humidity inside the experimental chambers.

### 2.4. Atmosphere conditions setup

When the fruit temperatures reached 2 °C, the experimental chambers were flushed with nitrogen until 5 kPa $O_2$. After that, the $pO_2$ was reduced by fruit respiration until 1.2 kPa for CA and 0.5 kPa for DCA conditions in a period of 7 d. The atmospheric conditions applied were: [1] CA - 1.2 kPa $O_2$ + 2.0 kPa $CO_2$; [2] DCA-CF; [3] DCA-RQ 1.1; [4] DCA-RQ 1.3; [5] DCA-RQ 1.5; [6] DCA-RQ 2.0; [7] DCA-RQ 1.1; 1.3; 1.5; [8] DCA-RQ 1.5; 1.3; 1.1; [9] DCA-RQ 1.1; 1.5; 2.0; [10] DCA-RQ 2.0; 1.5; 1.1. Fruit of treatments 7–10 were submitted to RQ level variations, in which RQ X; Y; Z represents the RQ levels used at each three-month storage period (Fig. 1B). The carbon dioxide partial pressure ($pCO_2$) was maintained at 1.2 kPa for all DCA conditions. The oxygen set point variations over nine months of storage for all atmosphere conditions are shown in Fig. 1.

### 2.5. Monitoring LOL with chlorophyll fluorescence (DCA-CF)

Two six-fruit batches were put into plastic baskets with chlorophyll fluorescence sensors in their upper sides to monitor fruit response to low oxygen levels with DCA-CF. These two sensors were placed inside the DCA-CF chamber to monitor the chlorophyll fluorescence in real time throughout the storage period. The $pO_2$ was changed according to the method proposed by Prange et al. (2003), as shown in Fig. 1A.

### 2.6. Monitoring LOL with respiratory quotient (DCA-RQ)

According to Weber et al. (2015), the DCA-RQ was performed twice a week, with a 13-h period of chamber closing to determine the fruit $O_2$ uptake and $CO_2$ production. The ratio between $CO_2$ production and $O_2$ uptake was the RQ. When the calculated RQ was below the set value, the $O_2$ set point was decreased, and if the RQ level was above the set value, $O_2$ set point was increased. The $O_2$ set point was increased or reduced at 0.01 – 0.03 kPa (Fig. 1B).

### 2.7. Atmosphere conditions maintenance

To maintain the $pO_2$ and $pCO_2$ at the set points, the atmospheric conditions were determined and corrected at least four times every day using an automatic CA and DCA control system (Valis®, Lajeado, RS, Brazil), which had a gas analyzer with two decimal cases (Siemens, Ultramat, Germany). The gas analyzer was calibrated twice a week with standard gas to ensure correct atmosphere measurements. If the oxygen was lower the set point, cold air was supplied. The excess $CO_2$ produced due to respiration was adsorbed with a lime scrubber.

### 2.8. Metabolism, overall quality, and volatile compound analyses

The analyses were carried out one day after harvest and after nine months of storage plus 7 d shelf life at 20 °C.

#### 2.8.1. ACC oxidase activity

The ACC oxidase activity was determined in the skin of the apples, according to Bufler (1986). The results were expressed as ng $kg^{-1}$ $s^{-1}$.

#### 2.8.2. Ethylene production

Ethylene production was determined by placing ~1.5 kg apples from each replicate into a 5-L glass and hermetically closing it for two hours.

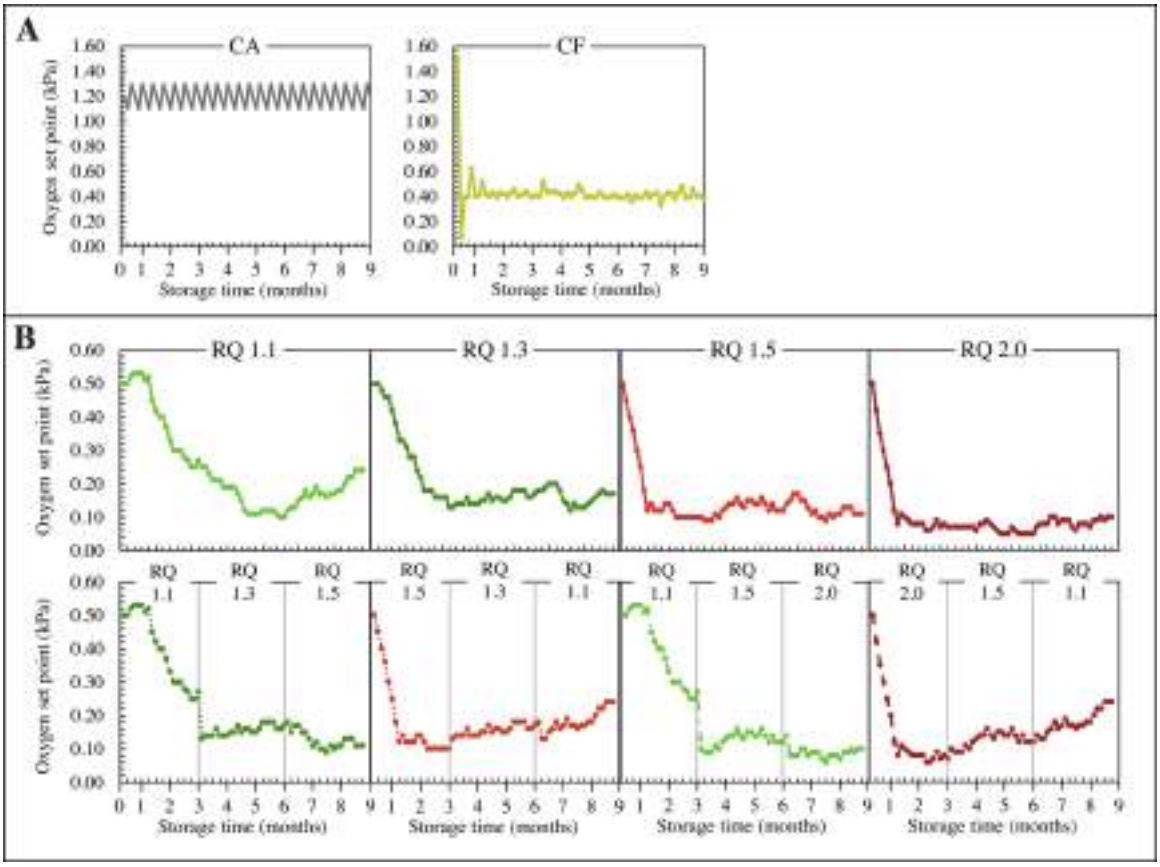



**Fig. 1.** Oxygen set point variation of 'Galaxy' apples during 9 months of storage under controlled atmosphere (CA), dynamic controlled atmosphere based on chlorophyll fluorescence (CF) (A), and dynamic controlled atmosphere based on respiratory quotient with four fixed RQs and RQ level variation during storage (B).

Then, two 1-mL samples of the headspace gas were taken and injected into a gas chromatograph (Varian®, Star CX3400, Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a Porapak N80/100 column to determine the ethylene concentrations inside the glass headspace. The injector, column, and detector temperatures were 140, 90, and 200 °C, respectively. The ethylene production rate was expressed as ng $kg^{-1}$ $s^{-1}$.

### 2.8.3. Respiration rates

Fruit respiration rates were determined in the same fruit sample that ethylene production was measured. The headspace gas of each glass was circulated using a gas analyzer (Schelle®, Germany), which determined the $CO_2$ concentration inside. The respiration rates were calculated by considering the difference of $CO_2$ concentrations inside the glass and ambient concentrations, fruit weight, and the time the glass was hermetically sealed for $CO_2$ accumulation. The results were expressed as μg $kg^{-1}$ $s^{-1}$.

### 2.8.4. Internal ethylene concentration

The internal ethylene concentration (IEC) was quantified by withdrawing the internal air of fruit, according to Mannapperuma et al. (1991). The internal air was injected into the same gas chromatograph used for ethylene production measurements. The IEC values were expressed as μg $L^{-1}$.

### 2.8.5. Electrolyte leakage

Electrolyte leakage was determined according to Gago et al. (2015), with modifications. In short, fifteen flesh discs were taken from 15 apples and put into 25-mL bi-distilled water for one hour, followed by electrical conductivity determination. The suspension was then placed into a water bath at 100 °C for 30 min and subsequently frozen at -30 °C. Thereafter, the samples were thawed (20 °C), and electrical conductivity was measured once again and taken as total conductivity. The results are expressed as a percentage.

### 2.8.6. Decay, pulp cracking, mealiness, flesh breakdown, and healthy fruit

The quality attributes of decay, pulp cracking, mealiness, flesh breakdown, and healthy fruit were determined according to Thewes et al. (2015). The healthy fruit percentage was determined by recording the number of fruit without decay, pulp cracking, mealiness, and flesh breakdown in relation to the total number of fruit per treatment. All variables were expressed as a percentage.

### 2.8.7. Flesh firmness

Flesh firmness was measured in two opposite sides of the fruit where the skin had been previously removed using an 11-mm tip penetrometer (Effegi Systems, FT 327, Milan, Italy). The results are expressed in Newton (N).

### 2.8.8. Volatile compound concentrations

The volatile compounds were identified and quantified according to the method proposed by Both et al. (2014). Fruit slices of the equatorial region were cooled down to 0 °C (pulp temperature) and, after cooling, the slices were processed to obtain the juice; this procedure was carried out under a low temperature (<5.0 °C) to avoid enzymatic activity. The juice was put into 100-mL amber flasks and frozen at -30 ± 1.0 °C until volatile compound analysis. On the day of the volatile compound analysis, the juice was thawed (2 °C). After that, a 10-mL juice aliquot was placed in a 20-mL amber vial with NaCl (3 g) and 3-octanol standard solution (10 μL; 82.2 μg $mL^{-1}$) and immediately hermetically closed with a PTFE-coated silicone seal. The vial was then put into a water bath at 35 °C for 5 min, followed by

divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS) fiber (Supelco, 50/30 μm ×20 mm) exposure to the vial headspace to adsorb the volatile compounds. The fiber was exposed to the headspace for 60 min under constant stirring for volatile compound extraction. Afterward, the VOC were thermally desorbed into the injection port of a DANI® (Dani Instruments Spa., Viale Brianza, Cologno Monzese, Italy-gas) gas chromatograph at 250 °C for 10 min in splitless mode. The volatile compounds were separated using a DN-WAX (60 m ×0.25 mm ×0.25 μm) capillary column. The carrier gas (nitrogen) was seated in a constant flow of 1.0 mL $min^{-1}$. For volatile compound separation, the following temperature program was employed: initial temperature of 35 °C held for 3 min, followed by a temperature ramp of 2 °C $min^{-1}$ up to 80 °C, and another ramp of 5 °C $min^{-1}$ up to 230 °C, which was held for 5 min. The FID detector temperature was set to 230 °C. A series of n-alkanes was analyzed under the same conditions described above to calculate the linear retention index (LRI). The volatile compounds were quantified in relation to the area of the internal standard, and the results are expressed in μg $L^{-1}$.

To identify the volatile compounds, the same method described above was performed using a Shimadzu QP2010 Plus gas chromatography coupled to mass spectrometry (GC/MS; Shimadzu Corporation, Kyoto, Japan) or by standard solutions (Supplementary Table 1). The detector was operated in the electron impact ionization, +70 eV ionization energy, and a scan mass from 35 up to 350 *m/z*. The mass spectra of each compound were compared with mass spectra available in the National Institute of Standards and Technology (NIST) library and comparing the LRI with data available in the scientific literature.

### 2.9. Statistical analysis

The experiment was conducted in a completely randomized design. An analysis of variance (ANOVA) at 5% of error probability was carried out ($p < 0.05$) using Sisvar software (version 5.6, Build 86). Treatment averages that showed a significant difference were subjected to a Skott-Knott test at a 5% error probability. Additionally, to show an overview of the results, data were submitted to a Principal Component Analysis (PCA) using MetaboAnalyst® 5.0 (https://www.metaboanalyst.ca/). All data were standardized (mean = 0 and variance = 1 for each variable) before the PCA was performed.

## 3. Results and discussion

### 3.1. ACC oxidase, ethylene production, and respiration rates as affected by DCA with RQ variation over the storage period

After 9 months of storage plus 7 d of shelf life at 20 °C, ACC oxidase activity was higher in fruit stored under CA, and there were no differences between DCA conditions and RQ level variations (Fig. 2A). The lower ACC activity of fruit under DCA is due to the lower $pO_2$ in the whole storage period (Fig. 1). Several studies also reported lower ACC activity in fruit stored under extremely low $pO_2$ (Anese et al., 2020; Both et al., 2017; Schmidt et al., 2020; Thewes et al., 2020a), albeit our study is the first to show that ACC oxidase is not affected by RQ level variations. The higher ACC oxidase activity under CA resulted in significantly higher ethylene production than all DCA treatments (Fig. 2C). By analyzing the effects of RQ level variations, high RQ levels (1.5 and 2.0) in the first three months of storage induced higher ethylene production compared to high RQ levels at the last three months of storage (Fig. 2C). These findings evidenced that high anaerobic metabolism at the beginning of the storage period induced ethylene production. This is perhaps because, at the beginning of the storage period, fruit are not adapted to the low oxygen levels and cannot metabolize the anaerobic metabolism compounds, including acetaldehyde and ethanol, thus resulting in metabolic stress.

Together with ethylene production, the IEC is important to induce fruit ripening during storage and shelf life as it represents the ethylene concentration around the flesh cells. Moreover, the IEC was higher when fruit were submitted to RQ level variations with high RQ (2.0) at the beginning of the storage period (Fig. 2B). Fruit submitted to CA, DCA-RQ 1.5, and DCA-RQ 2.0 had intermediary IEC values. These data demonstrate that fruit submitted to high RQ levels at the first three months of storage or throughout the storage period accumulate higher ethylene

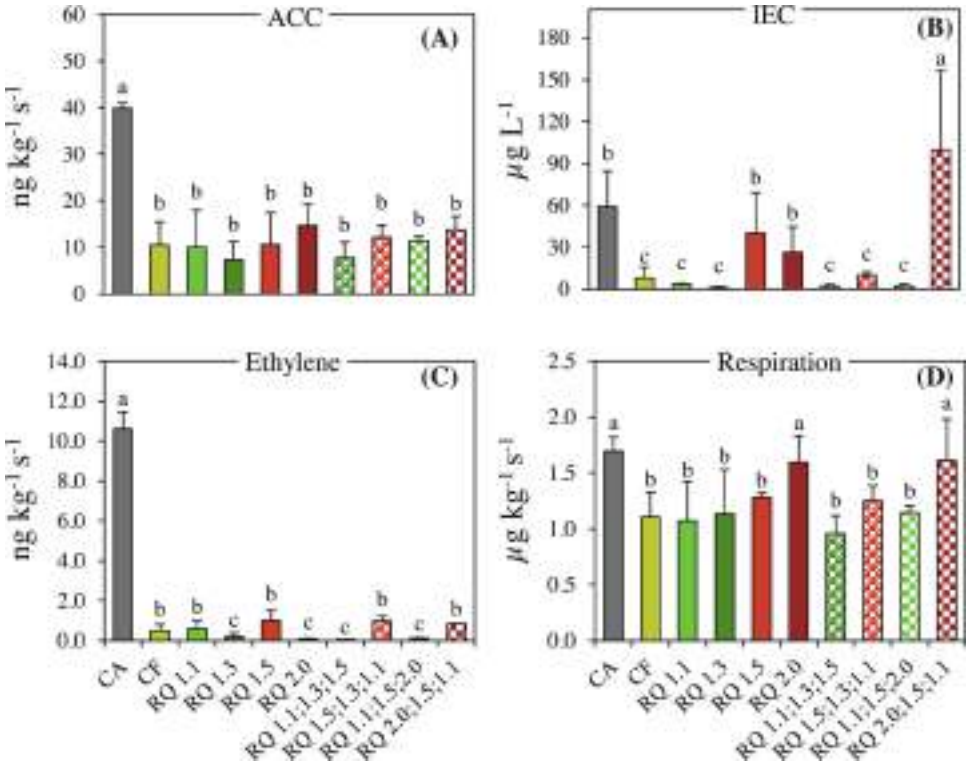



**Fig. 2.** ACC oxidase enzyme activity (A), internal ethylene concentration (IEC) (B), ethylene production (C), and respiration rate (D) of 'Galaxy' apples stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (CF) and on respiratory quotient (RQ) after 9 months of storage plus 7 d of shelf life at 20 °C. RQ X; Y; Z: means variation of RQ levels during storage; fruit remained in each RQ for 3 months. Bars with the same lowercase letter do not differ between CA and DCA conditions by the Scott-Knott test at 5% probability. Error bars mean standard deviation.

concentrations inside the pulp compared to lower RQ levels and DCA-CF. According to several studies, fruit with high IEC were riper and more susceptible to physiological disorders and flesh firmness loss (Brackmann et al., 2014; DeEll and Ehsani-Moghaddam, 2012; Koushesh Saba and Watkins, 2020; Thewes et al., 2017c).

In general terms, fruit with high ethylene production and IEC will have higher respiration rates (Saltveit, 2019). Fruit stored under CA, DCA-RQ 2.0, and DCA-RQ 2.0; 1.5; 1.1 had higher respiration rates during shelf life than all other storage treatments (Fig. 2D). The higher respiration rates under CA is due to the higher $pO_2$ in the storage period (Fig. 1), although in the case of DCA-RQ 2.0 and DCA-RQ 2.0; 1.5; 1.1, it is because of the damage induced by the high anaerobic metabolism of RQ 2.0 at the first three months or the whole storage period. In 'Braeburn' apples stored under DCA-RQ 1.7 and 2.0, higher respiration rates were also observed due to anaerobic metabolism damage (Schmidt et al., 2020; Weber et al., 2020). Nevertheless, the present study is the first to show that this is because of high RQ levels (2.0) in the first three months of storage (Fig. 2D), which is the period that fruit are adapting to low $pO_2$.

### 3.2. Accumulation of anaerobic metabolism compounds

The main challenge of storing fruit under extremely low $pO_2$ is defining the oxygen level that can induce anaerobic metabolism compounds to safe levels, which helps retain the maximum fruit quality without inducing off-flavors to the fruit. Acetaldehyde is the first compound produced during ethanolic fermentation, and it was accumulated in higher concentrations in fruit stored under DCA-RQ 2.0 (Fig. 3A). Fruit submitted to DCA-RQ 1.5, DCA-RQ 2.0; 1.5; 1.1, and DCA-RQ 1.1; 1.5; 2.0 had intermediary concentrations compared to the other storage conditions (Fig. 3A). Notably, storage under DCA-CF, DCA-RQ 1.1, DCA-RQ 1.3, DCA-RQ 1.1; 1.3; 1.5, and DCA-RQ 1.5; 1.3; 1.1 had the same acetaldehyde concentration as fruit under CA, although the $pO_2$ was significantly lower than CA (Fig. 1A and B). This suggests that fruit stored under such conditions had no significant anaerobic metabolism induction or could metabolize them, which avoids low oxygen damage.

As acetaldehyde is highly toxic to fruit cells (Ke et al., 1994; Pesis, 2005), it is actively converted to ethanol and ethanoic acid (Fig. 3B and C). Fruit stored under DCA-RQ 2.0 accumulated higher ethanoic acid and ethanol content, which are combined by AAT enzymes to form ethyl acetate (Fig. 3D). The higher quantity of anaerobic metabolism compounds under DCA-RQ 2.0 is due to the lowest $pO_2$ throughout the

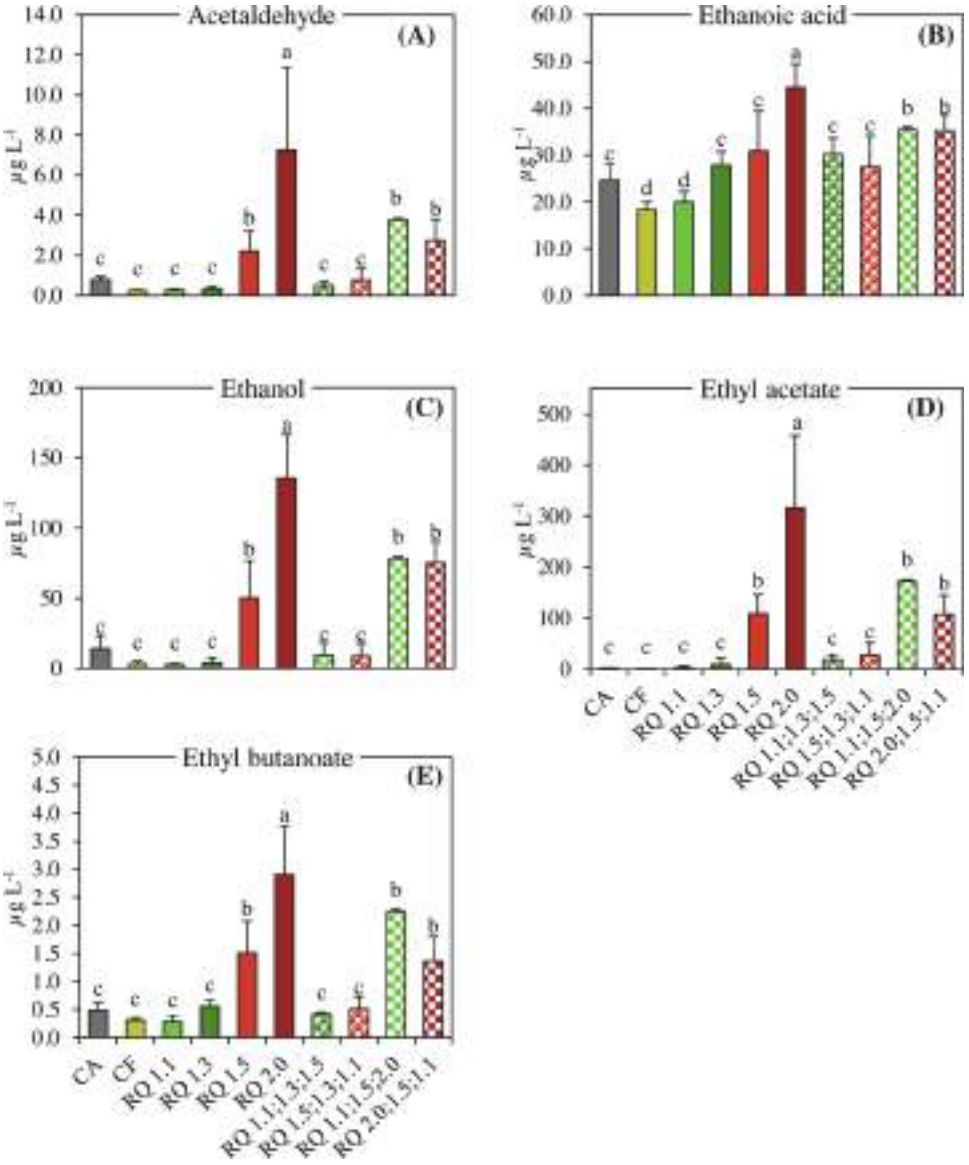



**Fig. 3.** Acetaldehyde (A), ethanoic acid (B), ethanol (C), ethyl acetate (D), and ethyl butanoate (E) of 'Galaxy' apples stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (CF) and on respiratory quotient (RQ) after 9 months of storage plus 7 d of shelf life at 20 °C. RQ X; Y; Z: means variation of RQ levels during storage; fruit remained in each RQ for 3 months. Bars with the same lowercase letter do not differ between CA and DCA conditions by the Scott-Knott test at 5% probability. Error bars mean standard deviation.

storage period (Fig. 1) and corroborates several studies (Both et al., 2017; Thewes et al., 2017b; Weber et al., 2020, 2017). Nonetheless, as demonstrated herein, a 3-month storage period of RQ 2.0 (DCA-RQ 1.1; 1.5; 2.0 and DCA-RQ 2.0; 1.5; 1.1) led to an anaerobic metabolism level similar to an entire storage period under DCA-RQ 1.5. Additionally, a 3-month storage period of RQ 1.5 (DCA-RQ 1.1; 1.3; 1.5 and DCA-RQ 1.5; 1.3; 1.1) did not induce higher anaerobic metabolism compared to CA. These findings show that fruit can metabolize a certain amount of anaerobic metabolism compounds, although it depends on when fruit are submitted to anaerobic metabolism. In fact, a recent study demonstrated that the capability of fruit to metabolize anaerobic metabolism compounds depends on the apple cultivar (Thewes et al., 2021a), albeit in the present study, it was also shown that it depends on the storage moment/period that fruit are submitted to anaerobic metabolism.

Ethyl acetate and ethyl butanoate are compounds that impact apple flavor, although ethyl acetate at high concentrations can be considered an off-flavor (Donadel et al., 2019; Wright et al., 2015). These volatiles accumulate more in fruit submitted to DCA-RQ 2.0, but the ethyl acetate concentration was far below the odor threshold (13,500 μg $kg^{-1}$) reported in the literature (López et al., 2007). Nevertheless, ethyl butanoate concentrations were above the odor threshold (1 μg $kg^{-1}$) when apples were submitted to DCA-RQ 1.5 and RQ 2.0 over three months or the whole storage period (Fig. 3E). These results evidenced that consumers would perceive its presence when apples were stored under these conditions, as this volatile compound (ethyl butanoate) confers a fruity flavor to apples (Echeverría et al., 2004; López et al., 2007; Mehinagic et al., 2006).

By analyzing anaerobic metabolites together, it is clear that the fruit accumulate significant levels of these compounds when submitted to RQ 1.5 and RQ 2.0 throughout the storage period or RQ 2.0 for three months (Fig. 3A–E). However, fruit can revert anaerobic metabolism compound accumulation when submitted to RQ 1.5 for three months or at RQ levels below 1.5. Additionally, the accumulation of anaerobic metabolism compounds directly depends on precursor availability (Fig. 3A–E), corroborating several literature studies (Donadel et al., 2019; Echeverría et al., 2004; Thewes et al., 2018).

### 3.3. Electrolyte leakage

One of the first symptoms of damage due to low oxygen is the loss of membrane selectivity, which can be attributed to low energy pool (Ho et al., 2013), anaerobic metabolism compound accumulation (Saquet et al., 2000), or fruit ripening (Thewes et al., 2021b). As reported herein, fruit stored under CA, DCA-RQ 2.0, DCA-RQ 1.5; 1.3; 1.1, and DCA-RQ 2.0; 1.5; 1.1 had higher electrolyte leakage (Fig. 4A), showing that fruit submitted to these storage treatments had cells with less selectivity, making the fruit more susceptible to physiological disorders. Nevertheless, the causes of higher electrolyte leakage under CA and DCA may vary, such as being due to fruit ripening under CA as a response to higher ethylene production (Fig. 2C). However, under DCA-RQ 2.0, DCA-RQ 1.5; 1.3; 1.1, and DCA-RQ 2.0; 1.5; 1.1, it is a damage caused by low oxygen, especially at the beginning of the storage period (first three months of storage) where the fruit are adapting to the extremely low oxygen partial pressures. This likely happened because fruit adaptation to low oxygen requires the expression of new genes, which takes some time to occur, and could be affected by oxygen rapidly decreasing at the beginning of storage. In avocados, Loulakakis et al. (2006) observed that the fruit induced a complex gene and protein expression pathway that promotes the adaptation of the fruit to low oxygen levels (<1.0 kPa), which requires time to occur.

### 3.4. Overall fruit quality as affected by DCA and RQ level variations

Fruit stored under DCA-RQ 2.0, throughout the entire storage period or the first three months, showed higher decay and pulp cracking incidence (Fig. 4B and C). These results evidenced that high anaerobic

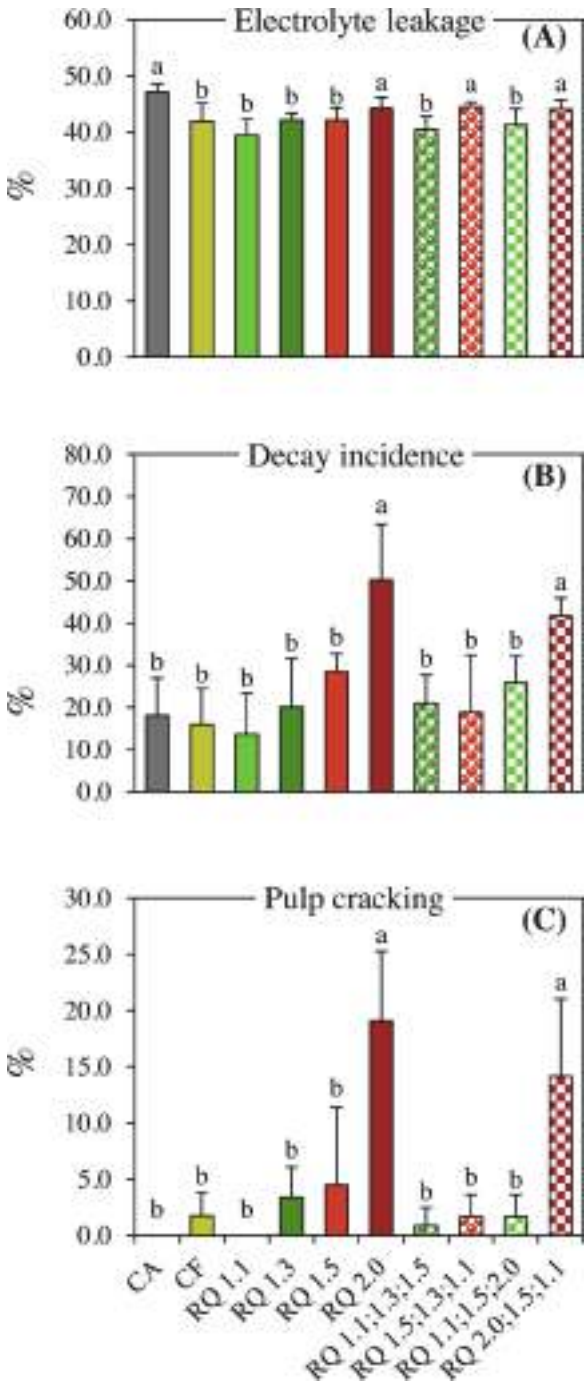



**Fig. 4.** Electrolyte leakage (A), decay incidence (B), and pulp cracking (C) of 'Galaxy' apples stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (CF) and on respiratory quotient (RQ) after 9 months of storage plus 7 d of shelf life at 20 °C. RQ X; Y; Z: means variation of RQ levels during storage; fruit remained in each RQ for 3 months. Bars with the same lowercase letter do not differ between CA and DCA conditions by the Scott-Knott test at 5 % probability. Error bars mean standard deviation.

metabolism during the whole storage period or the first three months reduces fruit defense against fungi. The high accumulation of anaerobic metabolism compounds likely damaged cells (Fig. 3), facilitating pathogen attacks and, consequently, increased decay incidence. In 'Galaxy' apples, storage under DCA-RQ 1.5 resulted in higher decay incidence, which the authors attributed to anaerobic metabolism damage (Both et al., 2018; Thewes et al., 2020a). External ethanol vapor application

(500 ppm) reduced the decay incidence of 'Nicoter' apples (Thewes et al., 2021b), showing an interaction between ethanol concentrations and apple cultivar. Nonetheless, the present study is the first to show that RQ 2.0 induced fruit cracking, and this is likely because of the low oxygen at the first three months of storage (Fig. 1), in which fruit are adapting to low oxygen (Thewes et al., 2017a); the cracks made fungal infection easier, increasing decay incidence.

The low oxygen could inhibit apple ripening because mealiness is one of the main ripening indexes of 'Gala' apples, and its incidence was the lowest in fruit stored under DCA-RQ, regardless of the RQ level or its variation over the storage period (Fig. 5A). Nonetheless, fruit stored under CA had the highest mealiness incidence (Fig. 5A), which is due to the high ethylene production (Fig. 2C), as ethylene is responsible for inducing cell-wall enzyme activity (Gwanpua et al., 2016, 2014; Payasi et al., 2009; Prasanna et al., 2007); these enzymes degraded the middle lamella and gave the mealy aspect of fruit stored under CA. By analyzing pulp cracking and mealiness incidence together, it is possible to observe higher pulp cracking incidence in fruit stored under DCA-RQ 2.0 and DCA-RQ 2.0; 1.5; 1.1 that is not due to fruit ripening, but because of damage induced by the low oxygen under these conditions (Figs. 4C and 5 A). Several studies have attributed higher pulp cracking to advanced fruit ripening (Brackmann et al., 2008; Weber et al., 2012), although this study has shown that it can also be induced by high anaerobic metabolism due to low oxygen levels (Figs. 1 and 3).

Generally, internal and/or external browning of fruit is the main symptom of low oxygen damage in apples (Thewes et al., 2021a,b; Weber et al., 2015). Flesh breakdown is characterized as pulp browning, and its incidence was higher when fruit were submitted to DCA-RQ 2.0 and DCA-RQ 2.0; 1.5; 1.1 compared to all other atmosphere conditions (Fig. 5B). This fact evidenced that flesh breakdown was a response to low oxygen damage of fruit stored under such extremely low oxygen conditions. Analysis of the effects of RQ level variation in the storage period revealed that a high RQ in the first three months of storage (RQ 1.5 and 2.0) leads to higher flesh breakdown than the same RQ levels at the last three months of storage (Fig. 5B). These results show that fruit are more susceptible to low oxygen damage at the beginning of the storage period, demonstrating that it is at this point in which the oxygen should be accurately supplied to avoid quality losses, as fruit are still not adapted to the extremely low oxygen conditions.

Flesh breakdown incidence of fruit stored under CA is due to the advanced ripening stage (Fig. 5C), which leads to higher electrolyte leakage (Fig. 4A), losing membrane selectivity, and, consequently, inducing internal browning. Some studies have attributed flesh breakdown incidence to advanced ripening (Thewes et al., 2017a), low-temperature damage (Anese et al., 2020; Both et al., 2018; Weber et al., 2019, 2015), and low energy availability (Ho et al., 2013; Saquet and Almeida, 2017; Saquet et al., 2000). However, under extremely low $pO_2$, flesh breakdown may result from anaerobic metabolism compound accumulation in a concentration that fruit cannot metabolize (Figs. 3 and 5B). Analyzing flesh breakdown and anaerobic metabolism compound accumulation (Figs. 3 and 5B) shows that it is in the last three months of storage that the fruit can be submitted to high RQ levels (1.5 and 2.0), thereby being capable of metabolizing and/or tolerating the anaerobic metabolism compounds and avoiding damage to fruit flesh. This may be a result of fruit adaptation to low oxygen partial pressures, producing lower amounts of anaerobic metabolism compounds. However, storage under DCA-CF, DCA-RQ 1.1, RQ 1.3, and RQ 1.5 over the entire storage period reduced flesh breakdown compared to CA, evidencing the beneficial effects of decreasing $O_2$ during storage, but at secure levels.

The higher decay incidence, pulp cracking, and flesh breakdown of fruit submitted to DCA-RQ 2.0 and DCA-RQ 2.0; 1.5; 1.1 resulted in fewer healthy fruit (Fig. 5C). Nonetheless, fruit stored under DCA-CF, DCA-RQ 1.1, DCA-RQ 1.3, and DCA-RQ 1.1; 1.3; 1.5 maintained a higher healthy fruit percentage because of the lower physiological disorders (Fig. 5C). When comparing RQ level variation over the storage period, fruit quality was better maintained when higher RQs were used at the end of the storage period. Several studies of the literature found higher healthy fruit amounts in storage under DCA-CF and DCA-RQ compared to CA (Anese et al., 2020; Schmidt et al., 2020; Thewes et al., 2020a, 2017a, 2017b; Thewes et al., 2021a,b; Weber et al., 2019, 2017). However, this study is the first to show that RQ level variation

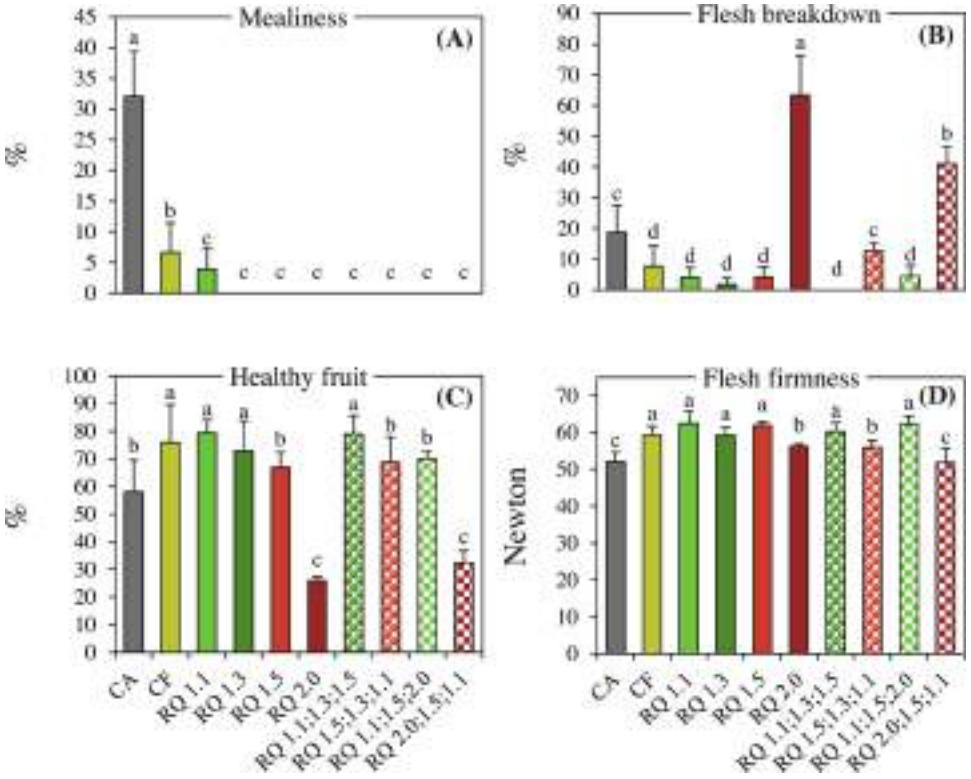



**Fig. 5.** Mealiness (A), flesh breakdown (B), healthy fruit (C), and flesh firmness (D) of 'Galaxy' apples stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (CF) and on respiratory quotient (RQ) after 9 months of storage plus 7 d of shelf life at 20 °C. RQ X; Y; Z: means variation of RQ levels during storage; fruit remained in each RQ for 3 months. Bars with the same lowercase letter do not differ between CA and DCA conditions by the Scott-Knott test at 5 % probability. Error bars mean standard deviation.

does not bring benefits to healthy fruit maintenance compared to static RQ levels in the entire storage period, especially compared to RQ 1.1 and 1.3 (Fig. 5C).

Healthy fruit amount is a critical quality parameter to know the number of fruit that can be commercially sold; flesh firmness is one of consumers' leading apple quality attributes (Bonany et al., 2013; Harker et al., 2008). Fruit stored under CA and DCA-RQ 2.0; 1.5; 1.1 had lower flesh firmness than all other storage conditions (Fig. 5D). The lower flesh firmness under these conditions is due to higher ethylene production under CA and higher IEC under DCA-RQ 2.0; 1.5; 1.1 (Fig. 2B and C), as cell wall degrading enzymes are started by ethylene (Gwanpua et al., 2016, 2014; Payasi et al., 2009; Prasanna et al., 2007). Nevertheless, fruit stored under DCA-CF, DCA-RQ 1.1, DCA-RQ 1.3, DCA-RQ 1.5, DCA-RQ 1.1; 1.3; 1.5, and DCA-RQ 1.1; 1.5; 2.0 maintained higher flesh firmness (Fig. 5D). Under these conditions, the higher flesh firmness is associated with lower overall metabolism (respiration rate) and ethylene production (Fig. 2C and D). A noteworthy fact is that fruit submitted to high RQ levels (1.5 and 2.0) in the first three months of storage had lower flesh firmness compared to fruit submitted to these high RQ levels at the last three months of storage (Fig. 5D). These findings emphasize that high anaerobic metabolism at the beginning of the storage period is detrimental for fruit quality maintenance; hence RQ levels above 1.3 are not recommended at the first three months of storage.

### 3.5. Main volatile compounds of 'Galaxy' apples

Several volatile compounds affect 'Gala' apple aroma, although some have a more significant impact on flavor, more notably, 2-methylbutyl acetate has the greatest impact on 'Galaxy' apple flavor (Both et al., 2014; Young et al., 1996). 2-Methylbutyl acetate concentrations were higher in fruit stored under CA, DCA-RQ 1.5, DCA-RQ 2.0, DCA-RQ 1.1; 1.5; 2.0, and DCA-RQ 2.0; 1.5; 1.1 compared to all other storage conditions (Fig. 6C). This is a result of higher precursor availability (Figs. 6A–B and 3B), corroborating other studies that attributed higher ester formation to precursor availability in extremely low oxygen (Both et al., 2017; Echeverría et al., 2004; Thewes et al., 2020a, 2017b, 2017c; Yang et al., 2016; Young et al., 1996), which are combined by AAT enzyme to form 2-methylbutyl acetate. Another study demonstrated that 'Galaxy' apples stored under DCA-RQ 1.5 had higher *MdAAT1* expression, explaining the higher ester accumulation (Thewes et al., 2020a). According to the literature, 2-methylbutyl acetate confers a fruity odor to apples (Komthong et al., 2006; Mehinagic et al., 2006; Niu et al., 2019). Another noteworthy fact is that storage under DCA-CF, DCA-RQ 1.1, and DCA-RQ 1.3 had low concentrations of this ester (Fig. 6C).

Furthermore, another volatile compound correlated to apple flavor is 1-butanol (Young et al., 1996), and its concentration was higher when fruit were stored under CA, DCA-RQ 1.5, DCA-RQ 2.0, DCA-RQ 1.5; 1.3; 1.1, and DCA-RQ 2.0; 1.5; 1.1 (Fig. 7A). The higher 1-butanol content under these conditions was combined with ethanoic acid (Fig. 3B) by the enzyme AAT forming butyl acetate, which is another important ester of 'Galaxy' apples (Fig. 7B). Once again, fruit stored under DCA-CF, DCA-RQ 1.1, DCA-RQ 1.3, and DCA-RQ 1.1; 1.3; 1.5 had the lowest concentrations of this ester (Fig. 7B). Recent studies also reported lower butyl acetate levels in 'Galaxy' apples stored under DCA-CF than CA and DCA-RQ 1.5 (Anese et al., 2020; Both et al., 2017; Thewes et al., 2017c). According to Thewes et al. (2020a), the lower ester biosynthesis of apples stored under DCA-CF is because of lower precursor availability and *MdAAT1* expression.

Together with 2-methylbutyl acetate and butyl acetate, hexyl acetate is a high-impact ester regarding apple flavor. Fruit stored under CA had higher hexyl acetate concentrations than all other storage conditions (Fig. 8C), which is associated with higher 1-hexanol concentrations (Fig. 8B). The higher hexyl acetate of fruit stored under CA is due to higher $pO_2$ (Fig. 1) and ethylene production (Fig. 2C), which is necessary for β-oxidation of fatty acid for its precursor production (Bangerth

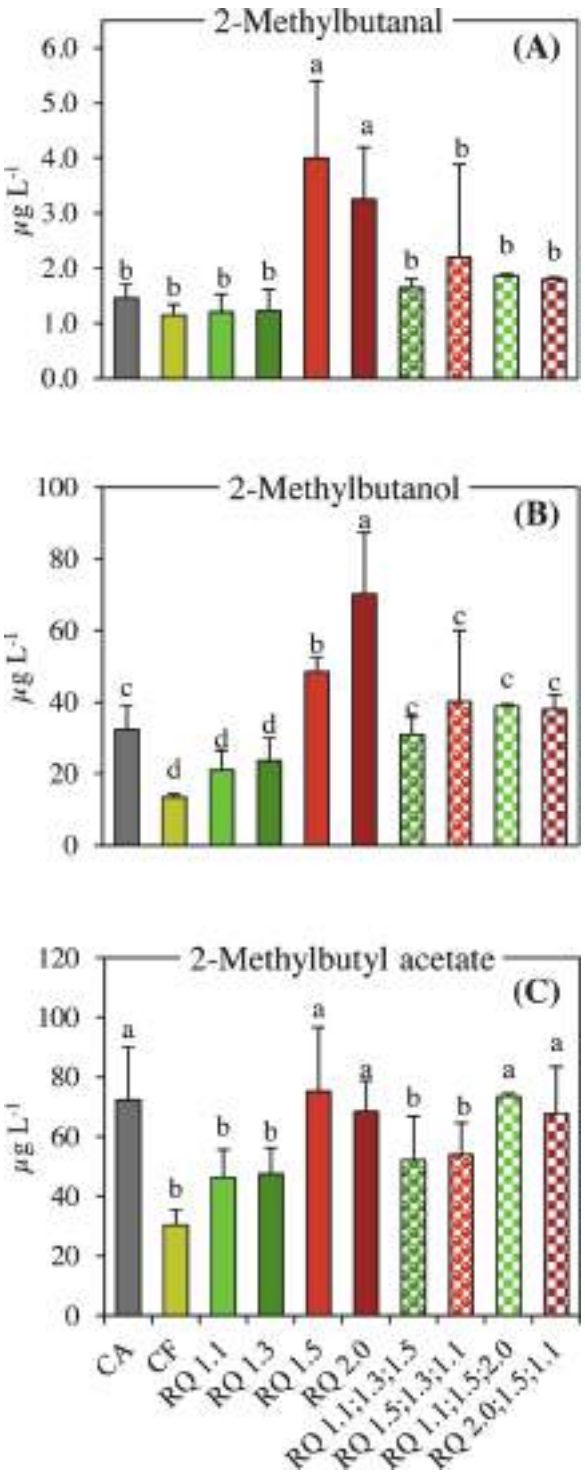



**Fig. 6.** 2-Methylbutanal (A), 2-methylbutanol (B), and 2-methylbutyl acetate (C) of 'Galaxy' apples stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (CF) and on respiratory quotient (RQ) after 9 months of storage plus 7 d of shelf life at 20 °C. RQ X; Y; Z: means variation of RQ levels during storage; fruit remained in each RQ for 3 months. Bars with the same lowercase letter do not differ between CA and DCA conditions by the Scott-Knott test at 5 % probability. Error bars mean standard deviation.

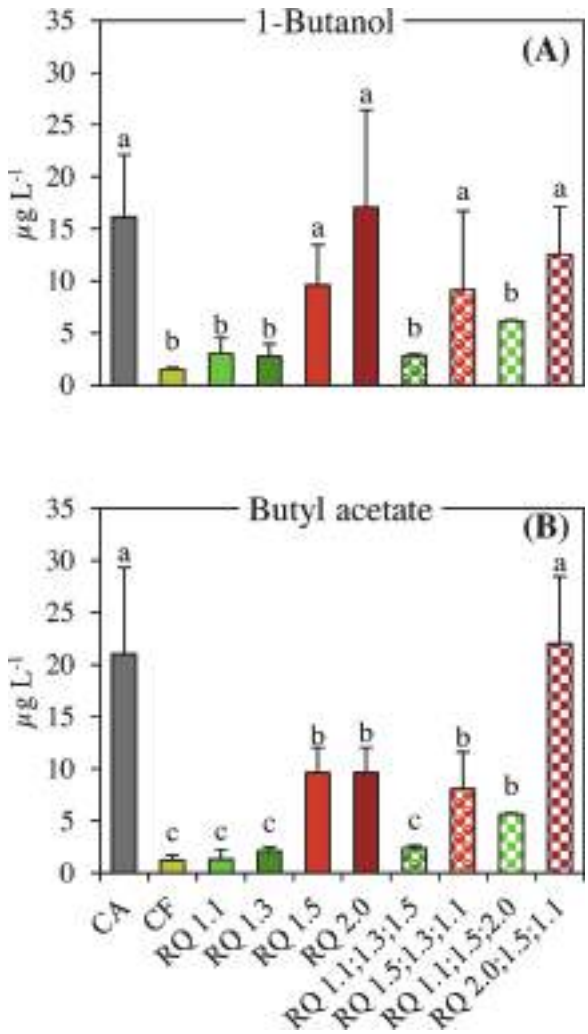



**Fig. 7.** 1-Butanol (A) and butyl acetate (B) of 'Galaxy' apples stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (CF) and on respiratory quotient (RQ) after 9 months of storage plus 7 d of shelf life at 20 °C. RQ X; Y; Z: means variation of RQ levels during storage; fruit remained in each RQ for 3 months. Bars with the same lowercase letter do not differ between CA and DCA conditions by the Scott-Knott test at 5% probability. Error bars mean standard deviation.

et al., 2012; Both et al., 2014; Brackmann et al., 1993; Song and Bangerth, 1996; Yang et al., 2016). When analyzing RQ level variations over the storage period, no benefit was observed compared to fixed RQ on hexyl acetate accumulation (Fig. 8C).

### 3.6. Joint analysis of the overall quality and volatile compounds by PCA

The PCA was performed to obtain an overview of the results and their relationships (Fig. 9). Principal components one (PC1) and two (PC2) explained 69.8 % of the overall variable variance (Fig. 9). The PC1 separated fruit submitted to high RQ levels (RQ 1.5 and RQ 2.0) throughout the storage period or first three months of storage from fruit submitted to DCA-CF, DCA-RQ 1.1, DCA-RQ 1.3, DCA-RQ 1.1; 1.3; 1.5, and DCA-RQ 1.1; 1.5; 2.0 (Fig. 9A). This treatment aggrupation was due to the higher accumulation of anaerobic metabolism compounds, decay incidence, and pulp cracking of fruit submitted to high RQ levels (RQ 1.5 and 2.0) (Fig. 9B). Associated to fruit stored under DCA-CF, DCA-RQ 1.1, DCA-RQ 1.3, DCA-RQ 1.1; 1.3; 1.5, and DCA-RQ 1.1; 1.5; 2.0 are higher flesh firmness and healthy fruit amount (Fig. 9A and B).

Together with the PC2, fruit stored under CA were separated from all

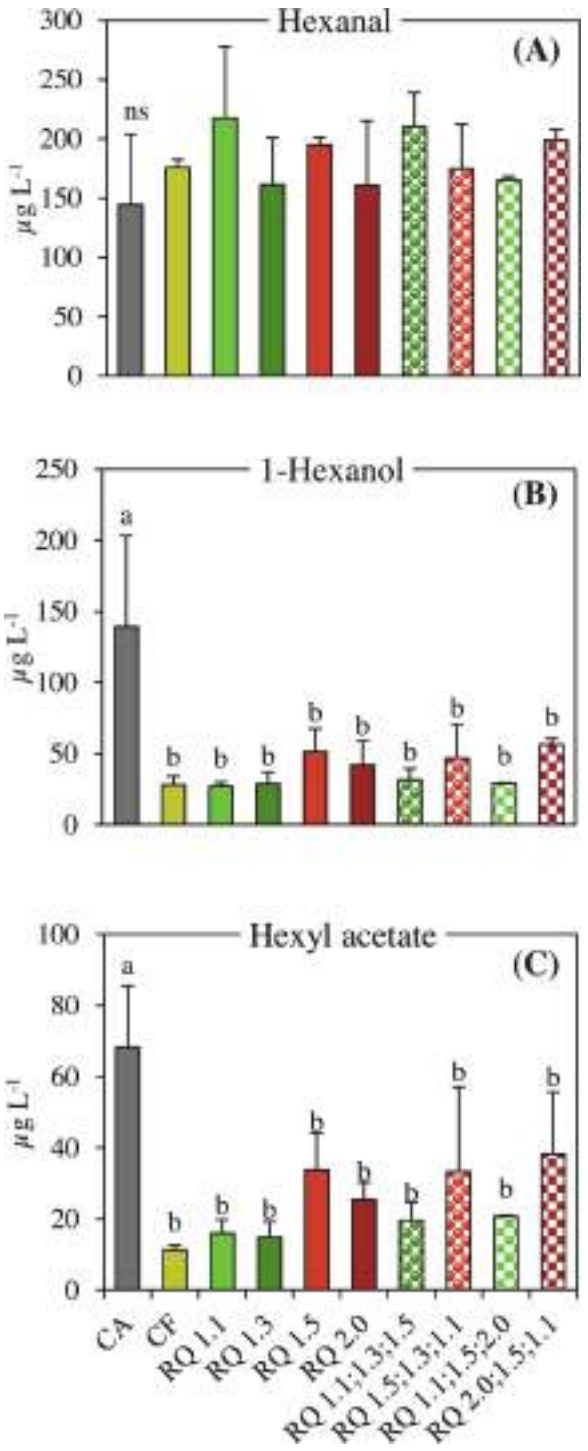



**Fig. 8.** Hexanal (A), 1-hexanol (B), and hexyl acetate (C) of 'Galaxy' apples stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (CF) and on respiratory quotient (RQ) after 9 months of storage plus 7 d of shelf life at 20 °C. RQ X; Y; Z: means variation of RQ levels during storage; fruit remained in each RQ for 3 months. Bars with the same lowercase letter do not differ between CA and DCA conditions by the Scott-Knott test at 5% probability. Error bars mean standard deviation.

DCA conditions, regardless of the RQ level used or its variation in the storage period (Fig. 9A). Correlated to fruit stored under CA are higher ethylene production, IEC, respiration rate, mealiness, and some important volatile compounds, including butyl acetate, hexyl acetate, 1-hexanol, and 1-butanol (Fig. 9B). However, when we analyze the main volatile compounds and overall quality, the storage conditions that

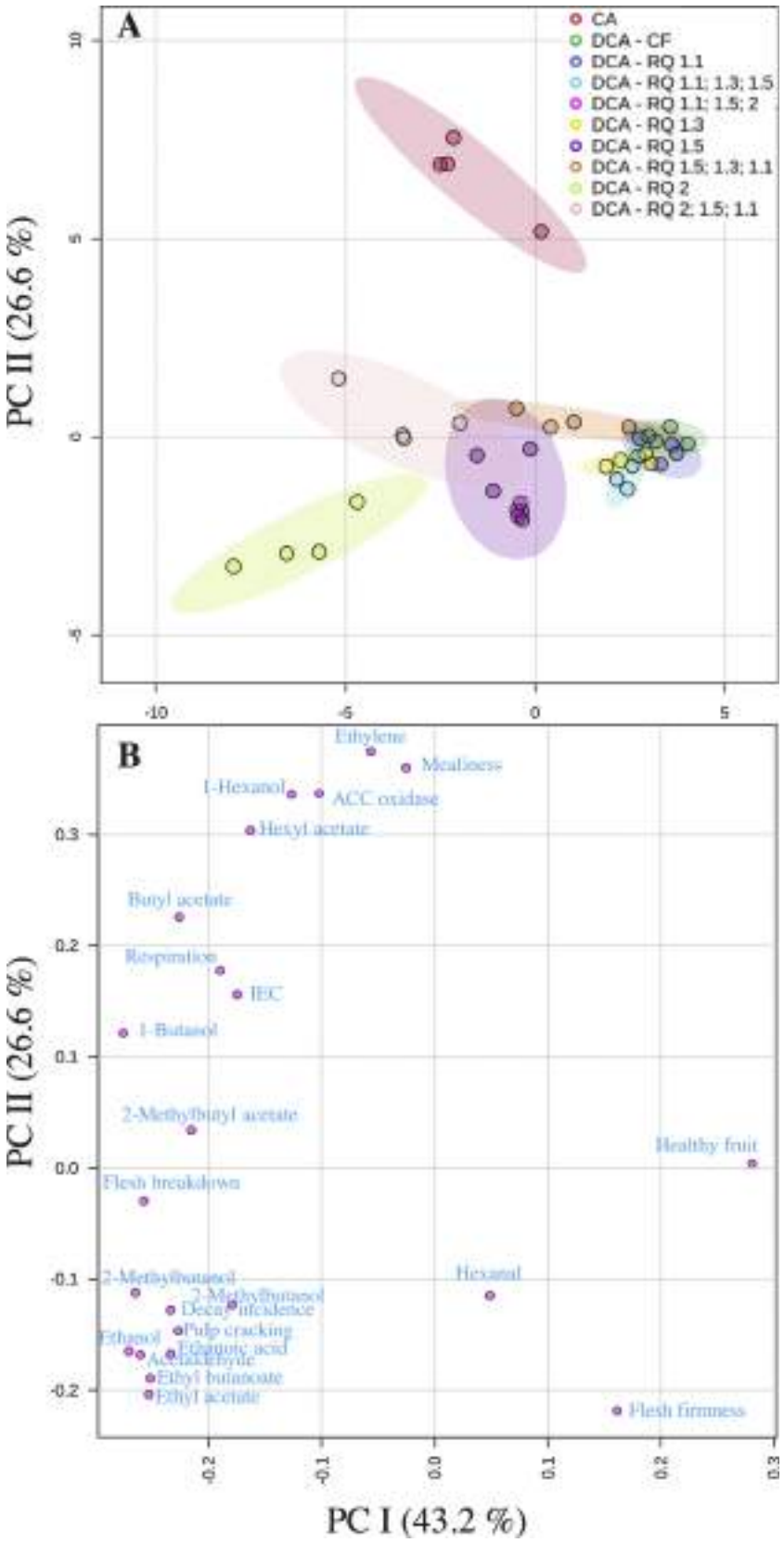



**Fig. 9.** Principal component analysis showing the score plot (A) and loadings of the variables (B) of 'Galaxy' apples stored under controlled atmosphere (CA) and dynamic controlled atmosphere based on chlorophyll fluorescence (CF) and on respiratory quotient (RQ) after 9 months of storage plus 7 d of shelf life at 20 °C. RQ X; Y; Z: means variation of RQ levels during storage; fruit remained in each RQ for 3 months. Shaded areas display 95 % confidence regions.

provide the best overall quality have the lowest ester accumulation, which highlights that even with RQ variation during the storage period, it was not possible to maintain the highest overall quality and induce high ester synthesis (Fig. 9A and B). These results evidence that the volatile compound induction due to anaerobic metabolism only occurs in RQ levels above 1.5, although in these RQs, the overall fruit quality can be reduced due to low oxygen damage such as flesh breakdown.

## 4. Conclusions

'Galaxy' apple storage under DCA with RQ 1.5 and 2.0 for nine months or the first three months of storage induces higher levels of anaerobic metabolism, which is detrimental to the overall fruit quality because of the higher flesh breakdown incidence and lower healthy fruit and flesh firmness maintenance. This shows that the critical period during storage for low oxygen damage is the first three months of storage, which is when RQ levels above 1.3 should be avoided.

Overall fruit quality is better maintained when fruit were stored under DCA-CF, DCA-RQ 1.1, DCA-RQ 1.3, and DCA-RQ 1.1; 1.3; 1.5 due to higher healthy fruit, flesh firmness, and lower physiological disorders, although the main ester accumulation is reduced under these storage conditions. 2-Methylbutyl acetate and butyl acetate increased in fruit stored under CA and high RQ levels, either throughout the storage period or the first three months of storage. This demonstrates that, even with RQ level variation over the storage period, it was not possible to obtain apples with the highest overall quality and high ester accumulation under the same atmosphere condition. Pulp cracking is induced by storage under DCA-RQ 2.0 and DCA-RQ 2.0; 1.5; 1.1, and this is likely a result of higher anaerobic metabolism compound accumulation.

## Authorship contribution statement

**Conceptualization, Methodology, Formal analysis, Data curation and Visualization:** Fabio Rodrigo Thewes, Auri Brackmann, Vanderlei Both, Rogerio de Oliveira Anese, Vagner Ludwig, Lucas MallmannWendt, Magno Roberto Pasquetti Berghetti, Flavio Roberto Thewes, Bruna Klein, Roger Wagner. **Writing - original draft:** Fabio Rodrigo Thewes. **Writing - review and editing:** Rogerio de Oliveira Anese, Flávio Roberto Thewes, Vagner Ludwig, Roger Wagner, Auri Brackmann and Vanderlei Both. **Funding acquisition, Project administration, Resources, Supervision:** Fabio Rodrigo Thewes, Auri Brackmann and Vanderlei Both.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.postharvbio.2021.111699.

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# Microencapsulated Lemongrass (*Cymbopogon flexuosus*) Essential Oil Supplementation on Quality and Stability of Silver Catfish Fillets during Frozen Storage

Cristine Rampelotto[a], Caroline S. Speroni[a], Lisiane Conte[a], Dirleise Pianesso[b], Isadora S. Machado[a], Renata F. Rodrigues[a], Natália M. Minuzzi[a], Taida J. Adorian[b], Bruna Klein[a], Roger Wagner[a], Bernardo Baldisserotto[c], Leila P. da Silva[b], Berta M. Heinzmann[d], Cristiano R. de Menezes[a], and Tatiana Emanuelli[a]

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**ABSTRACT**

There is growing interest for using essential oils (EOs) in fish feed as they can improve fish health, growth, and productive yield. But, there is little information concerning their effects on fish flesh quality and whether EO residues can be found in flesh. The EO of lemongrass was recently demonstrated to increase the productive yield of silver catfish. Thus, the aim of this study was to evaluate the impact of lemongrass EO supplementation on physico-chemical properties, sensory acceptance, and stability during frozen storage of fish fillets. Silver catfish (*Rhamdia quelen*) were fed diets containing 0, 1, or 3 mL/kg of EO for 20 days. Fish were slaughtered, and fillets were stored at −20°C for 12 months and evaluated every 90 days. Dietary supplementation with EO did not affect proximate composition, fatty acid composition, or sensory acceptance of fish fillets. EO constituents (especially citral) were not detected in fish fillets. EO supplementation at 1 mL/kg appears to be more suitable to maintain physico-chemical changes similar to control during frozen storage. These data support the safety of lemongrass EO for use in aquaculture for human consumption purposes.

**KEYWORDS**

*Rhamdia quelen*; citral; lipid oxidation; protein oxidation; color

## Introduction

There is growing interest in the use of plant essential oils (EOs) as anesthetics for fish handling (Souza et al. 2019), fish transportation (Becker et al. 2016; Daniel et al. 2016, 2014; Veeck et al. 2013b), or fish stunning/slaughtering (Veit et al. 2017). In addition, numerous studies propose the use of EOs as additives in fish diet to improve fish productive performance and promote fish health (Sutili et al. 2018), as they have been demonstrated to be able to improve immune response and modulate gut microbiota (Sutili et al. 2019). Dietary supplementation with EO has been shown to promote fish growth (Acar et al. 2015; Zeppenfeld et al. 2016), improve fish resistance against pathogenic micro-organisms (Baba et al. 2016) and parasites (Jensen et al. 2015), as well as their antioxidant status (Diler et al. 2017; Morselli et al. 2020; Saccol et al. 2013; Zheng et al. 2009). The use of EOs in pre-slaughter procedures, however, raises concern as to the presence of EO residues in fish flesh.

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Lemongrass belongs to the *Cymbopogon* genus that includes some grass species with special interest for EO production (Shah et al. 2011). *Cymbopogon flexuosus* and *Cymbopogon citratus* are widely used in folk medicine due to their citral-rich EO (Avoseh et al. 2015). Both species have been shown to improve animal productive performance. *C. citratus* EO has been patented as a growth promoter in pigs (Duarte et al. 2014), whereas *C. flexuosus* EO has been patented as a growth stimulant for fish (Baldisserotto et al. 2015). Moreover, we have recently demonstrated that supplementation of silver catfish (*Rhamdia quelen*) with microencapsulated *C. flexuosus* EO (1 mL EO/kg diet) increases protein deposition in the carcass and reduces gonadossomatic index, improving carcass yield (Rampelotto et al. 2018) with no damage to fish reproductive parameters (Souza et al. 2018). However, studies evaluating the impact of dietary EO on flesh quality are still scarce. Thyme EO and its components have been the most studied and have had their dietary effects characterized on physico-chemical, microbiological, and sensory qualities of fish flesh, demonstrating the potential to reduce lipid oxidation and to extend the shelf life of refrigerated fish (Álvarez et al. 2012; Giannenas et al. 2012; Hernández et al. 2015). However, there is some controversial data on this topic. Seabream (*Sparus aurata*) fed with carvacrol-rich thyme EO had lower lipid oxidation at 14 days of refrigerated storage (Álvarez et al. 2012; Hernández et al. 2015), whereas the same dose of thymol-rich thyme EO had no effect (Álvarez et al. 2012). Conversely, in rainbow trout (*Oncorhynchus mykiss*), dietary supplementation with carvacrol had no effect on lipid oxidation, whereas thymol reduced lipid oxidation after 5 days of storage at 4°C (Giannenas et al. 2012). Although lemongrass EO directly applied to processed seafood products has been shown to delay microbiological deterioration and lipid oxidation (Ahmad and Viljoen 2015; Masniyom et al. 2012), the effect of dietary supplementation with lemongrass EO on the quality of fish flesh has not been explored.

Silver catfish (*Rhamdia quelen*) is a freshwater fish that plays an important role for aquaculture in subtropical areas of Argentina, Uruguay, and Brazil (Valladão et al. 2016). It is well adapted to a wide range of temperatures, including the low winter water temperatures (Garcia et al. 2008).

The objective of this study was to evaluate the effect of dietary supplementation with microencapsulated *C. flexuosus* EO on the quality and stability of catfish fillets during frozen storage and to determine whether residues of EO constituents can be found in fish fillets. The supplementation was conducted during the termination stage, which is the final stage of growth for commercial slaughter.

## Material and methods

### Essential oil

The EO of *C. flexuosus* used in this study was supplied by Bio Natural Essenciais (Três Passos, Rio Grande do Sul, Brazil). To prevent degradation of EO, it was microencapsulated by complex coacervation using Arabic gum and gelatin (1:1, w/w) as wall material (Alvim and Grosso 2010), followed by lyophilization. The amount of EO contained in the microcapsules (about 365 mL/kg) was assessed by hydrodistillation using a Clevenger apparatus.

The chemical composition of essential oil (Table 1) was assessed using an Agilent 6890 gas chromatograph (GC) coupled to a mass spectrometry detector (MS) Agilent 5973 with a HP5-MS column (5% phenyl, 95% methylsiloxane, 30 m × 0.25 mm ID × 0.25 μm) (Garlet et al. 2016). The EO constituents were identified by comparison of the retention index and mass spectra with databases. EO constituents were quantified by gas chromatography with flame ionization detection (FID) on an Agilent 7890A GC (Figure 1a) and expressed as percentage based on peak area normalization.

### Diets

A basal (control) diet containing 34% of protein, 9% of fat, and 1345 kJ/100 g was formulated (Table 2) and supplemented with 1 or 3 mL/kg of *C. flexuosus* EO. The ingredients were manually homogenized, pelletized (6 mm), and then dried at 45°C in an air flow oven for 24 h. After drying, diets were stored at −18°C.

**Table 1.** Composition of the essential oil of *Cymbopogon flexuosus*.

| Compound | Experimental RI* | Literature RI[a,b] | Percent composition |
|---|---|---|---|
| Camphene | 946 | 946 | 0.65 |
| 6-Methyl-5-heptene-2-one | 988 | 984 | 0.61 |
| Limonene | 1028 | 1030 | 0.36 |
| Citronellal | 1153 | 1153 | 1.39 |
| *Z*-Verbenol | 1183 | 1188 | 2.35 |
| Citronellol | 1229 | 1228 | 1.97 |
| Neral | 1242 | 1242 | 32.11 |
| *Z*-Geraniol | 1255 | 1259 | 1.97 |
| Geranial | 1272 | 1270 | 45.68 |
| Neryl acetate | 1384 | 1381 | 0.87 |
| Caryophyllene | 1423 | 1418 | 1.68 |
| γ-Cadinene | 1517 | 1517 | 1.47 |
| δ-Cadinene | 1526 | 1528 | 0.25 |
| Elemol | 1552 | 1549 | 0.81 |
| Caryophyllene oxide | 1587 | 1582 | 1.01 |
| Identified compounds | | | 93.2 |

* RI = Retention index; [a]Adams (2009); [b]NIST (2010).

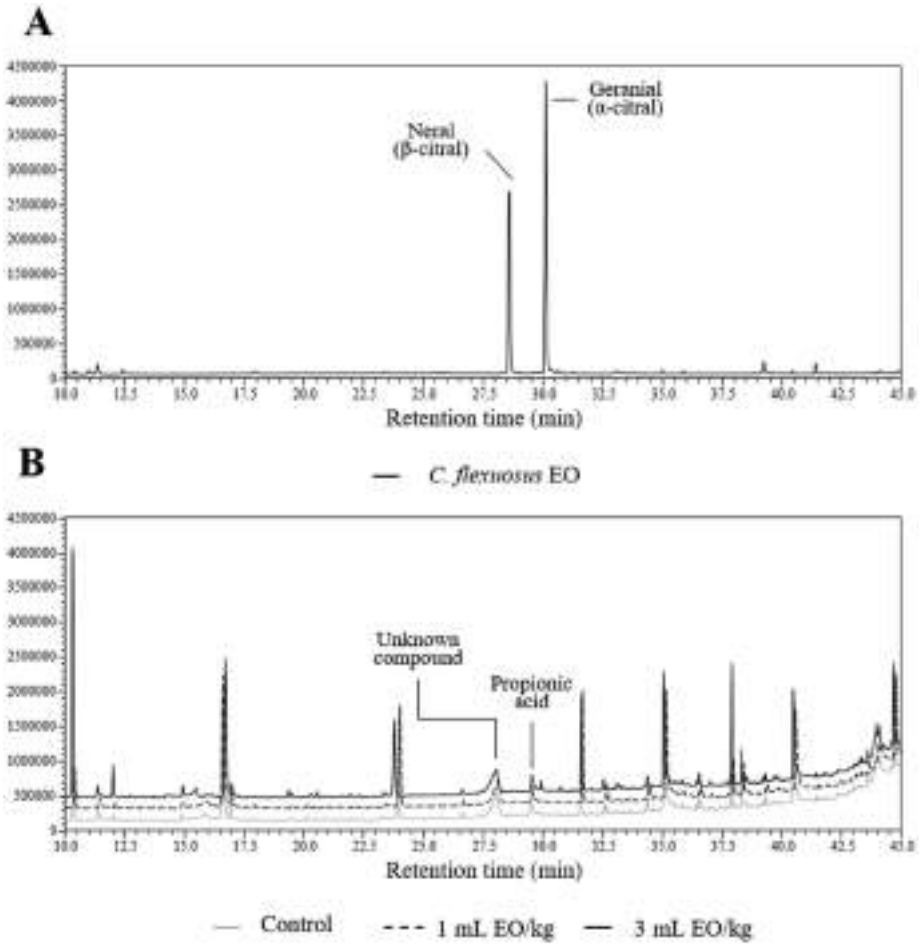



**Figure 1.** Representative chromatograms of volatile compounds of essential oil (EO) of *C. flexuosus* (a) and fillets from fish fed with diet supplemented with different levels of EO (b).

**Table 2.** Ingredients of diets.

| | Diets | | |
|---|---|---|---|
| Ingredients (g/kg) | Control | 1 mL EO/kg diet | 3 mL EO/kg diet |
| Meat and bone meal | 350 | 350 | 350 |
| Soybean meal | 300 | 300 | 300 |
| Broken corn | 150 | 150 | 150 |
| Rice bran | 120 | 120 | 120 |
| Canola oil | 30 | 30 | 30 |
| Vitamin and mineral premix | 30 | 30 | 30 |
| Sodium chloride | 10 | 10 | 10 |
| Dicalcium phosphate | 10 | 10 | 10 |
| Microcapsules of EO* | 0 | 2.74 | 8.22 |

*Containing 365 mL of *C. flexuosus* EO/kg of microcapsules.

### Fish treatment

Silver catfish (*Rhamdia quelen*) from both sexes (initial weight approximately 400 g; initial length approximately 35 cm) were obtained from a local fish farm (Santa Maria-RS, Brazil) and transported to the Laboratory of Fisheries at Federal University of Santa Maria in oxygenated water tanks. Fish were weighed and randomized into 21 tanks (270 L) with six fish per tank. Tanks were connected to a water recirculating system with two biological filters, an activated carbon filter, and a 2000-L water reservoir equipped with heating system. During the experimental period, water was maintained as follows: 21.3 ± 0.3°C, 8.5 ± 0.5 mg of dissolved oxygen/L; 0.32 ± 0.03 mg of $NH_3$/L and 0.17 ± 0.03 mg/L of $NO_2$.

Animals were fed the experimental diets twice a day (8:00 a.m. and 4:00 p.m.) to apparent satiation for 20 days. Apparent consumption of diet per tank was registered daily and used to calculate the amount of EO consumed/kg of fish/day. At the end of the experimental period, fish were fasted for 24 h and euthanized by spinal cord section. Fish were eviscerated, the skin was removed with pliers, and the fillet was removed with a cut perpendicular to the spine from the gills to the caudal fin. The fillets without bones and without skin were frozen at −18°C. All procedures were approved by the Ethical Committee on Animal Use from Federal University of Santa Maria (protocol number 120/2014).

### Proximate composition

Proximate composition was evaluated in four different fish fillets per treatment. Moisture content was obtained after oven-drying at 105°C until a constant weight (AOAC 2005). Ash content was determined at 550°C (AOAC 2005). Crude protein was determined by the micro-Kjeldahl method (N × 6.25) (AOAC 2005). Fat content was gravimetrically determined (Bligh and Dyer 1959).

### Fatty acid composition

Fat was extracted from fish fillets (Bligh and Dyer 1959), esterified (Hartman and Lago 1973), and then, fatty acid methyl esters were extracted with hexane and determined using an Agilent Technologies HP 6890 GC/FID with a capillary column (DB-23, 60 m × 0.25 mm ID × 0.25 μm, Agilent Technologies). The injection and detection temperature was 250°C, and nitrogen was used as carrier gas (0.3 mL/min). After the injection (1 μL, split ratio 50:1), column temperature was kept at 160°C for 4 min. Thereafter, the temperature was raised to 240°C at a rate of 4°C/min and maintained for 8 min. Fatty acid methyl ester standards (FAME Mix 37, Sigma) were analyzed under the same conditions, and their retention times were used for identification of fatty acids. The content of each fatty acid was expressed as percentage of total fatty acids identified.

### Sensory evaluation of cooked fish fillets

The acceptability attributes of cooked fillets were evaluated by a panel of 41 untrained judges recruited from the Federal University of Santa Maria. Sensory evaluation was conducted in individual booths under cool white fluorescent light. Samples were cooked individually in a microwave oven (20 g/1 min), without salt, wrapped in greaseproof paper, coded, and randomly offered to the judges in white plastic plates. Room temperature tap water and crackers were offered to the judges to clean the palate between samples. Panelists judged color, odor, taste, and texture of samples using a nine-point hedonic scale (1 – greatly dislike; 9 – greatly like) (Meilgaard et al. 2006). The protocol for this study was approved by the Research Ethics Committee of Federal University of Santa Maria (CAAE 36317414.3.0000.5346).

### Volatile compounds in fish fillets

Volatile compounds of fish fillets were isolated by solid-phase microextration (SPME) using a fiber polar phase sorbent (divinylbenzene/carboxen/polydimethylsiloxane, Supelco, 50/30 μm). Fillets were ground, and a 5-g sample was placed into a 20-mL vial that was sealed with PTFE septum and heated to 35°C for 15 min to release volatile compounds. Thereafter, volatile compounds were extracted at 35°C by exposing the fiber in the headspace of the sample for 45 min. Three fillets for each treatment were examined in each point of analysis (Zepka et al. 2013).

Analysis was conducted in a GC/MS QP-2010 Plus (Shimadzu Corporation, Kyoto, Japan). SPME fiber volatile compounds were thermally desorbed in a split/splitless injector operating in splitless mode for 1 min (split ratio 30:1) at 250°C. The carrier gas was nitrogen at a constant pressure of 30 psi. Compounds were separated using a capillary column of fused silica polar phase, CP-WAX 52 CB (Varian Chrompack, Palo Alto, CA, USA; 50 m × 0.25 mm ID x 0.25 μm). Oven temperature was set to 35°C for 2 min, then heated to 80°C at 2°C/min, then 150°C at 4°C/min, and then 230°C at 8°C/min. The interface between the GC and MS, as well as the ionization source, were maintained at 230°C. The quadrupole mass analyzer was operated in scan mode monitoring the masses 35–350 u.m.a. The identification of α-citral (geranial) and β-citral (neral) were done by comparing the experimental mass spectra and linear retention indices with authentic standards. The individual volatile compounds were quantified based on the intensity of their peak areas.

### Fillet quality during frozen storage

Immediately after filleting, fillets were packed in polystyrene trays, covered with polyvinyl chloride film, and stored at −18°C for 12 months. Instrumental color, instrumental texture, pH, lipid oxidation, and protein oxidation were evaluated every three months in fillets from four independent fish per treatment at each time point, totaling 60 fillets.

#### Instrumental color

Fillet color was assessed on the visceral side of fillets using a Minolta® CM-700d (Konica Minolta Sensing Americas Inc., Ramsey, NJ, USA), with a standard illuminant D65 and 2° observation angle. Reflectance measurement mode was used, and specular component was included (SCI). The measurement was of 3 mm (SAV mask).

Four fillets per treatment were evaluated at each storage point. Four color measurements were performed directly on each sample in distinct points along the fillet. The average of the four measurements represented the value of each repetition. The CIELAB coordinates L*, a*, and b* were obtained and used to calculate chroma (C*), hue angle (h*), and the numerical total color difference (ΔE) between fish fillets at 0 and 12 months as follows:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2} \quad (1)$$

$$h^* = \tan^{-1}\left\{\frac{a^*}{b^*}\right\} \quad (2)$$

$$\Delta E_{0-12} = \left[(L^*_{12} - L^*_0)^2 + (a^*_{12} - a^*_0)^2 + (b^*_{12} - b^*_0)^2\right]^{1/2} \quad (3)$$

### pH and water holding capacity

Flesh pH was measured on homogenates of sample and distilled water (1:10; w/v) using a Bel W3B (Bel Engineering, Monza, Italy) pH-meter. The water-holding capacity (WHC) was determined by centrifuging 2.5 g of grounded samples at 3500 × *g* for 15 min in 50 mL tubes. After centrifugation, the supernatant was discarded and samples were weighed again. WHC was expressed as the percentage of water retained in sample using the sample moisture as a reference.

### Instrumental texture

Instrumental texture was determined in a TA-XT.plus Texture Analyzer equipped with Texture Expert Exponent Software (Stable Microsystems, Surrey, England) and a 36-mm cylindrical probe. Fish fillets were fractionated into 10 × 10 mm portions. Texture measurement was performed by compressing samples to 60% of their original height in two consecutive compression trials at a constant rate of 1 mm/s with a 15 s interval between trials. Pre-test, post-test, and head speed were set at 1 mm/s, 5.0 mm/s, and 5.0 mm/s, respectively. Data of compressions were used to calculate hardness, springiness, cohesiveness, and chewiness of samples (Bourne 1978).

### Lipid oxidation

The lipid oxidation of fillets was assessed by monitoring the levels of conjugated dienes (CD), peroxide value (PV), and formation of hexanal. CD and PV were evaluated in the fat extracted with chloroform and methanol according to Bligh and Dyer (1959). Fat was diluted in cyclohexane, and its optical density at 233 nm was used to evaluate CD value (Recknagel and Glende 1984). PV was determined using the ferric thiocyanate method at 500 nm (Chapman and Mackay 1949), which is based on the ability of peroxides to oxidize ferrous ions to ferric ions.

Hexanal was isolated from fish fillets and analyzed as described for the analysis of volatile compounds in fish fillets, except that the identification of hexanal was done by comparing the experimental mass spectra and linear retention indices with the literature. The hexanal content was quantified based on its peak area.

### Protein oxidation

Protein oxidation of fillets was assessed by determining total reduced sulfhydryl groups (SH) and protein carbonyl (PC) content. Fillets were homogenized with phosphate buffered saline (1:4, w/v) for both determinations. SH content was determined at 412 nm after reaction with 5,5′-dithio-bis -(2-nitrobenzoic acid) (Ellman 1959). Cysteine was used to construct the standard curve. PC content was determined at 370 nm using 2,4-dinitrophenylhydrazine (Levine et al. 1990) and normalized to the protein content. Total protein was determined at 625 nm after reaction with Folin Ciocalteu and using bovine serum albumin as standard (Lowry et al. 1951).

### Statistical analysis

Proximate composition, fatty acid and volatile profile, color, and fish quality storage data were obtained from fillets of four independent fish per treatment, which were separately analyzed (n = 4). Levene's test was performed to check the homogeneity of variances for all data. Flesh composition,

fatty acids profile, and total color difference data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's test. Data from sensory analysis was analyzed by the non-parametric Kruskal–Wallis ANOVA.

The data obtained from fillets during storage was analyzed by a two-way ANOVA (diet treatment x storage time) followed by Tukey's test. Significance level was set at $p < .05$. Pearson's test was used to evaluate the correlation between variables assessed during storage. All data were analyzed using the package Statistica 9.0 (Copyright Stat Soft, Inc., Tulsa, OK, USA).

## Results

### Fillet composition and sensory analysis

Fish diet supplementation at 1 and 3 mL of *C. flexuosus* EO/kg diet yielded an average daily intake of 8 and 28 µL of *C. flexuous* EO/kg body weight, respectively. No significant difference in fillet proximate composition (Table 3) was observed among treatments. Fish fillets had an average composition of 76.9% moisture, 18.5% protein, 3.5% fat, and 1.2% ash. Dietary supplementation with EO did not modify the fatty acid composition of fillets (Table 4). The most abundant fatty acids in the fillets were oleic acid (C18:1n9), palmitic acid (C16:0), and linoleic acid (C18:2n6) (>10%). Monounsaturated (MUFA), polyunsaturated (PUFA), and saturated (SFA) fatty acids amounted to 45, 23, and 32%, respectively, of total identified fatty acids of fillets.

The sensory acceptance (Table 3) of fish fillets was similar for all treatments, showing no significant differences among groups by the Kruskal–Wallis ANOVA. Fillet texture was rated at 8 points in the hedonic scale (like very much), whereas color, odor, and taste were rated at 7 points (like moderately). Accordingly, no traces of geranial or neral, which are the major constituents of *C. flexuosus* EO, were detected in fish fillets (Figure 1b).

### Fillet stability during frozen storage

ANOVA demonstrated an interaction between time and treatment for L*, b*, and C* color coordinates ($p < .05$), whereas a* and h* values were not affected by diet or storage time (Table 5). At the beginning of storage, fillets from fish that received 3 mL EO/kg diet exhibited lower L* values than the other treatments. During storage, fillets from control and 1 mL EO/kg diet showed constant L* values, while those from fish that received 3 mL EO/kg diet had a linear increase in luminosity during the storage ($p < .05$).

**Table 3.** Proximate composition and sensory acceptance of fillets from silver catfish fed with diet supplemented with different levels of essential oil (EO) of *Cymbopogon flexuosus*.

| | Treatment | | |
|---|---|---|---|
| *Proximate composition (%)* | Control | 1 mL EO/kg diet | 3 mL EO/kg diet |
| Moisture | 77.2 ± 0.7 | 76.0 ± 1.3 | 77.5 ± 0.6 |
| Protein | 18.1 ± 0.8 | 18.0 ± 0.5 | 19.4 ± 0.2 |
| Fat | 3.4 ± 0.7 | 3.8 ± 0.8 | 3.2 ± 0.5 |
| Ash | 1.12 ± 0.07 | 1.19 ± 0.03 | 1.21 ± 0.05 |
| *Sensory acceptance* | | | |
| Texture | 8 (7–8) | 8 (7–8) | 8 (7–8) |
| Color | 7 (6–8) | 7 (6–8) | 7 (6–8) |
| Odor | 7 (5–8) | 7 (6–8) | 7 (6–8) |
| Taste | 7 (6–8) | 7 (7–8) | 7 (6–8) |

Proximate composition data are expressed as mean ± SE (n = 4). Sensory acceptance was evaluated using a hedonic scale (1 – greatly dislike and 9 – greatly like) and presented as the median (interquartile interval) of sensory scores from 41 judges.

**Table 4.** Fatty acid composition (% of total identified fatty acids) of fillets fromwhich we silver catfish fed with diet supplemented with different levels of essential oil (EO) of *Cymbopogon flexuosus*.

| | Treatment | | |
|---|---|---|---|
| Fatty acid | Control | 1 mL EO/kg diet | 3 mL EO/kg diet |
| C14:0 | 1.24 ± 0.18 | 0.68 ± 0.27 | 0.65 ± 0.24 |
| C14:1n5 | 0.06 ± 0.04 | 0.05 ± 0.01 | 0.21 ± 0.14 |
| C15:0 | 0.11 ± 0.04 | 0.08 ± 0.02 | 0.04 ± 0.03 |
| C16:0 | 23.75 ± 0.56 | 21.71 ± 0.61 | 22.32 ± 0.15 |
| C16:1n7 | 4.58 ± 1.54 | 5.41 ± 0.60 | 5.52 ± 0.69 |
| C17:0 | 0.20 ± 0.07 | 0.33 ± 0.04 | 0.28 ± 0.05 |
| C17:1n5 | 0.17 ± 0.04 | 0.18 ± 0.03 | 0.16 ± 0.03 |
| C18:0 | 7.38 ± 0.33 | 7.19 ± 0.34 | 7.71 ± 0.46 |
| C18:1n7 | 2.71 ± 0.16 | 2.88 ± 0.11 | 2.79 ± 0.12 |
| C18:1n9 | 35.46 ± 0.64 | 36.51 ± 0.40 | 34.82 ± 1.26 |
| C18:2n6 | 15.50 ± 1.10 | 15.11 ± 1.23 | 15.40 ± 0.32 |
| C18:3n3 | 1.00 ± 0.19 | 1.38 ± 0.09 | 1.15 ± 0.19 |
| C18:3n6 | 0.87 ± 0.19 | 1.07 ± 0.12 | 0.83 ± 0.06 |
| C20:0 | 0.20 ± 0.07 | 0.31 ± 0.08 | 0.19 ± 0.05 |
| C20:1n9 | 1.03 ± 0.08 | 1.16 ± 0.03 | 0.84 ± 0.09 |
| C20:2n6 | 0.28 ± 0.02 | 0.35 ± 0.09 | 0.33 ± 0.03 |
| C21:0 | 0.12 ± 0.04 | 0.33 ± 0.07 | 0.29 ± 0.05 |
| C20:3n3 | 0.49 ± 0.31 | 0.03 ± 0.02 | 0.03 ± 0.03 |
| C20:3n6 | 1.64 ± 0.28 | 1.45 ± 0.16 | 1.91 ± 0.40 |
| C20:4n6 | 0.98 ± 0.10 | 1.30 ± 0.20 | 1.51 ± 0.36 |
| C20:5n3 | 0.10 ± 0.01 | 0.13 ± 0.05 | 0.15 ± 0.01 |
| C22:5n3 | 0.29 ± 0.05 | 0.51 ± 0.09 | 0.55 ± 0.013 |
| C22:5n6 | 0.19 ± 0.04 | 0.26 ± 0.08 | 0.23 ± 0.06 |
| C22:6n3 | 1.40 ± 0.27 | 1.33 ± 0.18 | 1.90 ± 0.59 |
| C24:0 | 0.17 ± 0.05 | 0.24 ± 0.05 | 0.18 ± 0.03 |
| MUFA | 44.01 ± 2.18 | 46.19 ± 0.58 | 44.34 ± 1.70 |
| PUFA | 22.82 ± 1.63 | 22.93 ± 1.36 | 23.99 ± 1.13 |
| SFA | 33.17 ± 0.76 | 30.88 ± 0.89 | 31.66 ± 0.60 |
| n-3 | 4.99 ± 0.75 | 4.84 ± 0.50 | 5.69 ± 0.95 |
| n-6 | 19.47 ± 1.31 | 19.55 ± 1.16 | 20.20 ± 0.58 |
| n-9 | 36.50 ± 0.57 | 37.66 ± 0.43 | 35.66 ± 1.33 |
| n-3/n-6 | 0.25 ± 0.03 | 0.25 ± 0.03 | 0.28 ± 0.04 |

Results are expressed as mean ± SE (n = 4).

Coordinates b* and C* increased up to the 3rd month of storage in fillets from fish that received 1 and 3 mL EO/kg diet. Control fillets showed significant increase in b* and C* values only in the 6th month of storage. Dietary supplementation with 3 mL EO/kg diet yielded the greatest color change of fillets during storage ($\Delta E_{0\text{-}12\ months}$: 10.4) compared to control and 1 mL EO/kg diet (Figure 2a).

There was no significant effect of diet on the pH of fillets (Figure 2b) that were only affected by the storage time. The initial pH value of fillets was 6.36, and this value was reduced in the 3rd month of storage and thereafter increased reaching its maximum value at the 9th month of storage (6.68). The WHC of fillets was also affected by the storage time ($p < .05$), but diet had no effect (Figure 2c). The initial WHC was 96%, and it was reduced to 93% in the 6th month of storage and maintained this value until the end of storage.

Texture was evaluated by hardness, springiness, cohesiveness, and chewiness (Table 6). Cohesiveness and springiness were affected by the storage time independent of dietary treatment. Cohesiveness showed a small increase during the storage period, while springiness showed a small reduction (10% of initial time) at the end of storage. ANOVA revealed factorial diet treatment × time interaction for hardness and chewiness, which were reduced up to the 6th month and then increased until the end of storage. The increase in hardness and chewiness was more accentuated for fillets from fish that received 3 mL EO/kg diet, yielding higher hardness (at 9 and 12 months) and chewiness values (at 12 months) at the end of storage compared to the two other treatments.

**Table 5.** Changes in color coordinates during frozen storage of fillets from silver catfish fed with diet supplemented with different levels of essential oil (EO) of *Cymbopogon flexuosus*.

| | Storage (months) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 3 | 6 | 9 | 12 | Mean |
| Feed | | | L* | | | |
| Control | 50.6 ± 0.7$^{Aab}$ | 49.8 ± 1.3$^{Ab}$ | 52.6 ± 1.1$^{Aab}$ | 55.2 ± 1.4$^{Aa}$ | 52.4 ± 0.5$^{Bab}$ | 52.1 |
| 1 mL EO/kg diet | 53.0 ± 1.0$^{Aa}$ | 53.5 ± 1.4$^{Aa}$ | 55.5 ± 1.0$^{Aa}$ | 53.5 ± 1.0$^{Aa}$ | 53.1 ± 0.7 $^{Ba}$ | 53.7 |
| 3 mL EO/kg diet | 48.6 ± 0.9$^{Bc}$ | 51.9 ± 1.2$^{Acb}$ | 55.6 ± 0.6$^{Aab}$ | 55.4 ± 0.7$^{Aa}$ | 57.0 ± 0.5$^{Aa}$ | 53.7 |
| Mean | 50.7 | 51.7 | 54.5 | 54.7 | 54.2 | |
| | | | a* | | | |
| Control | −1.13 ± 0.30 | −2.01 ± 0.07 | −1.32 ± 0.20 | −1.45 ± 0.24 | −1.26 ± 0.47 | −1.43 |
| 1 mL EO/kg diet | −1.56 ± 0.42 | −1.80 ± 0.20 | −1.75 ± 0.32 | −1.40 ± 0.22 | −1.39 ± 0.35 | −1.58 |
| 3 mL EO/kg diet | −0.62 ± 0.51 | −0.69 ± 0.62 | −1.43 ± 0.42 | −2.06 ± 0.25 | −1.54 ± 0.28 | −1.27 |
| Mean | −1.10 | −1.50 | −1.50 | −1.64 | −1.40 | |
| | | | b* | | | |
| Control | 1.85 ± 0.43$^{b}$ | 4.32 ± 0.71$^{ab}$ | 6.41 ± 0.36$^{a}$ | 6.47 ± 0.75$^{a}$ | 6.19 ± 0.90$^{a}$ | 5.05 |
| 1 mL EO/kg diet | 1.53 ± 0.59$^{b}$ | 5.23 ± 0.37$^{a}$ | 6.65 ± 0.80$^{a}$ | 6.52 ± 0.77$^{a}$ | 6.15 ± 0.73$^{a}$ | 5.22 |
| 3 mL EO/kg diet | 2.57 ± 0.60 $^{c}$ | 6.61 ± 0.96$^{ab}$ | 4.11 ± 1.20$^{bc}$ | 4.57 ± 0.44$^{abc}$ | 7.95 ± 0.45$^{a}$ | 5.16 |
| Mean | 1.98 | 5.39 | 5.72 | 5.85 | 6.76 | |
| | | | C* | | | |
| Control | 2.30 ± 0.29$^{b}$ | 4.73 ± 0.58$^{ab}$ | 6.56 ± 0.32$^{a}$ | 6.66 ± 0.70$^{a}$ | 6.42 ± 0.79$^{a}$ | 5.33 |
| 1 mL EO/kg diet | 2.51 ± 0.11$^{b}$ | 5.53 ± 0.40$^{a}$ | 6.87 ± 0.70$^{a}$ | 6.70 ± 0.72$^{a}$ | 6.36 ± 0.65$^{a}$ | 5.59 |
| 3 mL EO/kg diet | 2.83 ± 0.49 $^{c}$ | 6.86 ± 1.06$^{ab}$ | 4.62 ± 0.90$^{bc}$ | 5.06 ± 0.35$^{abc}$ | 8.12 ± 0.40$^{a}$ | 5.50 |
| Mean | 2.55 | 5.71 | 6.02 | 6.14 | 6.97 | |
| | | | h* | | | |
| Control | 180.78 ± 1.29 | 180.37 ± 0.37 | 171.22 ± 7.57 | 180.33 ± 0.25 | 184.11 ± 4.98 | 179.36 |
| 1 mL EO/kg diet | 183.65 ± 6.01 | 181.45 ± 1.12 | 179.07 ± 0.87 | 179.86 ± 1.55 | 180.28 ± 2.09 | 180.86 |
| 3 mL EO/kg diet | 179.56 ± 0.66 | 178.13 ± 0.94 | 180.10 ± 1.10 | 180.08 ± 0.74 | 182.86 ± 1.39 | 180.15 |
| Mean | 181.33 | 179.98 | 176.80 | 180.09 | 182.42 | |

Results are expressed as mean ± SE (n = 4). Different lowercase letters indicate difference among the time points and different capital letters indicate difference among treatments within the same time point (Tukey´s test; $p < 0.05$).

Protein oxidation was assessed by the decrease in SH groups (Figure 3a) and formation of PC (Figure 3b). ANOVA revealed just effect of time on SH values, which decreased in all samples at the 12$^{th}$ month of storage (27% of initial time) ($p < .05$). There was a significant diet treatment × storage time interaction on PC content ($p < .05$). PC content showed a bell-shaped behavior during storage, but fillets from fish fed 3 mL EO/kg diet had delayed peak values (at 9 months of storage) compared to fillets from control and 1 mL EO/diet (at 6 months), showing higher PC content that the other groups at 9 months of storage ($p < .05$).

Lipid oxidation was evaluated by the formation of CD (Figure 3c), PV (Figure 3d), and hexanal (Figure 3e). ANOVA revealed only effect of time on PV and a significant diet treatment × storage time interaction on CD and hexanal content. PV had a slight reduction during storage for all treatments. CD had a bell-shaped behavior with peak values at the 9$^{th}$ month for all groups, but at the end of storage fillets from fish supplemented with EO had lower values of CD when compared to control ($p < .05$). Hexanal content, which was very low at the start of storage, was increased for all groups during the storage ($p < .05$). Although at 3 months of storage the content of hexanal was lower in fillets from fish that received EO than in control ($p < .05$), fillets from fish that received 1 mL EO/kg diet had faster increase in hexanal content thereafter, yielding higher hexanal content than the other groups at 9 months of storage ($p < .05$; Figure 3e).

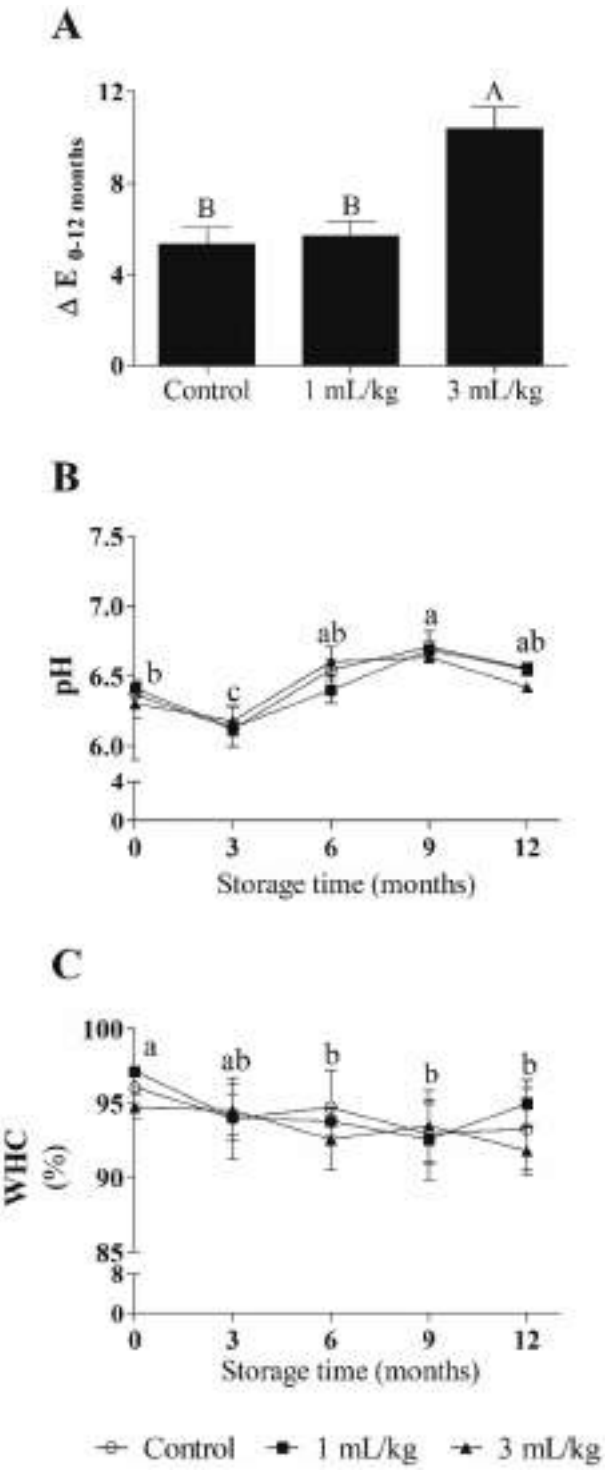



**Figure 2.** Total color difference (a), pH (b), and water holding capacity (c) during frozen storage of fillets from silver catfish fed with diet supplemented with different levels of essential oil of *Cymbopogon flexuosus*. Results are expressed as mean ± SE (n = 4). Different lowercase letters indicate difference among time points and different capital letters indicate difference among treatments (Tukey´s test; $p < .05$).

**Table 6.** Texture changes during frozen storage of fillets from silver catfish fed with diet supplemented with different levels of essential oil of *Cymbopogon flexuosus*.

| | Storage (Months) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 3 | 6 | 9 | 12 | Mean |
| Feed | Hardness (N) | | | | | |
| Control | 30.1 ± 3.8$^{Aa}$ | 21.6 ± 3.5$^{Aab}$ | 12.8 ± 1.4$^{Ab}$ | 16.8 ± 0.7$^{Bb}$ | 30.2 ± 3.1$^{ABa}$ | 22.3 |
| 1 mL EO/kg diet | 23.5 ± 2.3$^{Ab}$ | 17.3 ± 0.7$^{Ab}$ | 12.2 ± 0.9$^{Ac}$ | 20.1 ± 1.7$^{ABab}$ | 26.2 ± 2.3 $^{Ba}$ | 19.9 |
| 3 mL EO/kg diet | 18.5 ± 1.1$^{Aab}$ | 19.3 ± 2.6$^{Aab}$ | 11.5 ± 0.9$^{Ac}$ | 22.4 ± 0.7$^{Ab}$ | 40.8 ± 3.1$^{Aa}$ | 22.5 |
| Mean | 24.0 | 19.4 | 12.2 | 19.8 | 32.4 | |
| | Springiness (cm) | | | | | |
| Control | 0.36 ± 0.02 | 0.36 ± 0.01 | 0.34 ± 0.02 | 0.36 ± 0.00 | 0.29 ± 0.01 | 0.34 |
| 1 mL EO/kg diet | 0.34 ± 0.01 | 0.37 ± 0.01 | 0.33 ± 0.01 | 0.37 ± 0.02 | 0.31 ± 0.01 | 0.34 |
| 3 mL EO/kg diet | 0.34 ± 0.00 | 0.34 ± 0.01 | 0.33 ± 0.01 | 0.40 ± 0.01 | 0.33 ± 0.01 | 0.35 |
| Mean | 0.35$^{ab}$ | 0.36$^{ab}$ | 0.33$^{bc}$ | 0.38$^{a}$ | 0.31 $^{c}$ | |
| | Cohesiveness (dimensionless) | | | | | |
| Control | 0.25 ± 0.01 | 0.28 ± 0.01 | 0.30 ± 0.04 | 0.29 ± 0.01 | 0.28 ± 0.03 | 0.28 |
| 1 mL EO/kg diet | 0.27 ± 0.02 | 0.28 ± 0.02 | 0.31 ± 0.03 | 0.36 ± 0.04 | 0.30 ± 0.02 | 0.30 |
| 3 mL EO/kg diet | 0.25 ± 0.01 | 0.30 ± 0.01 | 0.26 ± 0.02 | 0.35 ± 0.03 | 0.31 ± 0.02 | 0.29 |
| Mean | 0.26$^{b}$ | 0.29a$^{ab}$ | 0.29$^{ab}$ | 0.33$^{a}$ | 0.30$^{ab}$ | |
| | Chewiness (N · cm) | | | | | |
| Control | 2.99 ± 0.63$^{Aa}$ | 2.23 ± 0.45$^{Aab}$ | 1.26 ± 0.07$^{Ab}$ | 1.80 ± 0.16$^{Aab}$ | 2.61 ± 0.34$^{Bab}$ | 2.18 |
| 1 mL EO/kg diet | 2.15 ± 0.35$^{Aa}$ | 1.81 ± 0.26$^{Aa}$ | 1.30 ± 0.21$^{Aa}$ | 2.46 ± 0.64$^{Aa}$ | 2.30 ± 0.39 $^{Ba}$ | 2.00 |
| 3 mL EO/kg diet | 1.58 ± 0.09$^{Abc}$ | 1.83 ± 0.21$^{Abc}$ | 0.96 ± 0.06$^{Ac}$ | 3.09 ± 0.28$^{Aab}$ | 4.52 ± 0.62$^{Aa}$ | 2.40 |
| Mean | 2.24 | 1.96 | 1.17 | 2.45 | 3.14 | |

Results are expressed as mean ± SE (n = 4). Different lowercase letters indicate difference among time points and different capital letters indicate difference among treatments in the same month by Tukey´s test ($p < 0.05$).

## Discussion

Fillet composition was not affected by lemongrass EO supplementation. Proximate composition of silver catfish fillets was similar to that found in other studies (Veit et al. 2017; Weber et al. 2008). Oleic acid, palmitic acid, and linoleic acid were the most abundant fatty acids in silver catfish fillets, as previously reported (Weber et al. 2008). These data demonstrate that dietary supplementation with lemongrass EO does not change the nutritional properties of silver catfish fillets, which present high-protein amount and fatty acids of good quality (unsaturated fatty acids >65%; >35% of oleic acid), important nutrients for human health (Pereira and Vicente 2013).

The sensory acceptance of fish fillets, assessed by texture, color, odor, and taste attributes, was not affected by dietary supplementation with EO. The EO used in this study is mostly composed of citral in its two isomeric forms, geranial (45.7%) and neral (32.1%). Citral has high odorific power and can be detected by human olfactory system at concentrations below 15 ppm (Plotto et al. 2004). Therefore, low amounts of citral deposited in fish fillets could have a negative impact on fillet flavor, as previously reported for other terpenoid compounds after fish anesthesia with eugenol (Cunha et al. 2010). However, analysis of the volatile profile of fish fillets revealed no traces of α and β-citral (Figure 1b). Accordingly, sensory analysis of fish fillets revealed no differences in the acceptance among treatments. EO components like citral have been shown to be rapidly excreted from mammals (Diliberto et al. 1990) and insects (Tak and Isman 2016), but this is the first study in fish. Fish volatile profile and sensory acceptance data indicate that dietary supplementation of adult fish with EO does not yield the accumulation of EO components in catfish muscle at sufficient amounts to be detected. Studies have already shown that dietary supplementation with thyme EO for 84 (Hernández et al. 2015) and 105 days (Álvarez et al. 2012) improved sensory acceptance of refrigerated seabream. On the other hand, anesthesia with *Lippia alba* EO did not affect the perception of taste and odor in fillets of silver catfish (Cunha et al. 2010), and meat sensory perception was not affected by dietary supplementation

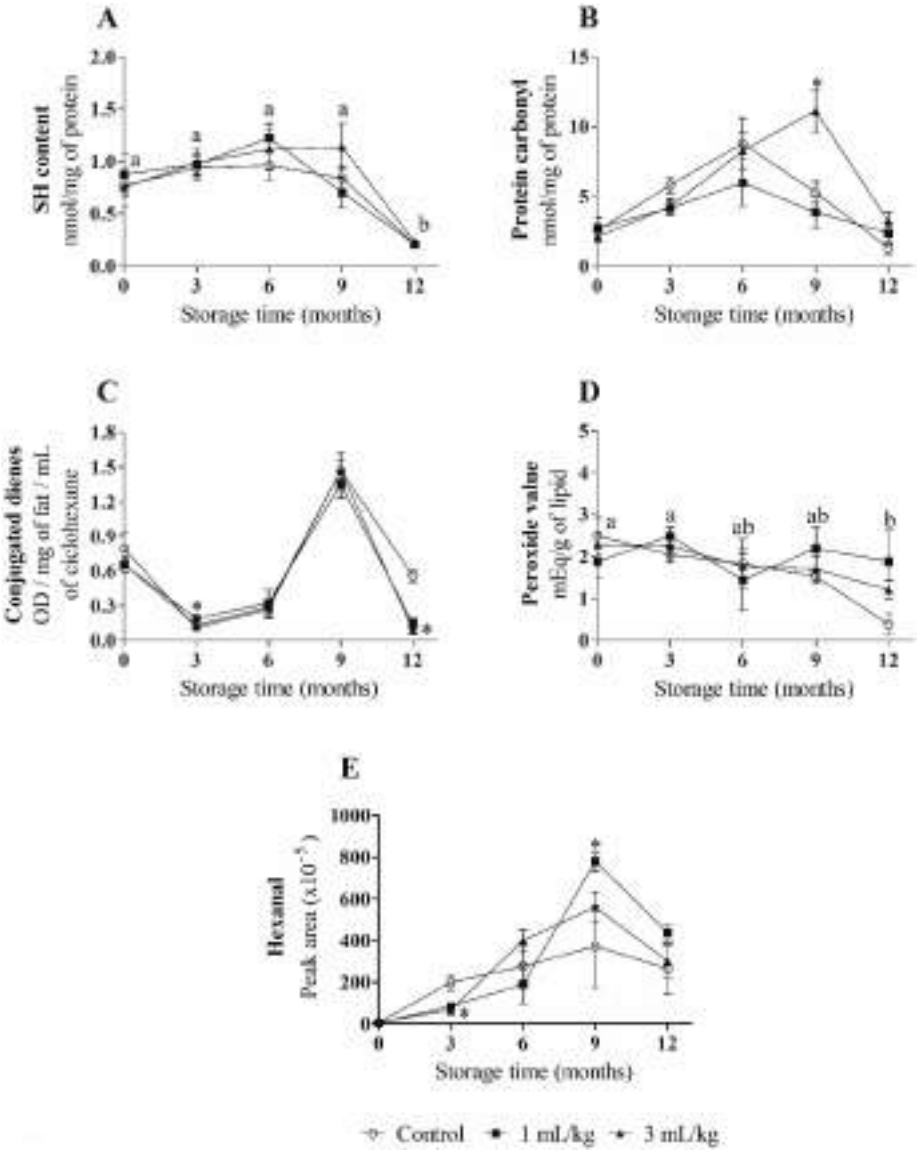



**Figure 3.** Protein and lipid oxidation during frozen storage of fillets from silver catfish fed with diet supplemented with different levels of essential oil of *Cymbopogon flexuosus*. SH content (a), protein carbonyl groups (b), conjugate dienes (c), peroxide value (d), and hexanal levels (e). Results are expressed as mean ± SE (n = 4). Different lowercase letters indicate difference among time points and * indicates difference from control group (Tukey´s test; $p < .05$).

with a blend of thyme, rosemary, and oregano EO in pigs (Yan et al. 2010) and oregano EO in chicken (Symeon et al. 2009). However, clove EO used as sedative in silver catfish impaired the acceptance of fillet taste (Cunha et al. 2010), suggesting that the high intensity of residual volatile compounds from OE may negatively affect sensory acceptance of fish fillets.

Data on the sensory acceptance of meat from animals exposed to EOs *in vivo* are still scarce and conflicting, emphasizing the relevance of studies to evaluate the acceptance of this kind of fish product in order to determine the feasibility of using EO and its components in fish production. In the present study, no traces of EO components were detected in the flesh, and the sensory attributes of fish fillets were not affected, suggesting that lemongrass EO is feasible as a feed additive for fish production.

Silver catfish flesh has low color saturation tending to a grayish color at the start of storage but had a marked increase in b* and C* values during the storage, which indicates that fillets were becoming yellowish and had increased color saturation over time (Pathare et al. 2013). This increase in fillet yellowness during prolonged frozen storage has been reported in other studies (Veeck et al. 2013a; Veit et al. 2017). Although lipid oxidation has been identified as the cause of color changes in fish muscle (Thanonkaew et al. 2006), in the present study markers of primary lipid oxidation, CD and PV, appear to be unrelated to color changes, whereas hexanal was positively correlated with L* ($R = 0.423$; $p < .01$), b* ($R = 0.420$; $p < .01$), and C* ($R = 0.441$; $p < .01$). The increase in yellowing during storage can also be related to the protein oxidation because products of protein oxidation, as carbonyl compounds, in complex food systems, interact with numerous food components and can undergo Maillard reaction (Estévez 2011), which produces colorless, yellow, and reddish to brown compounds that promote a progressive color change (Rizzi 1997). In addition, lipid oxidation products can also undergo non-enzymatic browning reactions with amino groups and give rise to pyrrole compounds, which are related to the increase of yellowness index in a food matrix model (Lu et al. 2015).

The numerical total color difference ($\Delta E_{0\text{-}12\ months}$) indicates the intensity of color change in fillets between the start and the end of storage. Samples of control and 1 mL EO/kg groups had evident color change during storage (ΔE 3–6), but this change was twice greater for fillets from fish fed 3 mL EO/kg diet compared to the other fillets, and the change of color of this group is classified as very clearly perceptible (ΔE 6–12) (Prändl et al. 1994). These data suggest that supplementation with EO at the highest level could negatively impact color of fillets by the end of storage, although sensory acceptance of fillets has not been evaluated during storage.

Hardness and chewiness of fish fillets decreased up to the 6$^{th}$ month of storage and then increased until the end of storage. Such behavior also has been described for fillets of Nile tilapia (Subbaiah et al. 2015). The activity of $Ca^{2+}$-activated ATPases has been shown to be reduced in fish flesh during the first months of frozen storage, indicating progressive degradation of myofibrillar proteins (Ganesh et al. 2006), which could explain the flesh softening. Protease activity has been suggested to underlie the degradation of myofibrillar proteins (Subbaiah et al. 2015). However, protein oxidation leads to the occurrence of intra and intermolecular cross-linking of meat proteins followed by protein polymerization, which has been implicated in the increase in meat hardness (Kim et al. 2010). The oxidation of protein thiol groups is mainly responsible for cross-links through the formation of disulphide bonds (Lund et al. 2011). In the present study, the content of SH reached the lowest level by 12 months of storage, indicating ideal conditions for protein cross-linking. In addition, the thiol content demonstrated an inverse correlation with hardness ($R = -0.531$; $p < .01$). Thus, protein oxidation is likely implicated in the increase in fillet hardness, which reached the peak value at 12 months of storage. Moreover, fillets from fish that received 3 mL EO/kg diet had higher protein oxidation and were firmer than control at 9–12 months of storage, which indicates greater changes in the protein structure of such fillets, even though the WHC did not differ among treatments. The parallel behavior of increased firmness and protein oxidation reinforces the hypothesis that the firmness increase was underlined by oxidative changes that led to protein aggregation/polymerization.

The pH value is closely linked to physical characteristics of meat, such as color and texture (Andrés-Bello et al. 2013); however, the greater color instability and final hardness of fillets from fish fed 3 mL EO/kg diet were not associated with differences in the flesh pH. The highest PC content of fillets from fish fed 3 mL EO/kg diet in the 9$^{th}$ month of storage reveals more substantial protein oxidation compared to the other samples. Frozen storage causes disruption of muscle tissue due to ice formation and increase in salt concentration of the unfrozen phase. Such events contribute to protein denaturation and exposure of hydrophobic protein residues in frozen fish. In addition, the oxidation of SH groups leads to protein polymerization through disulphide bond formation. Thiol oxidation along with other mechanisms of protein denaturation has been associated with physicochemical changes in fish meat (Benjakul et al. 2003). Additionally, carbonylation can affect protein functionality, causing, among other consequences, tenderness decrease and color change in meat (Estévez 2011).

Both *C. flexuosus* EO and citral have *in vitro* antioxidant activity, and this activity varies depending on EO concentration (Chandra and Ah 2014). In the present study, supplementation with EO did not protect against lipid (CD, PV, and hexanal) or protein oxidation (SH and PC) of silver catfish fillets, except in the third month, when EO treatments had lower hexanal content than control. However, in the 9$^{th}$ month of storage, fillets from fish supplemented with *C. flexuosus* EO had higher content of hexanal (1 mL EO/kg diet) and PC (3 mL EO/kg diet) than control. Hexanal is a volatile compound originating from the oxidative degradation of linoleic acid (Shahidi and Pegg 1994) and can be used as an indicator of fish quality (Secci and Parisi 2016). PC originates from direct metal-catalyzed oxidation of the side-chains of some amino acid residues (proline, arginine, lysine, or threonine) or by amino acid reaction with reactive aldehydes derived from fatty acid oxidation (Maisonneuve et al. 2009). In fact, the present study reveals a significant positive interaction between the contents of hexanal and PC ($R = 0.384$; $p < .01$). Thus, it is possible to infer that different doses of EO act by distinct pathways in the frozen fillet during storage. While the lowest dose of *C. flexuosus* EO appears to cause a slight increase in lipid oxidation, the highest one slightly increased protein oxidation after long-term frozen storage. Further studies are required to elucidate the mechanisms that underlie such effects and whether they would affect fish shelf life.

Recently, lemongrass EO has been shown to up-regulate the expression of heat shock proteins in canine hepatocyte cell culture (Zhang et al. 2015). Heat shock proteins are known to be upregulated in fish muscle as a response to protein denaturation (Kayhan and Duman 2010), oxidative stress, and depletion of endogenous antioxidants (Sahin et al. 2014). However, our lipid and protein oxidation data immediately after slaughtering (time zero) do not support the hypothesis that *C. flexuosus* EO caused *in vivo* oxidative stress.

Although there were some differences between treatments during frozen storage, no sufficiently expressive changes that would affect fillet shelf life were observed. These data support the feasibility and safety of lemongrass EO supplementation to increase the productive performance of fish without affecting the flesh quality. Previous study showed that the supplementation of silver catfish during fattening with a diet containing 1 mL/kg of lemongrass EO is more appropriate to improve protein deposition and carcass yield than 3 mL/kg lemongrass EO diet (Rampelotto et al. 2018). Thus, doses of lemongrass EO greater than 1 mL/kg may not be advantageous for productivity and fillet quality, since in the present study 3 mL/kg lemongrass EO caused more clear changes in color, texture, and protein oxidation of fillets.

## Conclusion

Dietary supplementation with *C. flexuosus* EO does not affect the composition or sensory acceptability of catfish fillets and does not leave citral residues in the fillets. Fillets from fish fed 3 mL EO/kg diet had greater color changes during frozen storage and higher firmness by the end of storage than control fillets, which appears to be caused by greater protein oxidation. However, fish feeding with the lowest EO dose does not cause relevant changes in fish fillets, demonstrating that the use of lemongrass EO on fish feed is viable and safe.

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## Disclosure statement

B. Baldisserotto and B. M. Heinzmann are coauthors of a patent application for the preparation and use of C. flexuosus essential oil as a growth stimulant for fish. BR 102015020931.

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C. Rampelotto conducted the experiments, collected data, analyzed and interpreted the results and wrote the first draft of the manuscript. T. Emanuelli, B. Baldisserotto and L.P. da Silva guided the experimental design, provided founds and structure for experiment and helped in the interpretation of data and revising the manuscript. C.S. Speroni, L. Conte, D. Pianesso I.S. Machado, R.F. Rodrigues, N.M Minuzzi, T.J. Adorian, P. Mombach, B. Klein and R. Wagner collected test data and collaborated with the experimental management of fish. B.M. Heinzmann, C.R. de Menezes characterized the essential oil and microcapsules. All of the authors contributed to the interpretation and discussion of the results related to their part of the study and approved the final version of the paper.

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# Metabolic properties of partially hydrolyzed pectin from passion fruit peel

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ABSTRACT

Partially degraded pectin can provide different metabolic effects and health benefits compared to native plant pectins, due to their lower molecular weight. These effects are mainly due to greater intestinal fermentation and production of short chain fatty acids promoted by non-digestible carbohydrates with low molecular weight. The aim of this work was to assess the metabolic effects of partially hydrolyzed pectin (PHP) from passion fruit peel. Forty weaned male Wistar rats (n = 8) received diets with PHP in crescent levels (Control, 0.25%, 0.50%, 0.75% and 1.00%) for 38 days. Feed intake, weight gain, pH and lipids in faeces, lipids and cholesterol in the liver, blood glucose response, TAG and lipoproteins of blood, and short chain fatty acids in the cecum content were investigated. PHP increased feed intake and weight gain in rats. Faecal pH decreased with consumption of PHP 1.00%, and the production of propionic and butyric acids in cecum was higher in treatments with PHP. These results confirm the metabolic effects and suggest a potential prebiotic activity of PHP, even at low doses.

## 1. Introduction

Dietary fibres, especially some soluble fibres and oligosaccharides, can be selectively fermented by the microbiota, are promising candidates for the prebiotic category (Chung et al., 2017; Gibson et al., 2017; Lam et al., 2018; Paesani et al., 2020). Prebiotic is 'a substrate that is selectively utilized by host microorganisms conferring a health benefit', being largely recognized for its role in the regulation of intestinal microbiota and influencing the local and systemic host physiology (Gibson et al., 2017; Neri-Numa & Pastore, 2020; Wang et al., 2020). Prebiotics are metabolized by microorganisms in the gut, releasing metabolites, among them the short chain fatty acids (SCFAs) such as acetic, propionic and butyric acids. (Slavin, 2013; Adebola et al., 2020; Neri-Numa & Pastore, 2020). These metabolites show hypocholesterolemic effect, modulate immune function and prevent colon cancer (Slavin, 2013; Adebola et al., 2020; Neri-Numa & Pastore, 2020).

Soluble arabinoxylans, beta-glucan and pectin are some examples of potential prebiotics (Chung et al., 2017; Lam et al., 2018; Paesani et al., 2020). Pectins are complex and heterogeneous polysaccharides found in several plants, including agro-industrial by-products such as apple pomace, sugar beet pulp, soy hull, citrus peel and passion fruit peel (Moura et al., 2017; Prandi et al., 2018; Zhang et al., 2018a,b; Wilkowska et al., 2019). Once obtained from these materials, pectin can be used to produce pectic oligosaccharides by controlled hydrolysis (Babbar et al., 2016; Gómez et al., 2019; Hu et al., 2019; Wilkowska et al., 2019).

However, non-digestible carbohydrates with lower molecular weight have greater prebiotic potential than more complex carbohydrates, such as polysaccharides (Babbar et al., 2016; Gibson et al., 2017). The most known non-digestible oligosaccharides with prebiotic activity are fructooligosaccharides, inulin and galactooligosaccharides (Gibson et al., 2017; Neri-Numa & Pastore, 2020). Recent studies have shown the prebiotic potential of pectin hydrolysates produced from sugar beet pulp, citrus peel and apple pomace (Zhang et al., 2018a,b; Gómez et al., 2019; Hu et al., 2019; Prandi et al., 2019; Wilkowska et al., 2019). Partially degraded pectin products provide specificities different from native pectins, such as lower water holding capacity and greater fat absorption capacity (Moura et al., 2020). In addition, partially hydrolyzed pectin has better prebiotic properties compared to native plant pectins (Gómez et al., 2016; Babbar et al., 2016; Chung et al., 2017). The

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health benefits of pectic oligosaccharides are determined by the source, nature and chemical structure of pectin, as well as the hydrolysis process used (Babbar et al., 2016).

Most studies on the properties of hydrolyzed pectin are carried out *in vitro* and have been shown to stimulate gut colonization by lactic acid bacteria and inhibit the development of pathogenic bacteria (Gómez et al., 2019; Prandi et al., 2019; Wilkowska et al., 2019). However, to be considered a prebiotic, their health benefits must be proven and supported by scientific evidences, including *in vivo* studies. Thus, the aim of this work was to assess the metabolic effects of partially hydrolyzed pectin from passion fruit peel.

## 2. Materials and methods

### 2.1. Materials

Pectin was extracted from the passion fruit peel according to the method described by Moura et al. (2017). The pectin extraction was carried out from the dry and ground passion fruit peel, which was treated with 0.05 N HCl at 90 °C for 45 min. After, the partial hydrolysis of passion fruit peel was performed with 0.25 N HCl at 60 °C for 2 h, at a ratio of 1:50 of sample (g) and solution (mL). Hydrolysis was stopped by adding KOH for neutralization (pH 7.0). The neutralized material was lyophilized and ground in a minimill (Moura et al., 2020). After these processes, the PHO presented the following composition: 2.7% of proteins, 3.8% of ashes, 0.3% of lipids, and total carbohydrate of 93.2%. The carbohydrate fraction had a sugar monomer composition of 6.1% of rhamnose, 2.4% of arabinose, 2.2% of xylose, 1% of mannose, 9.7% of galactose, 17.3% of glucose, and 61.6% of galacturonic acid. The average molar mass of PHP was $1.21 \times 10^5$ (g/mol) (Moura et al., 2020).

### 2.2. Methods

#### 2.2.1. Animals and diets

The animal experimental design and procedures was previously approved by the Ethics Committee on the Use of Animals of the UFSM, under document number 085/2012 (2).

Forty weaned male rats (*Rattus norvegicus* Wistar albino), 21 days old, were obtained from the "Biotério Central" of Federal University of Santa Maria (UFSM), which were allocated into five groups (n = 8), kept individually in metabolic cages with free access to feed and water. Diets for growing rat were formulated according Reeves, Nielsen, and Fahey Jr (1993), with addition of PHP at increasing levels to each of five animals groups: 0% of PHP (Control Group), 0.25% of PHP, 0.50% of PHP, 0.75% of PHP and 1.00% of PHP.

The biological assay lasted 38 days, with five days for adaptation. The room temperature was 22 ± 2 °C and the 12 h light/dark cycle was controlled throughout the experiment. The diet was daily offered to the rats in a feeder. Diet consumption was daily monitored and the faeces were collected in the last 13 days of the experiment for further analysis. The animals were weighed every week.

From the 28th day of experiment, eight animals were randomly selected, on four consecutive days, for analysis of postprandial blood glucose. Those submitted to the intervention were excluded in the next day selection, until all animals be tested. The rats remained 12 h without diet for blood glucose analysis at fasting. Next, each received 2 g of diet and blood glucose was measured at 15, 30, 60 and 120 min after feeding. The blood glucose was measured with capillary blood glucometer (Breeze2® - Bayer, Indiana - USA) from a drop of blood of the rat tail.

At the end of the biological assay (day 38) all animals were subjected to 12 h overnight fasting. Then, anesthesia was performed with intraperitoneal injection of 60–80 mg of ketamine +8–15 mg of xylazine/kg, for blood collection by cardiac puncture. After the procedure, the animals were euthanized using an overdose of anesthetic. The liver and epididymal fat from rats was immediately removed, weighted and freezing for further analysis. The epididymal fat/body weight ratio was calculated from epididymal fat and body weight values of each rat. Cecum content was also collected for further analysis of SCFAs content.

#### 2.2.2. Analysis of pH and lipids in faeces

The faeces collected were dried at 50 °C for 12 h and then ground in a minimill. To measure faecal pH, 1 g of sample was dissolved in 10 mL of distilled water and a pH-meter was used. The lipids content of the faeces was determined according to the method of Bligh and Dyer (1959).

#### 2.2.3. Liver analyses

The lipids content of the liver was analyzed by the method of the Bligh and Dyer (1959). To analyze total cholesterol, samples of 0.5 g of liver were homogenized in 2.5 mL of isopropyl alcohol. After 24 h at 4 °C, the homogenate was centrifuged at 2700 rpm for 5 min, and the supernatant was used to analyze total cholesterol by specific enzymatic test (Colesterol enzimático líquido - Doles®, Goiás, Brazil).

#### 2.2.4. Blood analyses

The blood collected was centrifuged to obtain the serum. Serum samples were used to determine the total cholesterol, high density lipoprotein cholesterol (HDL-c) and triacylglycerols (TAG) by specific enzymatic tests (Colesterol enzimático líquido - Doles®, Goiás, Brazil; Colesterol HDL - Doles®, Goiás, Brazil; Triglicérides enzimático líquido - Doles®, Goiás, Brazil, respectively).

#### 2.2.5. Analysis of short chain fatty acids in the cecum content

SCFAs in the cecum content were analyzed by gas chromatography according to Bianchi et al. (2011), with adaptations. The cecum content (0.5 g) and distilled water (5.5 mL) were placed in test tubes, with subsequent homogenization using a vortex mixer. Centrifugation was performed at 3500 rpm for 10 min, and then 1 mL of supernatant was transferred to a 5-mL hermetically closed vial. Samples were set to pH ≈ 2 with 1 M $H_2SO_4$ solution. The SCFAs were extracted using the solid-phase microextraction (SPME) technique into the headspace sample. The extractions were carried out with a Car/PDMS fiber (Supelco, 85 μm × 10 mm) at 40 °C under stirring for 30 min. Previous the extraction the samples were maintained without fiber exposition for 10 min to reach the temperature equilibrium. SPME fiber was desorbed into the injector at 230 °C, operating in split mode (split ratio 1:20) of a gas chromatograph equipped with a flame ionization detector (GC-FID Varian, Star 3400 CX). The separation of SCFAs were carried out in a fused silica capillary column ZBWax Plus (30 m × 0.25 mm; 0.25 μm of thickness film) using hydrogen as carrier gas (constant pressure of 15 psi and initial flow 1.3 mL/min). Gas chromatograph oven temperature program was started at 50 °C for 1 min, then, a ramp was activated with an increase of 5 °C/min until reached 110 °C and after 15 °C/min until to reach 230 °C, keeping in isotherm for 10 min. The injector temperature was 230 °C. SCFAs identification was carried out by comparing the retention times of the analyte with the authentic standards: acetic, propionic and butyric acids (Sigma Aldrich). A calibration curve was calculated in the range 350–3000 mg/L with the standards for quantification of each acid.

#### 2.2.6. Statistical analysis

The results were submitted to analysis of variance (ANOVA) and the means were compared using Tukey's test at the 5% level of significance. Statistical analyses were performed with Statistical Package for the Social Sciences (SPSS), version 18.0.

## 3. Results and discussion

### 3.1. Consumption, weight gain and epididymal fat/body weight ratio

Table 1 shows the daily feed consumption, weight gain and the ratio of epididymal fat/body weight of Wistar rats. Feed consumption and weight gain were higher in groups that received PHP. These results are

**Table 1**
Daily consumption, weight gain and epididymal fat/body weight ratio of rats fed with diets containing partially hydrolyzed pectin at increasing levels.

| | Diets* | | | | |
|---|---|---|---|---|---|
| | Control | PHP 0.25% | PHP 0.50% | PHP 0.75% | PHP 1.00% |
| Daily consumption (g) | $10.42^{b} \pm 0.96$ | $16.64^{a} \pm 1.45$ | $16.45^{a} \pm 1.68$ | $16.64^{a} \pm 0.82$ | $16.86^{a} \pm 1.26$ |
| Weight gain (g) | $110.00^{b} \pm 5.95$ | $169.37^{a} \pm 20.16$ | $164.6^{a} \pm 16.78$ | $161.05^{a} \pm 15.45$ | $168.03^{a} \pm 18.29$ |
| EF/BW** | $0.79^{b} \pm 0.14$ | $1.00^{ab} \pm 0.25$ | $1,32^{a} \pm 0.19$ | $1.02^{ab} \pm 0.17$ | $1.08^{ab} \pm 0.13$ |

The results are expressed as the mean ± standard deviation. Mean values followed by a different letter on the same line are significantly different using Tukey's test ($p < 0.05$), N = 8.
*Diets: Control – standard diet; PHP 0.25% – diet with 0.25% of partially hydrolyzed pectin; PHP 0.50% – diet with 0.50% of partially hydrolyzed pectin; PHP 0.75% – diet with 0.75% of partially hydrolyzed pectin; PHP 1.00% – diet with 1.00% of partially hydrolyzed pectin. **Epididymal fat/body weight ratio.

not expected as an effect for most prebiotics, however, Mao et al. (2016) and Chen et al. (2017) found similar results with the administration of apple pectic oligosaccharide to weaned rats and pigs. According to the authors, pectic oligosaccharides can improve growth performance by improving immune function and utilization of nutrients. In study by Mateos-Aparicio et al. (2020), the weight gain of rats that consumed a diet with 20% dietary fibre from apple pectin was slightly higher than the control group. Supplementation of pectic oligosaccharides in diets can improve the jejunum mucosal morphology and intestinal flora structure of rats (Mao et al., 2016). Therefore, the increased utilization of nutrients is possibly due to increased intestinal digestibility and nutrient absorption (Chen et al., 2017). Immune function is important to maintaining the health and growth of animals and humans (Chen et al., 2017). It is possible that dietary supplementation of pectic oligosaccharide increases the immune function via regulating the immunoglobulin production of whole body (Chen et al., 2017).

The epididymal fat/body weight ratio was higher in animals that consumed PHP 0.50% compared to the control group. However, the epididymal fat/body weight ratio of the PHP 0.50% group was statistically equal to the all other groups that received PHP. Greater adiposity is another unexpected result of prebiotic intake (García-Carrizo et al., 2020), but it is probably a consequence to the higher feed intake and weight gain of rats that consumed PHP. These metabolic effects of PHP and pectic oligosaccharides need further investigation. In this work, the administration of a balanced diet in nutrients with addition of PHP to growing animals was evaluated, which must be considered. In many available studies, prebiotics are administered for therapeutic purposes after/during the administration of high-fat or obesogenic diet (García-Carrizo et al., 2020; Mateos-Aparicio et al., 2020; Singh et al., 2018).

### 3.2. Liver and faeces analysis

In contrast to the results of higher epididymal fat, the liver of rats fed with PHP did not differ from the control group in adiposity or cholesterol content (Table 2). Fat elimination in the faeces also did not differ between treatments. Prebiotics can play an important role in lipids and cholesterol metabolism (García-Carrizo et al., 2020; Adebola et al., 2020; Mateos-Aparicio et al., 2020). Soluble dietary fibres, such as pectin, show a binding effect on bile acids in faeces, preventing their reabsorption in the intestinal lumen. Thus, the conversion of cholesterol to bile acids increases in the liver, showing a minor cholesterolemia. In addition, the oil holding capacity of pectins can decrease the absorption of lipids in the intestine and favors the faecal excretion of lipids, resulting in the reduction of plasma triglycerides and hepatic total lipids (Mateos-Aparicio et al., 2020). However, higher doses than those used in this study may be necessary to obtain more significant effects.

A decreased of the faecal pH was observed in treatment with PHP 1.00%, the highest tested concentration. Faeces acidification is a result of the prebiotic fermentation and production of SCFAs, mainly acetic, propionic and butyric acids (Macagnan et al., 2015; Neri-Numa & Pastore, 2020). The decrease in pH of the intestinal environment favors the absorption of minerals, such as calcium, phosphorus and magnesium, improving bone health (Bryk et al., 2016; Gibson et al., 2017).

**Table 2**
Analysis of liver and faeces of rats fed with diets containing partially hydrolyzed pectin at increasing levels.

| | Diets* | | | | |
|---|---|---|---|---|---|
| | Control | PHP 0.25% | PHP 0.50% | PHP 0.75% | PHP 1.00% |
| Liver lipids (%) | $4.29^{ab} \pm 0.18$ | $3.81^{b} \pm 0.27$ | $4.55^{a} \pm 0.30$ | $4.53^{a} \pm 0.41$ | $4.19^{ab} \pm 0.51$ |
| Liver total cholesterol (mg/g) | $1.38 \pm 0.34$ | $1.49 \pm 0.30$ | $1.70 \pm 0.44$ | $1.52 \pm 0.20$ | $1.62 \pm 0.37$ |
| Faecal lipids (%) | $5.00 \pm 1.00$ | $4.58 \pm 0.53$ | $4.08 \pm 0.38$ | $4.50 \pm 0.58$ | $4.29 \pm 0.64$ |
| Faecal pH | $5.94^{a} \pm 0.20$ | $5.78^{ab} \pm 0.08$ | $5.76^{ab} \pm 0.12$ | $5.76^{ab} \pm 0.16$ | $5.74^{b} \pm 0.08$ |

The results are expressed as the mean ± standard deviation. Mean values followed by a different letter on the same line are significantly different using Tukey's test ($p < 0.05$), N = 8.
*Diets: Control – standard diet; PHP 0.25% – diet with 0.25% of partially hydrolyzed pectin; PHP 0.50% – diet with 0.50% of partially hydrolyzed pectin; PHP 0.75% – diet with 0.75% of partially hydrolyzed pectin; PHP 1.00% – diet with 1.00% of partially hydrolyzed pectin.

### 3.3. TAG, total cholesterol and HDL-c of blood and postprandial blood glucose response

There was no difference in TAG, total cholesterol and HDL-c of blood and in the glucose response to fasting and feeding status (15, 30, 60 and 120 min postprandial) between the groups (Figs. 1 and 2). Prebiotics such as non-digestible oligosaccharides and polysaccharides can improve glucose tolerance and dyslipidaemia (Gibson et al., 2017).

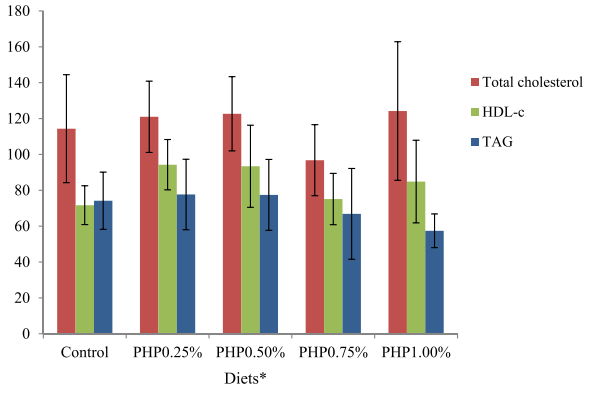



**Fig. 1.** Total cholesterol, HDL cholesterol and TAG levels of the serum from Wistar rats fed with diets containing partially hydrolyzed pectin at increasing levels of addition. *Diets: Control – standard diet; PHP 0.25% – diet with 0.25% of partially hydrolyzed pectin; PHP 0.50% – diet with 0.50% of partially hydrolyzed pectin; PHP 0.75% – diet with 0.75% of partially hydrolyzed pectin; PHP 1.00% – diet with 1.00% of partially hydrolyzed pectin.

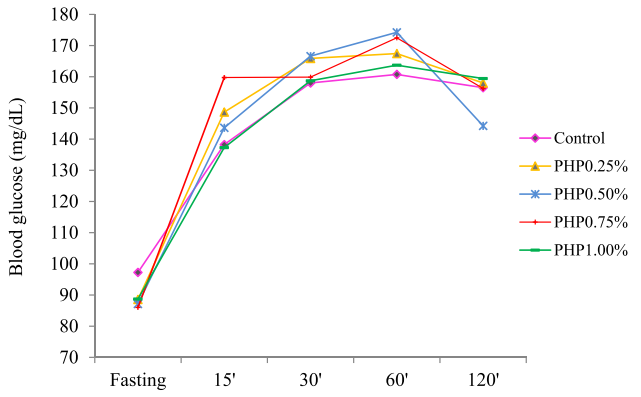



**Fig. 2.** Postprandial blood glucose response of Wistar rats after consuming a diet with partially hydrolyzed pectin at increasing levels of addition.
*Diets: Control – standard diet; PHP 0.25% – diet with 0.25% of partially hydrolyzed pectin; PHP 0.50% – diet with 0.50% of partially hydrolyzed pectin; PHP 0.75% – diet with 0.75% of partially hydrolyzed pectin; PHP 1.00% – diet with 1.00% of partially hydrolyzed pectin.

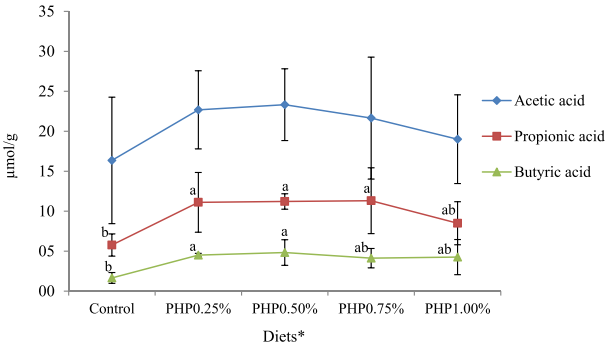



**Fig. 3.** Concentration of short chain fatty acids in the cecum content of Wistar rats fed with diets containing partially hydrolyzed pectin at increasing levels of addition.Different letter on the same line are significantly different using Tukey's test ($p < 0.05$), N = 8.
*Diets: Control – standard diet; PHP 0.25% – diet with 0.25% of partially hydrolyzed pectin; PHP 0.50% – diet with 0.50% of partially hydrolyzed pectin; PHP 0.75% – diet with 0.75% of partially hydrolyzed pectin; PHP 1.00% – diet with 1.00% of partially hydrolyzed pectin.

However, these effects are usually more pronounced in experiments with larger doses of the prebiotic and/or administration of unbalanced diets (Macagnan et al., 2015; Singh et al., 2018; Zhang et al., 2018a,b; Mateos-Aparicio et al., 2020). Inulin, for example, is a typical prebiotic that can improve glucose metabolism, in doses of 2.5%–25%, as in study of Singh et al. (2018) with high-fat-fed male rats; and 3 g of soluble dietary fiber (91% inulin)/kg body weight/day administered to a diabetic rat model, in study of Zhang et al. (2018a,b). It is observed that the doses used in these studies were much higher than those used in our study, from 0.25% to 1.00% of prebiotic (PHP).

According to Hu et al. (2019), pectin oligosaccharides exhibited hypocholesterolemic effects in mice. With the consumption of a high fat diet supplemented with 0.9 g/kg of body weight/day of pectin oligosaccharides, total cholesterol and LDL-c were the same as the control group, with a standard diet (Hu et al., 2019). In our study, all treatments with PHP showed a glycemic response, triacylglycerols and blood lipoproteins similar to the control group (characterized by a nutritionally balanced diet). Therefore, the results obtained can be considered good, since all animals remained healthy. The effects of higher doses of PHP and/or in context of hyperglycemic or hyperlipidemic diets need further investigation.

### 3.4. Short chain fatty acids in the cecum content

The production of SCFAs (acetic, propionic and butyric) in the intestine of Wistar rats is shown in Fig. 3. There was no difference in the acetic acid production between the groups. However, the production of propionic and butyric acid by the rats of groups with PHP was greater than the control, with significant differences in the production of propionic acid with consumption of PHP 0.25%, 0.50% and 0.75% and production of butyric acid with consumption of PHP 0.25% and 0.50%. These results suggest the prebiotic potential of the PHP, also already demonstrated by others studies with partially degraded pectin products, *in vitro* (Chung et al., 2017; Gómez et al., 2019; Prandi et al., 2019; Wilkowska et al., 2019; Zhang et al., 2018a,b) and *in vivo* (Hu et al., 2019; Liu et al., 2020).

SCFAs are metabolites produced in the intestine lumen by different colonic microorganisms, influencing the host physiology locally and systemically (Gibson et al., 2017; Neri-Numa & Pastore, 2020). Among the benefits attributed to the SCFAs are: improvement of colonocyte function and gut homeostasis, strengthening of the epithelial defense barrier and immune function, anti-inflammatory properties and improvement of chronic cardiovascular disease (Gibson et al., 2017; Guarino et al., 2020; Neri-Numa & Pastore, 2020).

The diet can influence the microbial production of SCFAs in the intestine. A high fat diet decreased the production of acetic, propionic and butyric acids, however, citrus pectin oligosaccharides were able to reverse this condition and increase the production of SCFA in the cecum and faeces of mice (Hu et al., 2019). According to Gómez et al. (2016), pectic oligosaccharides present better prebiotic properties than native pectins, and similar or better than fructooligosaccharides, proven in an *in vitro* study using human faecal inocula.

## 4. Conclusions

The metabolic effects of PHP from passion fruit peel added to the diet of Wistar rats were investigated in this work. The concentrations of PHP used were lower than most studies with partially degraded pectin. The PHP levels tested, 0.25%, 0.50%, 0.75% and 1.00%, promoted an increase of feed intake and weight gain in rats. It is important to highlight that the biological assay was carried out with weaned rats. Blood lipoproteins and triacylglycerols, as well as blood glucose response were not altered by the consumption of PHP. Nevertheless, the faecal pH decreased with consumption of PHP 1.00%, and the production of propionic and butyric acids in cecum was higher in treatments with PHP. These results confirm the metabolic effects and suggest a potential prebiotic activity of PHP. However, the effects of higher doses of partially degraded pectin, in adult experimental models and, perhaps, in the context of hyperglycemic or hyperlipidemic diets, for therapeutic purpose, need further investigation.

## CRediT authorship contribution statement

**Fernanda Aline de Moura:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Writing - original draft. **Fernanda Teixeira Macagnan:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Bruna Klein:** Formal analysis, Writing - review & editing. **Roger Wagner:** Formal analysis, Methodology, Writing - review & editing. **Leila Picolli da Silva:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft.

## Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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# Key aroma compounds of Canastra cheese: HS-SPME optimization assisted by olfactometry and chemometrics

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ABSTRACT

An analytical method was developed to determine volatile compounds (VC) that contribute to the aroma of cheese from Serra da Canastra (Brazil) and evaluate them in three ripening stages (fresh, short-ripened, and ripened) via headspace solid-phase microextraction (HS-SPME) combined with gas chromatography (GC). Proximate and fatty acid compositions were determined to observe whether there would be changes during ripening. Multivariate designs were applied to optimize the extraction parameters of volatile compounds and assisted by GC olfactometry (GC-O) and chemometrics. The adopted strategy revealed that the best extraction condition requires 10 min of equilibration, 75.2 min of fiber exposure at 40 °C, and 1 g of sample. The data obtained evidenced the alteration of the abundance of volatile compounds, fatty acids, and proximate composition of Canastra cheese during ripening. The fatty acid profile of the samples was mainly composed of palmitic, oleic, and stearic acids. This dairy product is rich in volatile compounds and formed primarily by alcohols (n = 14), acids (n = 13), and esters (n = 11). Olfactometry indicated that the VCs that most affected the aroma of ripened Canastra cheese were acetic acid, isobutyric acid, butanoic acid, and ethyl hexanoate. The method developed effectively discriminated against Canastra cheeses at their different ripening stages.

## 1. Introduction

Artisanal cheeses are among the leading dairy products produced in European and American countries (González-Córdova et al., 2016). In recent years, these products have been in the spotlight due to their social, cultural, and economic values and peculiar characteristics that depend on the region they are produced (Bemfeito et al., 2016). Canastra artisanal cheese is produced in the Brazilian mountain range of Serra da Canastra (Minas Gerais State), obtained from raw milk and "*pingo*" (a natural starter culture made of whey collected from the previous production day and a group of specific microorganisms) and marketed mainly with 22 days of ripening (Campos et al., 2021; Kamimura et al., 2019; Fialho et al., 2018). Unlike other typical Brazilian cheese, such as Serro, Marajó, Campo das Vertentes, Minas Padrão and Coalho (Kamimura et al., 2019; Matera et al., 2018), Canastra cheese has a longer ripening period and, lower water content, which contributes to this product presenting exclusive characteristics. Factors such as weather, altitude, solar incidence, native pasture, and the water of the region contribute to the unique flavor of this cheese (Resende et al., 2011; Silva, Abreu, Magalhães, Pinto, & Piccoli, 2013).

Aroma is considered one of the most important quality attributes of cheese, with consumers using it to either accept or reject the product (Bezerra et al., 2017). During processing, several chemical and biochemical reactions occur simultaneously (Bezerra et al., 2017), and these reactions are catalyzed by endogenous milk enzymes and microorganisms of the native culture responsible for producing many volatile organic compounds that contribute to the aroma. During ripening, glycolysis, proteolysis, and lipolysis are the main biochemical reactions (McSweeney et al., 2017). Secondary events promote the formation of volatile compounds, and the elements that lead to their production are fatty acids and amino acids (Sulejmani et al., 2016).

The volatile profile of cheese can consist of different classes of compounds, including acids, esters, aldehydes, alcohols, ketones, hydrocarbons, terpenes, and sulfur compounds (High et al., 2021; Pluta-Kubica et al., 2021; Bezerra et al., 2017; Ozturkoglu-Budak et al., 2016; Sulejmani et al., 2016). These analytes are present in food matrices at low concentrations, which are quite complex, and present distinct characteristics regarding polarity, vapor pressure, and reactivity (Kataoka et al., 2000). Given the above, sample preparation techniques that are properly optimized to isolate and preconcentrate volatile

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compounds are pivotal.

The headspace solid phase microextraction (HS-SPME) technique is used to extract VCs in different types of cheese (Lopes et al., 2021; Teter et al., 2020; Salum et al., 2017), integrating the extraction, concentration, and introduction of the sample into a single device bypassing the use of organic solvents. The main advantages of this technique are reduced sample use, sample preparation automation and chromatographic analysis, simplicity, high selectivity, and speed. However, several factors that affect the extraction efficiency should be considered for its application, such as the temperature and extraction time, balance of the sample in the vial and desorption of the fiber analyte, ionic force, pH, agitation, sample quantity, sample dilution, and fiber coating material. Hence, the variables to be evaluated depend on the characteristics of the matrix being studied (Moreira et al., 2019; Arcari et al., 2017).

Given the numerous aspects that can influence the response of the HS-SPME system, optimizing this technique is necessary as it ensures that all tested variables contribute to achieving the best analytical performance. For this reason, one can evaluate the effects of one variable at a time, while all other variables are kept constant in analytical optimizations (Bernardi et al., 2014). Nevertheless, this is a time-consuming approach, and there is no information on the interaction among the variables tested in the extraction process. Therefore, multivariate statistical methods, including the Plackett-Burman method (PB) and Central Composite Rotatable Design (CCRD), have been used to optimize analyte extraction (Arcari et al., 2017). This method enables all variables to be simultaneously studied and acquires individual and interactional information, thus reducing the number of runs, time, and cost of analysis (Semren et al., 2018).

Little is known about the multivariate optimization of the extraction conditions of volatile compounds in food using HS-SPME, and there are no studies regarding its application in Brazilian artisanal cheeses using olfactometry and chemometrics. Olfactometric methods enable researchers to successfully identify the active volatile compounds of food (Xiang et al., 2020; Barba et al., 2018; Franitza et al., 2016). Thanks to the application of sensory science, although numerous volatile compounds can be found in food, only a small number is detected by human olfactory receptors that lead to aroma perception (Poisson and Schieberle, 2008). Moreover, even in large quantities, some volatiles do not affect the aroma, whereas others can be significant even in small quantities (D'Acampora Zellner et al., 2008). In this sense, the information on the key-odor volatile compounds in food obtained from GC-O, associated with chemometric techniques seems to be a valuable tool to assist the sample preparation development in foods.

Therefore, the purpose of this study was the multivariate optimization of HS-SPME to extract VCs that affect the aroma of Canastra cheese. This optimization was assisted by chromatographic techniques with olfactometry and chemometric applications. Additionally, the method developed was employed to characterize the volatile fraction of Canastra cheese in three ripening stages (fresh, short-ripened, and ripened) and evaluated for proximate composition and fatty acid profile.

## 2. Materials and methods

### 2.1. Reagents and standards

A series of $C_7$–$C_{30}$ alkanes were acquired from Supelco (Bellefonte, PA, USA), hexane from Alphatec (Macaé, RJ, Brazil), ethyl alcohol, chloroform, potassium hydroxide, and sodium sulfate (PA) from Vetec Química Fina (São Paulo, Brazil), hydrochloric acid and sodium hydroxide from Êxodo científica (São Paulo, Brazil), and sulfuric acid and 2% boric acid solution from Fmaia (São Paulo, Brazil). The C23:0 (methyl tricosanoate) and fatty acid methyl esters (FAME) standards were obtained from Sigma-Aldrich (USA).

### 2.2. Products

The cheese used were produced on a farm in Medeiros (19°59′44″S, 46°13′34″W) in the Serra da Canastra mountain range (Minas Gerais State, Brazil). The production unit was selected according to the criteria of favorable installation characteristics, herd health, milking hygiene, and good manufacturing practices - which represents the traditional cheese-making process - and because it is registered at the Instituto Mineiro de Agropecuária (IMA) and the Associação de Produtores de Queijo Canastra (APROCAN). Furthermore, the expressive production of the region was also considered, as it is one of the largest producers of this type of cheese.

### 2.3. Sample preparation and sampling

Cheese samples (≈ 1 kg each) were manually cut with knives into smaller portions (50 g), packed in opaque polyethylene vacuum packs, and stored at –22 °C until analyses. The entire sample preparation procedure was carried out in a refrigerated environment to avoid oxidation reactions. The cheese was evaluated in three ripening stages (treatments): fresh cheese (no ripening process), short-ripened cheese (10 days of ripening), and ripened cheese (22 days of ripening) (Supplementary Material 1). For each process stage two cheeses were homogenized together in a Philips Walita mixer. The homogenized samples were then used for the analyses in triplicate. Each repetition referred to one processing run (n = 6).

### 2.4. Volatile compound analysis

#### 2.4.1. Optimization of the volatile compound extraction method by HS-SPME

The conditions employed to extract the volatile compounds of the ripened Canastra cheese samples were optimized using headspace solid phase microextraction (HS-SPME) through the PB design to select the variables that influence the extraction. The variables selected for this stage were: sample quantity (g), equilibration time (min), extraction time (min), and extraction temperature (°C). The factor levels and experimental domain are listed in Supplementary Material 2. The experimental planning consisted of eight runs, four more than the number of independent variables, to have enough degrees of freedom to estimate the standard error, and four additional central points to evaluate the repeatability of the experiment, totaling 12 experiments (Rodrigues and Iemma, 2014).

The variables that had significant effects ($p < 0.10$) on extracting volatile compounds from the cheese were evaluated using CCRD. The variables used in this stage were extraction time (min) and extraction temperature (°C). The design was employed to determine the best conditions to extract volatile compounds and consisted of a factorial $2^2$ design with four axial points and three central points, totaling 11 runs performed in random order. The experimental variables, levels, and results of the design are presented in Table 1.

The volatile compounds were isolated from the matrix using a 1-cm SPME fiber (50/30 μm) with a mixed divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS) coating from Supelco (Bellefonte, PA, USA). This fiber was previously chosen in optimization studies of cheese aroma, due to the suitable for the adsorption of volatile compounds from different chemical classes important for this product (Di Donato et al., 2021; Bezerra et al., 2016). The fiber was previously conditioned according to the manufacturer's recommendations. The vials (20 mL) containing the samples were immersed in water at 25 °C for 10 min in order for all samples to be in the same conditions before treatment. The samples were then exposed to the system at the desired equilibration time and temperature.

#### 2.4.2. Chromatographic conditions

After extraction, the analysis was carried out in a Varian Star 3400CX

**Table 1**
Experimental variables, levels, and results for the central composite rotatable design (CCRD) used to optimize the best experimental conditions of HS-SPME.

| Coded variables | | Real variables | | Responses | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $X_1$ | $X_2$ | Temperature (°C) | Time (min) | 1 | 2 | 3 | 4 | 5 | 6 |
| −1 | −1 | 30 | 45 | 6,821,889 | 58,270 | 928,129 | 197,825 | 2,926,920 | 3,064,904 |
| +1 | −1 | 50 | 45 | 7,982,968 | 41,483 | 526,195 | 319,084 | 1,847,596 | 5,439,526 |
| −1 | +1 | 30 | 70 | 7,762,792 | 81,649 | 1,170,761 | 418,932 | 2,835,376 | 3,651,854 |
| +1 | +1 | 50 | 70 | 8,462,052 | 45,488 | 588,557 | 415,363 | 1,709,524 | 5,892,597 |
| −1.41 | 0 | 25.86 | 57.50 | 7,555,073 | 76,544 | 1,194,730 | 283,098 | 3,106,936 | 3,305,515 |
| +1.41 | 0 | 54.14 | 57.50 | 8,155,933 | 33,712 | 533,395 | 354,720 | 1,713,239 | 5,680,008 |
| 0 | −141 | 40 | 39.82 | 7,444,013 | 60,191 | 875,370 | 301,902 | 2,376,775 | 4,086,269 |
| 0 | +1.41 | 40 | 75.18 | 8,713,068 | 72,067 | 982,755 | 420,619 | 2,338,193 | 5,158,224 |
| 0 | 0 | 40 | 57.50 | 7,112,924 | 44,160 | 540,759 | 162,251 | 2,152,166 | 4,591,001 |
| 0 | 0 | 40 | 57.50 | 7,164,959 | 55,315 | 657,938 | 288,630 | 1,724,699 | 4,473,263 |
| 0 | 0 | 40 | 57.50 | 7,355,536 | 65,421 | 738,717 | 145,358 | 2,200,841 | 4,472,538 |

1: Sum of the area of all volatile compounds; 2: Sum of the total area of aldehydes; 3: Sum of the total area of ketones; 4: Sum of the total area of esters; 5: Sum of the total area of alcohols; 6: Sum of the total area of carboxylic acids.

gas chromatograph (CA, USA) equipped with a flame ionization detector (GC-FID) to obtain the arbitrary areas referring to the identified compounds, since this detector presents a wide response range, comprising all analytes in a single chromatographic analysis. The fiber was thermally desorbed in the injector vaporization chamber at 250 °C for 10 min in splitless mode for 1 min, followed by opening the splitter valve at a 1:20 ratio. Hydrogen was used as the carrier gas and kept under constant pressure (15 psi). The chromatographic separation of the compounds was performed on a ZB-WAX plus column (30 m × 0.25 mm, 0.25 μm stationary phase thickness). The initial temperature of the column was adjusted to 35 °C and maintained for 3 min. Then, a temperature rise was initiated, rising 4 °C $min^{-1}$ to 80 °C, followed by a rise of 5 °C $min^{-1}$ to 230 °C, where it was maintained in isothermal conditions for 5 min, totaling 49 min of analysis. The detector temperature was maintained at 250 °C. The chromatographic peaks were integrated, and their arbitrary areas were observed in the stage of method development and comparison between ripening points.

Qualitative analyses were carried out to confirm the identity of the compounds extracted from the samples and conducted in a gas chromatograph (Shimadzu QP 2010 Plus) connected to a mass spectrophotometer (GC/MS) (Shimadzu Corporation, Kyoto, Japan). Analyte desorption was performed as described above. The chromatographic separation of the compounds was performed on a polar fused silica capillary column ZB-WAX Plus (60 m × 0.25 mm; 0.25 μm stationary phase thickness; Zebron, USA) using helium as the carrier gas at the constant pressure of 30 psi and an initial flow of 2.2 mL $min^{-1.}$ The MS was operated in electron ionization mode (70 eV), and the simple quadrupole analyzer adjusted to scan mode (35–350 *m/z*). The chromatographic conditions were the same as those used in the GC-FID. The volatile compounds were identified by comparing the experimental mass spectra with the data available in the National Institute of Standards and Technology (NIST) library, considering over 80% of similarity by comparing the linear retention indices (LRI) calculated with those available in the literature, and a series of homologous n-alkanes ($C_7$–$C_{30}$) were analyzed under the same chromatographic conditions to calculate the retention index. Additionally, some compounds were identified by comparing the retention times of the analytes with those of the authentic standards.

### 2.4.3. GC-Olfactometry (GC-O) analysis

The experiment followed all ethical standards according to Resolution No. 196/96 of the Brazilian National Health Council. The study was approved by the research ethics committee of the Universidade Estadual de Campinas (São Paulo, Brazil) under number 91238418.9.0000.5404.

Volatile compounds in Canastra cheese samples were determined by GC-O. The effluents were evaluated using a Varian 3400 Star gas chromatograph and modified as described by Garruti et al. (2006). The chromatographic separation conditions were the same as those of item 2.4.2, with the modification in the detector. In this case, the column effluent was loaded with compressed air, purified on an activated carbon column (3.5 L $min^{-1}$), and with temperature control (28 °C) up to the analyzer nose. The VC extraction conditions were obtained by the central point (CP) of the CCRD (10 min of equilibration of the headspace of the sample; 1 g of the sample; exposure of the fiber to the sample at 40 °C for 57.5 min; and thermal desorption of the VCs in the GC injector for 10 min).

The evaluators were selected in advance based on interest, consumption frequency of ripened cheese, availability, experience in olfactory analysis, and the ability to recognize and discriminate of odor intensity through sensory tests. The evaluators who were right in at least 80% of the tests were selected. Then, the selected individuals were trained with the food matrix of the study and, sensory description tests of typical cheese compounds through solutions containing pure substances (butanoic acid and ethyl hexanoate) and intensity scales. A total of fifteen participants were recruited, and from them ten evaluators (ages 22–39) were selected to evaluate the chromatographic effluent and instructed to verbally describe the odorous stimuli perceived and mark the start and end times of each stimulus. The evaluators were also asked to rate the intensity of the perceived odor using a scale (0 = no odor; 1 = poorly recognized odor; 2 = moderate odor; and 3 = strong odor). The sample evaluation time was approximately 49 min divided into two parts in order that two participants could evaluate for 24.5 min in each run, thus avoiding olfactory fatigue. All evaluators analyzed the volatile compounds of two chromatographic runs, smelling in different moments the first and the second half chromatographic. According to the data obtained, it was possible to calculate the normalized modified frequency (MF) (Equation (1)) of each compound, rank of the volatile compounds as to their odorous importance (expressed as a percentage), as proposed by Dravnieks (1985) and applied in studies such as Carrascon et al., (2014) and Culleré et al., (2013). The compounds corresponding to each odorous stimulus were identified by comparing their experimental retention indices acquired by GC-FID and compared with those obtained by GC/MS.

$$\mathrm{MF}(\%) = \sqrt{F(\%)\ x\ I(\%)} \quad (1)$$

Where: F (%) is the detection frequency of the volatile compound, and I (%) is the average intensity of the perceived odor.

### 2.5. Proximate composition

The analyses were carried out according to AOAC procedures (2005). The moisture content was determined by weight loss in a vacuum oven (AOAC 925.09/17), ash by muffle furnace incineration at 550 °C (AOAC 923.03), and protein by the Kjeldahl method (AOAC 960.2). The lipid content was determined according to Bligh and Dyer (1959) and carbohydrates by difference. Additionally, the pH was measured by the

potentiometric method.

### 2.6. Fatty acid determination

The lipids extracted by the Bligh and Dyer method (1959) (~20 mg) were transferred to 10 mL test tubes, and 250 μL of the methyl ester solution of tricosanoic acid (C23:0; Sigma-Aldrich, USA) were added in an isooctane solution (4 mg $mL^{-1}$) as an internal standard. The solvents were dried with a stream of nitrogen and a heating block (TE-021, Technal, Brazil) at 40 °C. Acylglycerols were derivatized using the method described by Hartman and Lago (1973). The lipids were added to a 1.5-mL potassium hydroxide solution (0.4 mol/L) in methanol and subjected to a water bath at 100 °C for 10 min. Afterward, the tubes were cooled with running water, followed by the addition of 3.0 mL of sulfuric acid solution (1 mol/L) in methanol. The tubes were placed in a water bath again under the same conditions of time and temperature. Next, the tubes were cooled and received 2 mL of hexane, being homogenized in a vortex (MX-S, SCILOGEX, USA) for 20 s. The upper part of the tube containing the FAMEs dissolved in hexane was removed for chromatographic analysis.

The FAMEs were analyzed using a GC-FID (Varian, Star 3600CX), and 1 μL of the esterified sample was introduced into the injector operating in split mode with a 1:30 ratio and a temperature of 240 °C. Hydrogen was used as the carrier gas at constant pressure (40 psi). The FAMEs were separated in an SP-2560™ capillary column (100 m × 0.25 mm; 0.20 μm stationary phase thickness; Supelco, Inc., Bellefonte, PA, USA). The column heating program started at 140 °C, remaining for 1 min, then increased to 180 °C at 8 °C $min^{-1}$, followed by increasing at 4 °C $min^{-1}$ to 210 °C, and finally increasing at 20 °C $min^{-1}$ until 250 °C, remaining in isothermal conditions for 7 min. The FID was maintained at 250 °C. The fatty acids were identified by comparing the retention times of the analytes with the FAME Mix-37 standards (Sigma-Aldrich, USA). The results were expressed in mg of fatty acid 100 $g^{-1}$ of fat using C23:0 as an internal standard (Visentainer, 2012).

### 2.7. Statistical analyses

The optimization study (PB and CCRD) was evaluated using the Protimiza Experimental Design software. In addition, an exploratory analysis of the data was performed using principal component analysis (PCA) to observe the correlation between the experimental tests of the CCRD and volatile compounds using the statistical program Pirouette 3.11 (Infometrix, 2003). This analysis was performed by normalizing and self-scaling the data to obtain the same magnitude of response. The data ($n = 3$) of proximate composition, fatty acid profile, and volatile compounds were analyzed by analysis of variance (ANOVA) and Tukey's test ($p < 0.05$) using the SISVAR software version 5.6.

## 3. Results and discussion

### 3.1. Variable screening

The variables applied to HS-SPME were evaluated for VC extraction in Canastra cheese. Initially, preliminary assays were carried out to determine the more suitable sample for the optimization study (data not shown). The ripened Canastra cheese was used due to its highly complex volatile profile. Therefore, the PB design was applied to select the variables that affected VC extraction. The response used was the sum of the total area of the chromatographic peaks obtained from each planning test. The effects of the tested parameters on the response variable were assessed using a significance level of $\alpha = 10\%$ to prevent important factors from being excluded from the CCRD (Rodrigues and Iemma, 2012).

Only the extraction time and temperature had significant effects ($p < 0.10$), with the extraction time having a more significant influence in this analysis (Fig. 1). The equilibration time and sample quantity are

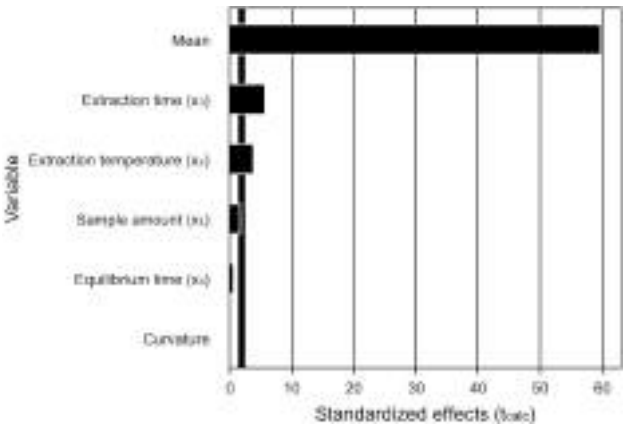



**Fig. 1.** Pareto chart of the standardized effects of the variables studied in selecting the conditions to extract the volatile compounds from Canastra cheese using the Plackett-Burman design.

variables that must be considered when analyzing volatile extraction using HS-SPME (Arcari et al., 2017). Given that the equilibration time and sample quantity had no significant effects ($p > 0.10$) in the studied range, they were fixed at the lowest level (10 min and 1 g, respectively). Some studies have suggested that extraction time differentially influences VC extraction in shorter times, thus favoring extracting VCs of hydrophilic character while exposing the fiber for extended periods and favoring hydrophobic ones (Salum et al., 2017). When considering the effects of temperature, Alpendurada (2000) reported that the behavior is strongly dependent on the properties of the analytes, which can have opposite effects in certain temperature ranges. By increasing the temperature, the diffusion coefficient of the compounds increases; nonetheless, since the extraction is an exothermic process, it also reduces the distribution coefficient of the analytes. Therefore, the temperature and extraction time parameters must be optimized. This information corroborates the results reported herein and other studies that demonstrated that temperature and extraction time are the factors that most affect VC extraction (Semren et al., 2018; Salum et al., 2017).

Desorption conditions must also be assessed to prevent the analyte from being transported to the next stage (Arcari et al., 2017). This variable was not included in the experimental design, although it was tested in isolation in previous experiments. Blank injections were performed in different treatment samples to prevent a residual concentration of the fiber analytes. Thermal desorption in the GC injector occurred for 10 min at 250 °C.

### 3.2. Central composite rotatable design

A CCRD was used to optimize the independent variables selected in the PB design. The response variables considered were: the sum of the total area and the areas of the analytes grouped according to chemical class (aldehydes, ketones, esters, alcohols, and carboxylic acids). The experimental error was calculated using the repetition values of the central point. For the sum of the total area, the coefficient of variation (CV) was 1.77%, and for the sum of the areas of each class of analytes, the average CV was 6.98%.

Regarding the sum of the total area of the peaks, the quadratic model obtained showed an $r^2$ of 90.89%. Analysis of variance showed that the regression was significant ($p < 0.05$), and the model did not present a lack of fit ($p > 0.05$). The regression coefficients were calculated, and the linear and quadratic parameters showed significant positive effects ($p < 0.05$). The extraction time had a stronger influence than the temperature, and the interaction between both variables was not significant ($p > 0.05$).

Considering the results obtained from ANOVA for the sum of the area of each chemical class, the regression was significant ($p < 0.05$),

although the lack of fit was not significant ($p > 0.05$) for all groups studied. Aldehydes, ketones, and alcohols were only negatively influenced by temperature ($p < 0.05$), with an $r^2$ of 67.75, 73.06, and 84.30%, respectively. There were notable effects ($p < 0.05$) of time (linear) and time and temperature (quadratic) in relation to the area of the peaks corresponding to the esters, with an $r^2$ of 74.79%, while the acid extraction efficiency had a solely linear effect in both variables; however, the temperature had a greater effect on volatile extraction ($r^2 = 97.10\%$). All models generated for each chemical class investigated had no significant influence ($p < 0.05$) from the interaction between temperature and extraction time.

Volatile compound extraction in Canastra cheese samples was more efficient at higher temperatures and extraction times (Fig. 2A). Nonetheless, the classes of volatile organic compounds behaved differently in terms of the best extraction conditions (Fig. 2B-2F). Given our findings, the response variables studied presented different optimal regions, and high extraction temperatures appeared to contribute to the formation of compounds from lipid oxidation (Li et al., 2019). This scenario favors compounds with low vapor pressure; therefore, the GC-O technique was used as one of the weighting criteria to select the best extraction conditions for volatile organic compounds that affect the aroma of Canastra cheese.

### 3.3. GC-Olfactometry

The relationship between the volatile profile and elucidation of the main compounds responsible for the aroma can be achieved using gas chromatography combined with different techniques, including GC-O. Hence, it is possible to determine which compounds can affect the aroma of cheese. For this analysis, the extraction conditions of the central point of the CCRD were employed since the volatile compounds profile was not reasonably different from those obtained in other experimental runs.

An exploratory multivariate analysis (PCA) was used to determine the correlation between volatile compounds that influenced the aroma of Canastra cheese and extraction conditions of these analytes in each

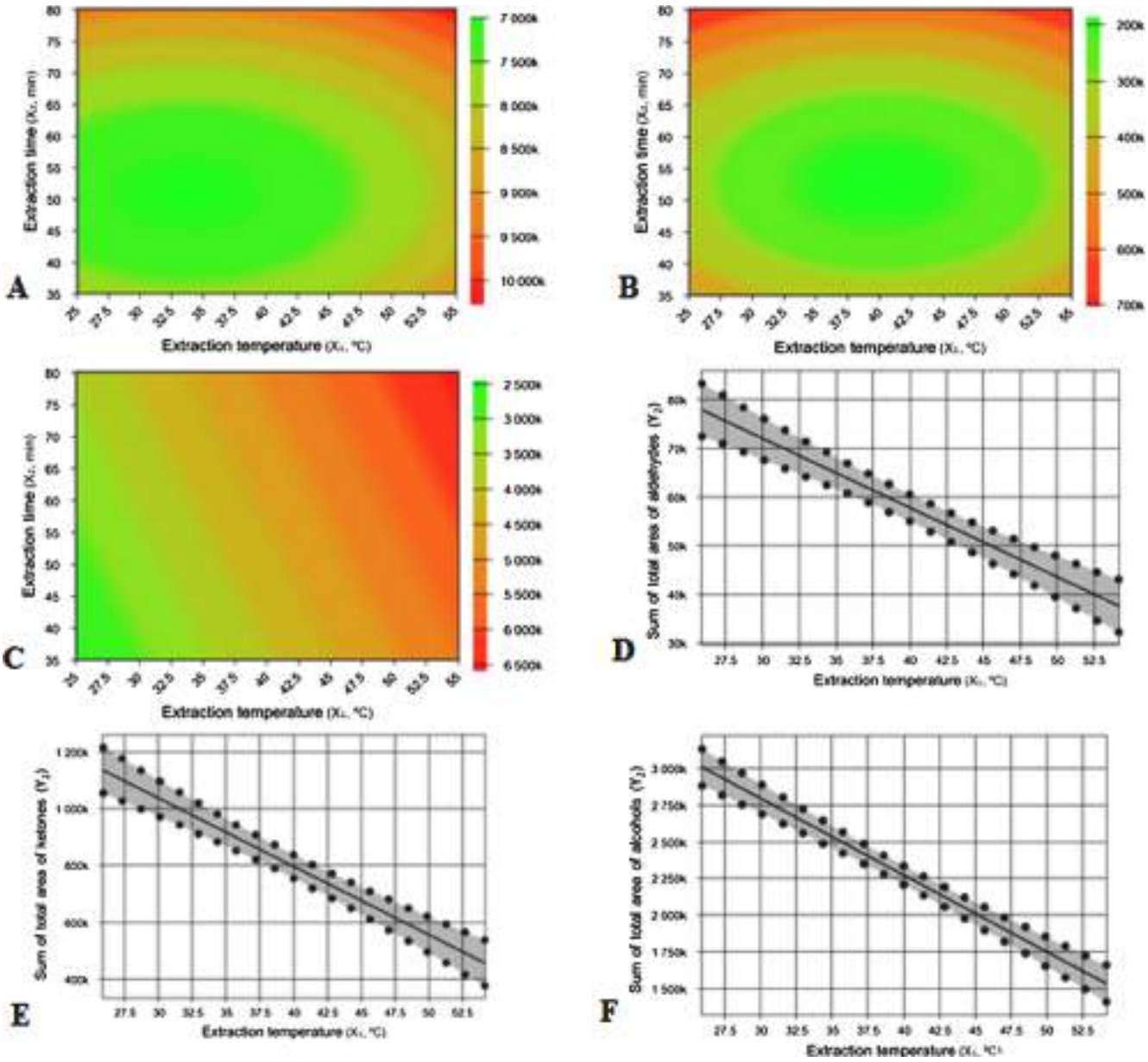



**Fig. 2.** Contour surface chart of temperature and extraction time as a function of the sum of the total area of volatile compounds (A), esters (B) and carboxylic acids (C). Chart of the total area of aldehydes (D), ketones (E) and alcohols (F) as a function of temperature extraction.

experimental run. The data matrix consisted of the areas of the 44 volatile compounds detected in the olfactometric analysis and the 11 runs of the CCRD, referring to the different temperatures and extraction time. The PCA explained 57.9% of the data variance using two principal components (PC), with PC1 explaining 43.72% and PC2 explaining 14.18% of this variation (Fig. 3). In the same figure, the results of modified frequency obtained by the GC-O analysis are presented. With this analysis, it was possible to order the volatile compounds by their odorous importance. Notably, butanoic, acetic, and isobutyric acids and ethyl hexanoate were considered the volatiles that most affected the aroma of Canastra cheese since they had a modified frequency of over 90%. Table 3 lists the results of the GC-O in detail, and these are discussed below in relation to their odorous importance.

The PC1 mainly distinguished the volatile compounds from runs 3 and 5 from runs 4 and 6. The first run were related to the compounds ethyl acetate, 2-butanone, 3-methylbutanal, and 3-methyl-2-butanone, among other compounds allocated in the left quadrants and that have high vapor pressure as a common characteristic. However, runs 4 and 6, which had the highest temperatures, were correlated with the organic acids of linear chains from C3 to C9, isovaleric and phenylacetic acids, ethyl octanoate ester, among others, whose compounds have lower vapor pressure in common.

When evaluating the PC2, the correlation of run 1 with guaiol and 2-ethylhexanoic and 2-methyl-2-butenoic acids can be noted, while run 8 had a more significant correlation with isoamyl acetate. Nonetheless, the volatile compounds of odorous importance were distributed differently at the limits of the chart, presenting median weight in all runs that were allocated to the center (PC1) and positive quadrants (PC2). Therefore, if the choice were for the run that had the largest total area of the chromatogram, the importance of some volatile compounds, such as alcohols, would have been overestimated, and the contribution of less volatile compounds (e.g., 3-methylbutanal, and the *sec*-butyl acetate ester) would have been underestimated.

Based on these findings, the conditions considered ideal for extracting volatile compounds that affect Canastra cheese aroma were those corresponding to run 8 (temperature: 40 °C; extraction time 75.18 min; equilibration time: 10 min; 1 g sample). Given the above, the chosen experimental run employs a mild temperature and enough time to extract the compounds with the lowest vapor pressure (carboxylic acids) while also maintaining good efficiency in extracting the most volatile compounds.

### 3.4. Physicochemical characteristics and fatty acid composition of Canastra cheese during ripening

The average data of pH, water content, proteins, carbohydrates, lipids, ash content, and fatty acids in Canastra cheese samples are listed in Table 2. All variables showed a significant effect ($p < 0.05$) of ripening. The pH decreased during the aging process and varied between 5.12 and 4.60, and researchers have already reported this behavior. Xu et al. (2020) observed that pH values in Xinjiang Kazak cheese oscillated between 4.2 and 3.6 with 20 days of ripening, while Delgado et al. (2012) obtained average pH values between 5.14 and 4.71 in goat cheese ripened for 60 days. This is associated with the catabolic lactose process during the initial stage of cheese ripening through glycolysis. In addition, when lactic acid is produced, fat is degraded by lipase into fatty acids, such as butanoic, octanoic, and hexanoic acids, thus contributing to pH reduction (Delgado et al., 2010).

Loss of water was observed during the ripening process of cheese, with water content varying from 41.04 to 36.87%. In Canastra cheese

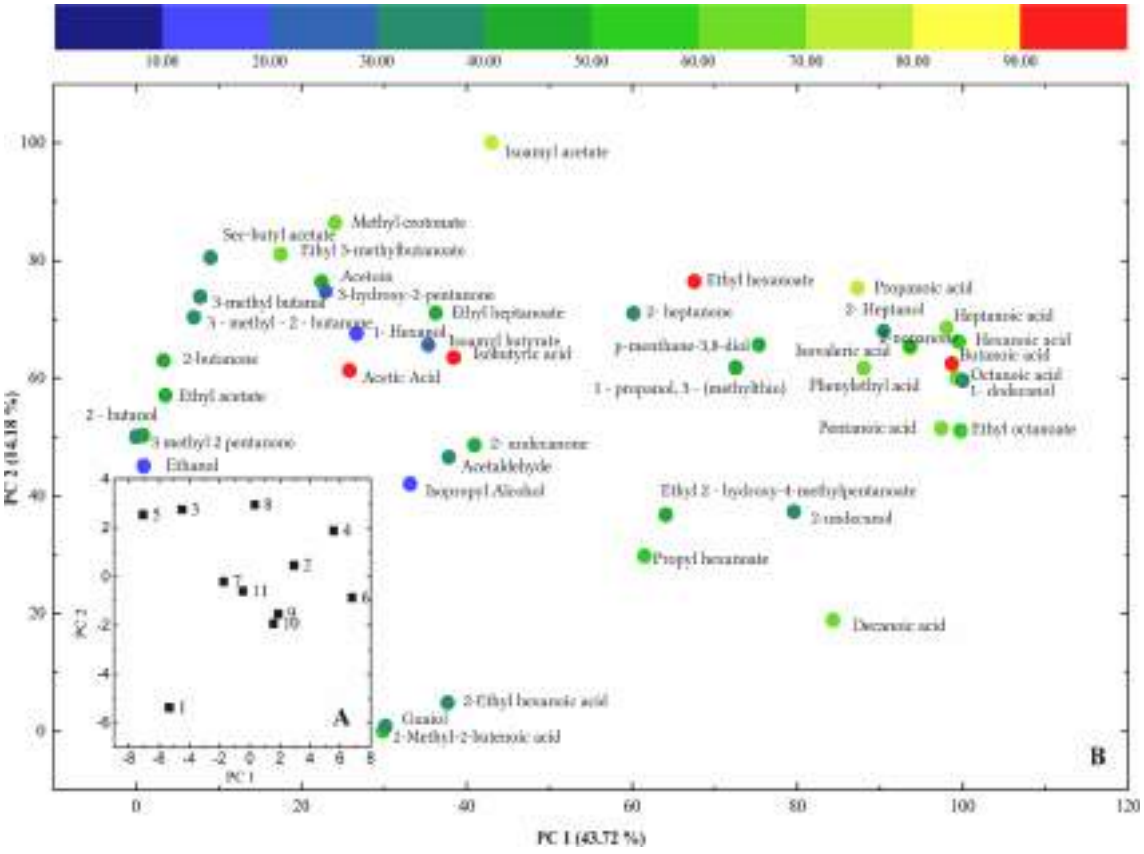



**Fig. 3.** Principal component analysis (PCA) in relation to the experimental runs used in the CCRD and the volatile compounds extracted from Canastra cheese, in addition to the respective range in which the modified frequency value (%) for the different volatiles was found. Scores (A) (extraction conditions) and loadings (B) (volatile compounds) representing the two main components.

**Table 2**
Proximate composition and fatty acid profile of Canastra cheese at different ripening stages.

| | Ripening stage | | |
|---|---|---|---|
| | Fresh | Short-ripened | Ripened |
| pH | 5.12 ± 0.02 [a] | 4.80 ± 0.03[b] | 4.60 ± 0.03[c] |
| Water content (%) | 41.04 ± 0.11[a] | 39.84 ± 0.22[b] | 36.87 ± 0.19[c] |
| *Ash content (%) | 5.57 ± 0.04[c] | 7.53 ± 0.02[a] | 5.87 ± 0.03[b] |
| *Proteins (%) | 41.89 ± 0.46[c] | 43.20 ± 0.63[b] | 45.70 ± 0.23[a] |
| *Carbohydrates (%) | 6.81 ± 0.12[a] | 1.57 ± 0.10[b] | 0.69 ± 0.05[c] |
| *Lipids (%) | 45.73 ± 0.12[b] | 47.70 ± 0.13[a] | 47.74 ± 1.87[a] |
| Fatty acids (mg.100 $g^{-1}$ of fat) | | | |
| C4:0 | 16.0 ± 0.20[c] | 23.0 ± 0.50[b] | 52.0 ± 0.70[a] |
| C6:0 | 13.0 ± 0.19[c] | 19.0 ± 0.35[b] | 25.37 ± 0.29[a] |
| C8:0 | 19.0 ± 0.90[c] | 30.0 ± 0.50[b] | 46.00 ± 0.10[a] |
| C10:0 | 29.0 ± 0.23[c] | 32.20 ± 0.35[b] | 42.70 ± 0.67[a] |
| C11:0 | 0.10 ± 0.03[c] | 0.15 ± 0.02[b] | 0.19 ± 0.01[a] |
| C12:0 | 10.7 ± 0.34[c] | 24.6 ± 0.47[b] | 28.9 ± 0.50[a] |
| C13:0 | 0.12 ± 0.01[c] | 0.16 ± 0.01[b] | 0.18 ± 0.03[a] |
| C14:0 | 56.63 ± 1.92[c] | 68.29 ± 1.01[b] | 99.12 ± 1.72[a] |
| C14:1 | 0.56 ± 0.05[c] | 0.82 ± 0.03[b] | 0.89 ± 0.04[a] |
| C15:0 | 9.90 ± 0.06[c] | 12.1 ± 0.10[b] | 20.8 ± 0.28[a] |
| C16:0 | 190.09 ± 2.58[c] | 212.0 ± 3.37[b] | 260.2 ± 2.30[a] |
| C16:1 | 0.93 ± 0.03[c] | 1.20 ± 0.20[b] | 1.35 ± 0.34[a] |
| C17:0 | 0.25 ± 0.05[c] | 0.33 ± 0.03[b] | 0.44 ± 0.04[a] |
| C17:1 | 0.10 ± 0.01[c] | 0.24 ± 0.02[b] | 0.39 ± 0.03[a] |
| C18:0 | 79.9 ± 2.04[c] | 98.9 ± 2.95[b] | 119.9 ± 1.40[a] |
| C18:1n-9 t | 0.53 ± 0.03[c] | 0.67 ± 0.09[b] | 1.54 ± 0.08[a] |
| C18:1n-9c | 107.3 ± 0.03[c] | 122.25 ± 0.09[b] | 199.64 ± 0.08[a] |
| C18:2n-6c | 0.99 ± 0.27[c] | 1.52 ± 0.22[b] | 1.93 ± 0.45[a] |
| C18:3n-3 | 0.09 ± 0.07[c] | 0.12 ± 0.01[b] | 0.18 ± 0.03[a] |
| ∑ SFA | 424.69 | 520.73 | 695.80 |
| ∑ MUFA | 109.42 | 125.18 | 203.81 |
| ∑ PUFA | 1.08 | 1.64 | 2.11 |

Average ± standard deviation (n = 3). SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids. Averages followed by different letters on the same line differ statistically ($p < 0.05$) using Tukey's test. *Data expressed on a dry basis.

processing, ripening occurs without temperature and humidity control. Therefore, the longer the cheese is exposed to room temperature, the more water content is lost by evaporation, which justifies changes in the texture of the cheeses during this process (Delgado et al., 2012). Regarding ash content, variations were detected in the three treatments studied, with values ranging from 5.57 to 7.53%. This may be related to the sodium content added during the cheesemaking process (Figueiredo, 2006) and how sampling was made (e.g., if the milk used in the cheesemaking process came from different batches, among other factors).

The data on the carbohydrates revealed a reduction in the samples analyzed (6.81–0.69%). The cheese curd contains low residual lactose levels that rapidly metabolize into lactate at the beginning of ripening (McSweeney et al., 2017). Thus, it is plausible that carbohydrate levels reduced due to the degradation of that sugar during ripening. The protein content varied from 41.89 to 45.70% and lipid content from 45.73 to 47.74%. McSweeney et al. (2017) reported that lipolysis and proteolysis reactions occur since the beginning of ripening, although the speed of these biochemical processes depends on the quantity and activity of enzymes, water activity of the product, pH, presence of substrates, and lipolytic action originating, for example, from milk, "*pingo*," and the initiating bacteria, which consequently intensify with ripening.

Regarding fatty acid levels, higher concentrations were observed in the ripened samples compared to fresh cheese, and the total content of saturated fatty acids was much higher than in monounsaturated and polyunsaturated fatty acids. These results corroborate Tekin and Güler (2019) and Vélez et al. (2017), who evaluated cheese made from the milk of different species (cows and sheep). Nineteen fatty acids were detected in Canastra cheeses, the highest concentrations being C16:0 (190.09 – 260.2 $mg.100^{-1}$ of fat), C18:1n9c (107.3 – 199.64 $mg.100^{-1}$ of fat), and C18:0 (79.9 – 119.9 $mg.100^{-1}$ of fat), respectively (Table 3). Seguel et al. (2020) found a similar fatty acid profile in Chanco and Ricotta cheeses produced from cow milk and detected the same predominant fatty acids.

### 3.5. Volatile compounds profile responsible for the Canastra cheese aroma

The composition of the volatile compounds by the chemical group present in Canastra cheese in three ripening stages are listed in Table 3. There were 52 volatile compounds identified: 2 aldehydes, 8 ketones, 11 esters, 14 alcohols, 13 acids, and 4 compounds from other classes.

There was a significant effect ($p < 0.05$) of ripening in acids, with an increase in the area of all analytes during the process. Acetic and hexanoic acids, respectively, were the major volatile compounds in the samples at the three ripening stages. The first is perceived with acid and vinegar notes, and its abundance is the result of the activity of lactic acid bacteria, in addition to being synthesized through the catabolism of citrate and fatty acids (Ianni et al., 2020). The second compound is described as sour, rancid, and goat-like and comes from the lipolytic action. This phenomenon can be explained by the autolysis extension of the starter cells during cheese ripening, with the consequent release of enzymes, especially lipases, which promote lipolysis by cleavage of the ester link between the fatty acid and glycerol in the triacylglycerol (Collins et al., 2003). Therefore, the results obtained were similar to Sulejmani et al. (2016), as acetic acid was one of the main volatile compounds found. Additionally, Ozturkoglu-Budak et al. (2016) also determined acetic acid and butanoic acid as the most abundant during cheese ripening. Sulejmani et al. (2016) determined the volatile profile of Kashkaval cheese and reported that butanoic acid was the most prevalent volatile compound, which was also the case of the present study (Table 3). Additionally, this compound showed significant odorous importance in Canastra cheese due to the rancid and cheesy aroma, in addition to being considered the third most abundant acid in the evaluated samples (Table 3).

2-Butanone was the most abundant compound and one of the most important ketones that impacted the Canastra cheese aroma, being described aromatically as buttery and milky, followed by acetoin (buttery, baked bread, fermentation). There was a decrease in the peak chromatographic area of acetoin during ripening, whereas that of 2-butanone increased ($p < 0.05$). According to Sulejmani et al. (2016), probably the concentration of this analyte is reduced due to the reduction to butanone. Contrary to this study, Domingos et al. (2019) found that the main compounds identified in Prato cheese were diacetyl and acetoin. These discrepant results are likely associated with ripening time and conditions, microbial diversity, chemical composition of the milk, processing method, and whey protein concentration in the raw material during production. The formation of these compounds comes from the oxidation of free fatty acids into keto acids and their consequent decarboxylation into ketones by microbial enzyme action (Bontinis et al., 2012).

Alcohols were the largest group of volatiles detected in Canastra cheese. There was a significant increase ($p < 0.05$) of these analytes at the different ripening stages. Most of these compounds had already been reported by researchers in other types of cheese (Pluta-Kubica et al., 2021; Ozturkoglu-Budak et al., 2016; Sulejmani et al., 2016). Ozturkoglu-Budak et al. (2016) determined the volatile compounds profile of Turkish cheese and, although the extracted alcohols showed the same behavior as the Canastra cheese, the levels tended to decrease after 30 days of ripening. This may be due to the more extended ripening period evaluated (120 days) since, in the present study, only cheese ripened up to 22 days were investigated. 2-Butanol, ethanol, and 3-methyl-1-butanol were the major volatiles during the entire ripening period of Canastra cheese. 2-Butanol is a secondary alcohol formed from diacetyl (2,3-butanedione), especially in cheese made from raw milk (Bontinis et al., 2012). Ethanol originates from the metabolism of lactic acid bacteria and yeasts from milk and the processing environment (Bontinis

**Table 3**
Profile of volatile compounds that contribute to the aroma of canasta cheese in three ripening stages.

| Chemical group | Compounds | $LRI_E$ | $LRI_R$ | Area (uV*s) × $10^3$ Fresh | Short-ripened | Ripened | Aroma description* | MF (%) |
|---|---|---|---|---|---|---|---|---|
| Aldehydes | Acetaldehyde [b] | 871 | 876 | 1.16 ± 0.30[c] | 2.54 ± 0.45[b] | 3.01 ± 0.30[a] | fresh, fruity, fatty | 39 |
| | 3-Methylbutanal [b] | 909 | 909 | 20.39 ± 2.15[c] | 34.68 ± 4.15[b] | 54.65 ± 4.35[a] | fruity, almondy | 32 |
| Ketones | 2-Butanone [b] | 900 | 900 | 239.70 ± 9.56[c] | 458.22 ± 11.99[b] | 629.63 ± 20.75[a] | buttery, milky | 45 |
| | 3-Methyl-2-butanone [b] | 967 | 956 | 10.99 ± 0.99[c] | 16.4 ± 1.53[b] | 21.28 ± 1.56[a] | buttery | 32 |
| | 3-Methyl-2-pentanone [b] | 1006 | 1011 | 15.00 ± 2.66[c] | 20.04 ± 3.05[b] | 46.93 ± 5.04[a] | buttery, rancid | 42 |
| | 2-Heptanone [b] | 1163 | 1164 | 9.82 ± 0.87[c] | 18.35 ± 3.01[b] | 26.97 ± 3.02[a] | soap, roasted | 32 |
| | Acetoin [b] | 1283 | 1282 | 98.56 ± 6.88[a] | 78.95 ± 5.77[b] | 61.30 ± 3.46[c] | buttery, baked bread, fermentation | 42 |
| | 3-Hydroxy-2-pentanone [b] | 1325 | 1327 | 0.65 ± 0.00[c] | 1.01 ± 0.20[b] | 3.01 ± 0.22[a] | buttery, hay, wax, smoke | 26 |
| | 2-Nonanone [b] | 1345 | 1342 | 2.37 ± 0.60[c] | 6.67 ± 0.45[b] | 11.87 ± 0.90[a] | coffee, fruity, earthy, soap, oil | 45 |
| | 2-Undecanone [b] | 1621 | 1623 | 0.12 ± 0.00[c] | 0.33 ± 0.10[b] | 0.48 ± 0.10[a] | mold, dust | 42 |
| Esters | Ethyl acetate [ab] | 875 | 872 | 19.68 ± 2.05[c] | 24.89 ± 3.33[b] | 27.80 ± 4.44[a] | sweet, pineapple | 45 |
| | Sec-Butyl acetate [b] | 975 | 974 | 0.98 ± 0.10[c] | 2.27 ± 0.55[b] | 3.85 ± 0.64[a] | fruity | 39 |
| | Methyl crotonate [b] | 1044 | 1050 | 2.34 ± 0.19[c] | 5.57 ± 0.88[b] | 8.80 ± 0.73[a] | sweet | 63 |
| | Ethyl 3-methylbutanoate [b] | 1076 | 1077 | 1.47 ± 0.24[c] | 4.89 ± 0.69[b] | 8.24 ± 0.99[a] | citric fruity, fruit peel | 63 |
| | Isoamyl acetate [ab] | 1086 | 1096 | 5.79 ± 0.74[c] | 10.95 ± 0.97[b] | 14.19 ± 1.12[a] | banana, sweet | 76 |
| | Ethyl hexanoate [b] | 1321 | 1325 | 98.70 ± 4.44[c] | 156.79 ± 10.48[b] | 208.77 ± 9.45[a] | fruity, sweet, fragrant | 91 |
| | Isoamyl butyrate [b] | 1263 | 1263 | 13.46 ± 3.80[c] | 19.85 ± 2.13[b] | 25.47 ± 1.00[a] | fruity | 26 |
| | Propyl hexanoate [b] | 1307 | 1306 | 6.79 ± 0.55[c] | 10.98 ± 0.88[b] | 12.94 ± 0.69[a] | fruity, floral | 50 |
| | Ethyl heptanoate | 1321 | 1325 | 5.56 ± 0.45[c] | 7.35 ± 0.90[b] | 10.18 ± 1.0[a] | sweet, fruity, fruit peel | 45 |
| | Ethyl octanoate [b] | 1434 | 1435 | 15.42 ± 1.45[c] | 20.06 ± 2.45[b] | 26.99 ± 2.49[a] | fruit, fat | 50 |
| | Ethyl-2-hydroxy-4-methylpentanoate [b] | 1555 | 1545 | 8.76 ± 0.75[c] | 12.45 ± 0.94[b] | 14.40 ± 0.44[a] | fruity, sweet | 42 |
| Alcohols | Isopropyl alcohol [b] | 929 | 921 | 0.99 ± 0.00[c] | 2.05 ± 0.35[b] | 3.59 ± 0.85[a] | alcohol | 18 |
| | Ethanol [b] | 934 | 934 | 127.78 ± 13.45[c] | 223.45 ± 18.33[b] | 503.25 ± 27.89[a] | poignant | 18 |
| | 2-Butanol [b] | 1019 | 1019 | 653.79 ± 11.22[c] | 1073.86 ± 79.45[b] | 1455.04 ± 65.00[a] | oily, alcohol | 32 |
| | 2-Pentanol [b] | 1100 | 1107 | 7.23 ± 0.31[c] | 10.64 ± 1.01[b] | 16.51 ± 1.21[a] | oregano, herbaceous, fruity, cheese | 32 |
| | 3-Methyl-2-butenol [ab] | 1136 | 1126 | 3.33 ± 0.23[c] | 5.68 ± 0.53[b] | 8.13 ± 0.91[a] | – | – |
| | 2-Methylbutanol [b] | 1210 | 1210 | 0.07 ± 0.00[c] | 0.09 ± 0.00[b] | 0.12 ± 0.00[a] | – | – |
| | 3-Methyl-1-butanol [b] | 1216 | 1217 | 102.23 ± 2.45[c] | 193.33 ± 4.54[b] | 246.82 ± 7.43[a] | – | – |
| | 2-Heptanol [b] | 1316 | 1318 | 12.27 ± 1.50[c] | 15.66 ± 2.45[b] | 23.14 ± 2.02[a] | oregano, herbaceous, fruity, cheese | 32 |
| | 1-Hexanol [b] | 1331 | 1336 | 0.84 ± 0.0[c] | 1.09 ± 0.2[b] | 1.31 ± 0.1[a] | green | 18 |
| | 2-Undecanol [b] | 1720 | 1717 | 0.98 ± 0.0[c] | 1.57 ± 0.1[b] | 3.38 ± 0.3[a] | wax, oily | 32 |
| | 1-Propanol, 3-methylthio [b] | 1728 | 1727 | 1.36 ± 0.2[c] | 2.83 ± 0.3[b] | 3.39 ± 0.3[a] | acid, bitter, sulfury | 43 |
| | 1-Dodecanol [b] | 1976 | 1977 | 4.58 ± 0.45[c] | 6.68 ± 0.56[b] | 8.85 ± 0.67[a] | fat, wax | 32 |
| | Guaiol [b] | 2072 | 2065 | 1.26 ± 0.20[c] | 3.48 ± 0.30[b] | 6.27 ± 0.99[a] | oily, floral, fresh, green | 32 |
| | p-Menthane-3,8-diol [b] | 2191 | 2189 | 0.87 ± 0.00[c] | 2.04 ± 0.13[b] | 4.02 ± 0.50[a] | fruity, nuts, flower | 42 |
| Acids | Acetic acid [ab] | 1450 | 1450 | 346.79 ± 10.45[c] | 998.53 ± 36.56[b] | 1421.86 ± 43.23[a] | acid, vinegar | 95 |
| | Propanoic acid [ab] | 1544 | 1540 | 67.89 ± 3.56[c] | 107.37 ± 9.87[b] | 126.35 ± 12.34[a] | poignant, rancid, fermented, boiled potatoes | 78 |
| | Isobutyric acid [ab] | 1574 | 1574 | 45.36 ± 5.45[c] | 96.56 ± 15.67[b] | 116.18 ± 15.98[a] | rancid, cheese | 95 |
| | Butanoic acid [ab] | 1633 | 1633 | 221.00 ± 15.45[c] | 567.33 ± 17.87[b] | 961.03 ± 19.98[a] | rancid, cheese | 95 |
| | Isovaleric acid [ab] | 1676 | 1676 | 123.57 ± 8.45[c] | 345.89 ± 17.78[b] | 587.40 ± 25.65[a] | acid, oxidized oil | 88 |
| | Pentanoic acid [b] | 1749 | 1749 | 2.89 ± 0.33[c] | 4.68 ± 0.95[b] | 8.64 ± 1.12[a] | sweet, sugary, poignant, sour | 65 |
| | 2-Methyl-2-butenoic acid [b] | 1807 | 1804 | 17.59 ± 2.23[c] | 25.99 ± 3.24[b] | 38.55 ± 3.34[a] | burnt, old yerba mate, fermented, acid | 58 |
| | Hexanoic acid | 1859 | 1857 | 547.00 ± 29.98[c] | 963.58 ± 55.56[b] | 1029.47 ± 59.66[a] | sour, rancidity, goat-like | 58 |
| | Phenylacetic acid [b] | 1917 | 1915 | 0.57 ± 0.00[c] | 1.13 ± 0.10[b] | 1.38 ± 0.10[a] | sweet, honey, caramel, earth, floral | 65 |
| | 2-Ethylhexanoic acid [b] | 1930 | 1928 | 1.69 ± 0.10[c] | 2.48 ± 0.2[b] | 4.70 ± 0.4[a] | sour, fermentation, mushrooms | 36 |
| | Heptanoic acid [b] | 1967 | 1966 | 9.85 ± 0.56[c] | 23.57 ± 1.13[b] | 30.80 ± 3.54[a] | fat, sour, rancid | 63 |
| | Octanoic acid [b] | 2090 | 2089 | 90.73 ± 8.96[c] | 113.57 ± 8.86[b] | 219.45 ± 10.23[a] | sweet, cheese | 60 |
| | Decanoic acid [b] | 2282 | 2281 | 1.59 ± 0.2[c] | 4.47 ± 0.5[b] | 7.26 ± 0.5[a] | rancidity, fat | 62 |
| Hydrocarbons | Undecane [b] | 1093 | 1088 | 3.60 ± 0.23[c] | 6.67 ± 0.54[b] | 8.42 ± 0.35[a] | – | – |
| | Decane | 988 | 989 | 0.89 ± 0.0[c] | 1.22 ± 0.1[b] | 2.88 ± 0.25[a] | – | – |
| Sulfur compounds | 3,4-Propylenedioxythiophene [b] | 1004 | 1009 | 8.88 ± 0.78[c] | 12.66 ± 0.98[b] | 16.43 ± 1.45[a] | – | – |
| Ether | Octaethylene glycol monododecyl ether [b] | 2436 | 2427 | 2.56 ± 0.98[c] | 5.54 ± 0.54[b] | 8.39 ± 0.45[a] | – | – |

Average ± standard deviation (n = 3).
*Description of the aroma obtained from GC-olfactometry.
$LRI_E$ = Experimental linear retention index using a capillary column (ZB-WAX Plus).
$LRI_R$ = Reference linear retention index obtained from National Institute of Standards and Technology (NIST).
MF: Percentage of modified frequency.
Averages followed by different letters on the same line differ statistically ($p < 0.05$) using Tukey's test.
[a] Mass spectrum and retention time comparable to the analytical standard.
[b] Mass spectrum; LRI comparable with data from the literature and NIST library.

et al., 2012). 3-Methyl-1-butanol is a branched-chain alcohol resulting from leucine degradation. Alcohols play important roles in the aromatic profile of cheese; however, compared with other chemical groups, these compounds had less influence on aroma perception in the Canastra cheese samples.

Among the volatile compounds that make up the aroma of cheese, in general, esters and aldehydes are easily volatile and have a low perception threshold (Bezerra et al., 2017; Irigoyen, Ortigosa, Juansaras, Oneca, & Torre, 2007), significantly influencing the aroma of foods. Esters have fruity, floral, sweet, and fragrant aromas (Table 3), minimizing the perception of undesirable odors caused by some aldehydes, carboxylic acids, and amines (Bontinis et al., 2012). Among the 11 esters identified, ethyl hexanoate had a larger area, and ripening influenced most esters significantly ($p < 0.05$). Hence, our findings corroborate Domingos et al. (2019), who studied volatile compound formation during the ripening of Prato cheese with reduced fat and co-aggregated with whey protein for 210 days. According to McSweeney (2004), ethyl hexanoate formation occurs through the esterification of hexanoic acid with alcohol. This confirms the increase in the concentration of this fatty acid concomitantly with the increase in the corresponding ester concentration during ripening. The aldehydes acetaldehyde and 3-methylbutanal were identified in the cheese samples. The first volatile compound presented fresh, fruity, and fatty notes, and the second one had fruity and almondy traits. There was a significant increase ($p < 0.05$) in the peak area for aldehydes during the ripening process. It is believed that this behavior may be associated with a short ripening time (22 days) because, in other studies that used long ripening times, aldehyde content increased up to a certain point, but, as they were transitory compounds, they were converted into primary or oxidized alcohols corresponding to the acids in the last ripening stages (Bontinis et al., 2012).

## 4. Conclusion

This is the first study to present data on the behavior of volatile compounds during the ripening of Canastra cheese and report the aroma descriptors of these analytes in this food matrix. Multivariate optimization, with the aid of sensory information by olfactometry and chemometrics, was adequate to determine the best extraction condition of compounds that affected Canastra cheese aroma: extraction time (75.18 min) and temperature (40 °C), equilibration time (10 min), and sample amount (1 g). The method extracted and identified 52 volatile compounds that were mostly esters, alcohols, acids, aldehydes, and ketones. Considering the abundance of volatiles present in cheese and the study of olfactometry, acetic acid, isobutyric acid, butanoic acid, and ethyl hexanoate were considered the key aromatic compounds, attributing notes of rancidity, cheese, vinegar, fruit, sweetness, and fragrance to the aroma. The volatile and fatty acid profiles and proximate composition of the analyzed samples were modified during ripening. Nevertheless, further research is required to better assess the metabolomic changes of Canastra cheese during longer ripening periods.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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# Fungal growth, patulin accumulation and volatile profile in 'Fuji Mishima' apples under controlled atmosphere and dynamic controlled atmosphere

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**ABSTRACT**

The objective of this study was to evaluate fungal and patulin contamination, together with its correlation with the volatile compounds (VCs), in 'Fuji Mishima' apples (up to 25% decayed) under controlled atmosphere (CA) and dynamic controlled atmosphere with respiratory quotient (DCA-RQ) of 1.3 combined with different partial pressures of carbon dioxide (0.8, 1.2, 1.6 and 2.0 $pCO_2$). Fruits were stored under the above conditions for 8 months at 0.5 °C plus 7 days shelf life at 20 °C. Toxigenic fungi and patulin accumulation were found in apples from all treatments. *Penicillium expansum* was the most prevalent species. For all storage conditions, patulin concentrations were above the maximum level allowed in Brazil (50 μg $kg^{-1}$) with an exception of DCA-RQ1.3 + 0.8 kPa $CO_2$. This condition, with lower $pCO_2$, showed the lowest patulin accumulation, below the legal limit. The CA provided the highest patulin concentration (166 μg $kg^{-1}$). It was observed that fungal growth could also contribute to changes in the volatile composition. Styrene and 3-methyl-1-butanol are considered *P. expansum* markers in some apple cultivars and were detected in the samples. However, it was not possible to identify volatile organic compounds (VOCs) that are biomarkers from *P. expansum*, because there were other fungi species present in all samples. In this study, styrene, n-decanoic acid, toluene, phenol and alpha-farnesene were the compounds that showed the most positive correlation with patulin accumulation. On the other hand, a negative correlation of patulin with acids has been shown, indicating that in treatments with a higher patulin concentration there were less acidic compounds.

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## Introduction

Apple is an important crop worldwide, with an estimated production of 75.8 million tons in the 2019/2020 harvest (FAO 2020). Factors such as adapted varieties' production, some regions with favourable climate conditions, as well as concerns about productivity and conservation have made Brazil a major world producer. According to FAO (2020), in the 2019/2020 harvest, Brazilian apple production was 1.3 million tons.

Apple industries usually process the fruit throughout the year, which requires storing the fruit for several months. In order to prevent fruit decay, increased shelf-life and preserve organoleptic characteristics, controlled atmosphere (CA) storage strategies have been developed. Conventional CA storage of apples consists of fruit preservation under low temperature, relative humidity control and the static maintenance of low oxygen ($pO_2$) and higher carbon dioxide ($pCO_2$) partial pressures in the storage chambers. However, after prolonged storage under static CA quality losses occur, such as higher decay incidence compared to modern technologies (Both et al. 2017). To solve this problem, a new method for apple storage using dynamic controlled atmosphere (DCA) conditions, based on the respiratory quotient (DCA-RQ) was recently developed and studied in Brazil and other countries (Weber et al. 2015; Bessemans et al. 2016; Both et al. 2017; De Anese et al. 2020). DCA-RQ monitors the lowest oxygen limit (LOL) by the ratio between $CO_2$ released and $O_2$ consumed during respiratory metabolism. Some studies have evaluated

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DCA-RQ and showed high-quality maintenance after long-term storage when compared to CA and DCA-CF (chlorophyll fluorescence) (Brackmann et al. 2015; Thewes et al. 2017).

However, even in CA and DCA, losses occur during prolonged storage, especially by fungal decay (Watkins 2010). The $pO_2$ and $pCO_2$ are both important for the growth of fungi as they are aerobic organisms. Some fungi could grow in an atmosphere containing $pO_2$ from 0.1 to 0.2 kPa, or in an atmosphere containing more than 80 kPa of $CO_2$ (Taniwaki et al. 2009).

Postharvest diseases cause significant economic losses in apple production, estimated to range from 30 to 40% in developing countries, reaching 60% in more extreme cases (Naets et al. 2018). Blue mould decay caused by *Penicillium expansum* is one of the main causes of postharvest losses in stored apples (Tannous et al. 2018). During growth on fruit, *P. expansum* can produce patulin, a secondary metabolite with mutagenic, carcinogenic, teratogenic and embryotoxic potential effects on humans (Puel et al. 2010). The International Agency for Research on Cancer (IARC) classifies patulin as a carcinogen in group 3 – confirmed animal carcinogen with unknown relevance to humans (IARC, 2019).

Mycotoxins legislation differs not only according to country but also according to the food. In Brazil, the legislation states a maximum patulin level of 50 μg $kg^{-1}$ for apple juices and pulps (ANVISA 2011).

Recently, results published by dos Santos et al. (2018) showed that in 'Fuji Kiku' apples, there was no significant difference among CA, DCA-CF and DCA-RQ 1.3 for patulin accumulation. All decayed fruits of this cultivar presented patulin contamination with values below the maximum limit of patulin, 50 μg $kg^{-1}$, after 9 months of storage plus 7 days of shelf life, undertaking with 1.2 kPa $pCO_2$. The literature does not describe completely the effects of the $CO_2$ concentrations on fungal growth, for the reason that these effects are very specific to each species. According to (Taniwaki et al. 2009), depending on the $CO_2$ concentrations, it can become an inhibitor in fungal development. Thus, it is important to study the effect of different $pCO_2$ on the decay incidence and patulin accumulation of apples stored under a dynamic controlled atmosphere, especially for recent technologies of DCA-RQ.

Even though the practice of processing mouldy fruit is avoided in many countries, spoilt fruit can still be incorporated into the process line, especially when fungal decay is incipient or not detectable by the standard quality controls performed by apple industries (Patriarca 2019). Thus, contaminated apples are directed to industrialisation, mainly for the preparation of juices that are exported as concentrate. Several studies reported patulin concentrations in apple juices and other apple by-products exceeding the limits established by regulatory agencies (Oteiza et al. 2017).

In addition, for mycotoxins development, fungal growth can lead to changes in appearance and colour as well as to off-flavours production (Samson et al. 2004). Some specific volatile organic compounds (VOCs) are identified in infected fruits (Vikram et al. 2004a, 2004b; López et al. 2015). Vikram et al. (2004a) demonstrated that *P. expansum* infection modified the volatile profile from 'McIntosh' and 'Cortland' apples. The total ester content of the fruit was increased by infection, while the alcohol and aldehyde content decreased (Vikram et al. 2004b). López et al. (2015) reported that (Z)-3-hexenyl 2-methylbutanoate was only detected in 'Golden Smoothee' apples when infected by *P. expansum* and *Rhizopus stolonifer*. The volatile profile of microorganisms can reveal their metabolic specificity, and it provides the potential for discovery of unique metabolic products that are host-specific markers.

However, there is little information on the capability of *P. expansum* and other fungi to infect and produce patulin, as well as VOCs in apples stored under DCA-RQ combined with several $pCO_2$. So far, the published works about fungi, mycotoxins, VOCs and storage conditions studied specific inoculated strains presented no data regarding 'real apple samples', with several fungal populations, that would be destined for industrialisation. In this work, fungal presence, patulin production and VOCs metabolic specificity were investigated in 'Fuji Mishima' apples under CA and DCA-RQ with different $pCO_2$.

## Material and methods

### *Plant material and treatments*

The experiment was carried out at the postharvest research centre of the Federal University of Santa Maria (UFSM), Brazil. The experimental materials

consisted of 'Fuji Mishima' apples harvested in 2018 from commercial orchards in Vacaria city (Rs, Brazil). The fruit that presented any kind of visible injury or defect were excluded, and the remainder were randomised into groups. Treatments were applied in experimental CA/DCA chambers with a volume of 233 L.

The experiment was conducted in a randomised design with four replications per treatment. The treatments were composed of five storage conditions: i) Controlled atmosphere (CA) with 1.0 kPa $O_2$ and 1.2 kPa $CO_2$; ii) DCA – RQ1.3 and 0.8 kPa $CO_2$; iii) DCA – RQ1.3 and 1.2 kPa $CO_2$; iv) DCA – RQ1.3 and 1.6 kPa $CO_2$; and v) DCA – RQ1.3 and 2.0 kPa $CO_2$. Four replications of 25 fruits were used for each treatment. The fruits were stored for 8 months.

### CA and DCA establishment, monitoring and controlling

During the entire storage period, the temperature was daily monitored by a mercury thermometer inserted into the tissue of individual fruit. For this purpose, additional apples were used in parallel with the test groups (maintained at 0.5 ± 0.1 °C). Psychrometers with mercury thermometers were used to monitor the relative humidity in the chambers and maintained at 94 ± 2%. The $pO_2$ of each mini-chamber was reduced inside the experimental chambers by flushing them with $N_2$ until 5 kPa $O_2$ was reached. The $pO_2$ was decreased stepwise over a period of 5 days until reaching 1.2 kPa for CA stored apple, and 0.8 kPa $O_2$ for DCA-RQ stored apple. Gas concentrations were monitored and corrected using a device for automatic control of the atmosphere (Valis®, Lajeado, RS, Brazil). This equipment corrected $pO_2$ by injecting cold atmospheric air and absorbing excess $CO_2$ via circulation through a $CO_2$ absorber containing hydrated lime until the desired concentrations were reached.

In DCA-RQ1.3 treatments, concentrations were determined twice a week during 8 months of storage. For that, partial pressure of $O_2$ and $CO_2$ were measured immediately before and after the devices for automatic control of the atmosphere from these chambers were turned off. After 13 h, the RQ was calculated as the ratio between $CO_2$ produced and $O_2$ consumed within this period. The $pO_2$ was controlled by RQ variation. If the calculated RQ was higher or lower than the pre-established value (RQ1.3), $O_2$ levels were increased or decreased by ± 0.02 kPa to reduce or induce anaerobic metabolism and RQ, respectively. After RQ measurement, the $pO_2$ (with updated set-point) was controlled automatically by the device as controlled on CA storage (Weber et al. 2015). The $pCO_2$ of each DCA-RQ treatment was adjusted as described above for CA.

### Sampling protocol for fungal identification, patulin and COVs determination

After 8 months of storage plus 7 days' shelf-life at 20 °C, decay incidence and severity were evaluated in order to standardise the sampling for subsequent quality analysis. Decay incidence was evaluated by counting fruit showing typical fungal lesions larger than 5 mm in diameter, according to Brackmann et al. (2015) and severity was evaluated according to fruit area affected indexes by decay: index 1: 0–25% of the surface area of decayed fruit; 2: 26–50% of the surface area of decayed fruit; 3: 56–75% of the surface area of decayed fruit and 4: 76–100% of the surface area of decayed fruit. In order to simulate apples that can go to the processing of the industry, fruits of index 1 (0–25%) were chosen to carry out the quality analysis.

### Fungal identification

Aseptically, 10 g of each triturated apple sample was weighed, 90 mL of 0.1% peptone water added and then homogenised in a Smasher (BioMérieux, Brazil), for 1 min. Subsequently, serial dilutions were made, and aliquots of 100 µL were inoculated by using the technique of surface plating in Petri dishes containing 18% Dichloran Glycerol Agar with chloramphenicol (DG18). Following this, plates were incubated at 25 °C for 7 days.

After the incubation period, the colonies were counted, and results were expressed in logarithm of colony-forming units per gram of sample (log CFU/g). Finally, taxonomic identification of genera was performed on plates containing specific culture media for identification. After following the regimen of cultivation (time, temperature and culture media) recommended by each identification manual, macro and microscopic colonies characteristics (colony size, ornamentation, colour, colony

exudate, pigment production, etc.) were observed and appropriate identification keys were used. *Penicillium* spp. were identified according to Frisvad and Samson (2004) and Pitt (2000). *Aspergillus* spp. and other genera were identified according to Pitt and Hocking (2009).

### Patulin determination

Patulin determination was conducted using the method developed and validated by Dias et al. (2019). Apples (up to 25% decayed fruit) were homogenised in a multiprocessor (whole fruit) and 10 ± 0.1 g extracted with ethyl acetate (10 mL) containing 30 µL of acetic acid followed by addition of 4 g of sodium sulphate in a 50 mL polypropylene tube. Mechanical shaking was performed for 5 min, and after centrifugation (4500 rpm for 5 min), an aliquot of 4.0 mL upper layer was pipetted into a tube containing 0.6 g sodium sulphate and 1.2 g of Bondesil silica (40 µm), used as a dispersive cleanup step. Manual shaking was performed for 1 min and centrifuged at 4500 rpm for 5 min. An aliquot of 2.0 mL was evaporated to dryness at 65 °C and 0.5 mL of aqueous acetonitrile solution (25%) was added to reconstitute the extract, which was submitted to chromatographic analyse. Each storage treatment was analysed in three replicates, and each replicate was extracted in triplicate.

An ultra-performance liquid chromatography model Acquity, coupled to a mass spectrometer model Xevo TQS (UPLC-MS/MS) from Waters (Milford, USA) using electrospray ionisation in negative mode, was employed. A BEH C18 (100 mm × 2.1 mm i.d.; 1.7 µm particle size) the chromatographic column was used at a constant temperature of 30 °C. Mobile phases were water with 2 g $L^{-1}$ ammonium formate and acetonitrile (80:20 v/v) using isocratic elution and a constant flow rate of 0.3 mL $min^{-1}$. A volume of 10 µL sample was injected into the chromatographic system. Run time was 5 min and patulin retention time was 2.4 min. Mass spectrometer ion source parameters were: capillary voltage 3.0 kV, source temperature 150 °C and the desolvation gas temperature 400 °C. The flows rates for desolvation gas and cone gas ($N_2$) were set at 500 and 150 L $h^{-1}$, respectively. Two mass transitions were monitored for quantification (153→109) and for confirmation (153→125) with cone voltage 35 V and collision energy 9 eV. Ions were fragmented by collision with argon at 0.15 mL $min^{-1}$. For instrument control, data acquisition and processing MassLynx and TargentLynx software 4.1 version were used. Patulin concentration was expressed in µg $kg^{-1}$ of apple analysed.

### Volatile organic compounds profile

The VOCs of decayed apples (up to 25%) were extracted by the headspace solid-phase micro extraction (HS-SPME) technique, according to Both et al. (2014). First, whole apple samples were homogenised in a multiprocessor and centrifuged. The juice was immediately stored at −30 °C until GC analyses. A 4.0 mL aliquot of juice apple, 20 µL of a 3-octanol standard solution (8.22 µg $mL^{-1}$) and 1 g of NaCl were transferred to a 20 mL glass vial. Divinylbenzene/carboxen/polydimethylsiloxane fibre (DVB/Car/PDMS, 50/30 µm × 20 mm, Supelco, Bellefonte, USA) was used for extraction. VOCs extractions were performed at 35 ± 0.2 °C in a water bath for 60 min, under stirring. Prior to exposure of the fibre to headspace, the vial containing the samples was maintained at the same temperature as extraction for 5 min to reach the extraction temperature. VOCs were thermally desorbed into the GC injection port.

The determination was carried out using a Shimadzu QP2010 Plus gas chromatography coupled to a mass spectrometer (GC/MS). The injector operated in a splitless mode for 1 min with an SPME liner (0.75 mm i.d.). The injector temperature was set to 250 °C. The carrier gas was helium at an initial flow rate of 2 mL $min^{-1}$ (constant pressure of 68.9 kPa). A ZB-WAX plus capillary column (60 m × 0.25 mm, 0.25 µm film thickness; Phenomenex, Palo Alto, CA, USA) was used for VOCs separation. The oven temperature was held at 35 °C for 2 min and raised to 80 °C at 2 °C $min^{-1}$, then programmed to rise to 150 °C at 4 °C $min^{-1}$, and then to 230 °C at 8 °C $min^{-1}$ and held in this temperature for 5 min. MS was operated in electron ionisation (EI) mode at 70 eV, scanning from m/z 35 to 350. A series of homologous *n*-alkanes was analysed under the same conditions to calculate the linear retention index

(LRI). The VOCs were identified by comparison to experimental mass spectra to those of commercial spectra databases (NIST 14) and by comparing experimental retention index (RI) with the literature. VOCs were semi-quantified using internal standardisation with addition of 3-octanol as described by Both et al. (2014) and results were expressed in μg $L^{-1}$.

### Statistical analysis

Data were submitted to analysis of variance (ANOVA), and means of treatments were compared by Tukey test ($p < .05$). This analysis was performed using the Statistica 7.0 software (StatSoft Inc., Tulsa, OK, USA). In addition, data were submitted to a Pearson correlation to find VOCs correlated with patulin using the MetaboAnalyst 5.0 software.

## Results and discussion

### Fungal identification

Regarding identification of fungal contamination, a total of 6 genera and 3 filamentous fungi species were identified (as well as yeasts) in the decayed apple samples after 8 months of CA and DCA storage plus 7 days shelf-life at 20 °C. *Penicillium expansum* was the most prevalent species in 'Fuji Mishima' apples (73%). *Cladosporium* spp., *Fusarium* spp., *Alternaria* spp. and *Wallemia* spp. were also found (Table 1).

Some fungal genera detected in this study are potential mycotoxin producers and should be considered as biological hazards (Pitt et al. 2013). Patriarca (2019) reported that when mycotoxigenic species such as *Penicillium* spp., *Alternaria* spp. or *Fusarium* spp., are the causal agents of apple spoilage, the potential risk of mycotoxin contamination is high, and it intensifies if these toxigenic species are able to grow during storage under refrigeration temperatures.

The main interest in identifying *Penicillium* sp. and/or *P. expansum* strain is because this fungal species is directly related to the contamination and deterioration of apple and apple products, besides patulin production. *P. expansum* is considered the most significant postharvest pathogen of apples (Tannous et al. 2018). Thereby, apple contamination with mycotoxigenic fungal species entails risk for consumers. Our results show that in all treatments *Penicillium* sp. and/or *Penicillium expansum* were found.

**Table 1.** Fungal contamination in 'Fuji Mishima' decayed apples (n = 15).

| Fungi | (FO%)* |
|---|---|
| *Alternaria* sp. | 6.7 |
| *Cladosporium* sp. | 73.3 |
| *Fusarium* sp. | 13.3 |
| *Mycelia sterilia* | 13.3 |
| *P. expansum* | 73.3 |
| *Penicillium* sp. | 66.7 |
| *Wallemia* sp. | 6.7 |
| Yeasts | 100.0 |

*FO: frequency of occurrence: number of samples that the microorganism was present / total number of apple samples × 100.

Regarding *Alternaria* sp., species from this genus can synthesise a high number of secondary metabolites (around 70) (López et al. 2016). In spite of this fungus being found in only two samples in this study (Table 1), it is necessary to emphasise its importance. Among mycotoxins produced by *Alternaria* sp., the most relevant are the alternariols (alternariol, alternariol monomethyl ether) and tenuazonic acid. These toxins represent a new challenge for the apple industry since its main disease, mouldy core (inner decay), goes unnoticed until reaching consumers or processing (Patriarca 2019). So far, there are currently no limits for *Alternaria* toxins presence in apple-based products, and no information is available on how processing steps affect their prevalence in them. The European Food Safety Authority (EFSA) has recently drawn attention to *Alternaria* toxins by demanding occurrence data in food products in order to make decisions on the need to establish regulatory limits (Arcella et al., 2016). Therefore, these fungi and toxins need more studies to provide more detailed information.

The presence of other fungi like *Fusarium* spp., *Cladosporium* spp., *Wallemia* spp., and *Mycelia sterilia*, can be associated with field contamination. In relation to yeasts presence (found in 100% of samples), it can be associated with the production of VOCs (Oro et al. 2018).

The majority of the works already published regarding new storage technologies, including DCA-RQ, report data only of decay incidence (Thewes et al. 2017; Donadel et al. 2019). In this study, in addition to decay incidence, severity and

fungal quantification were determined in decayed apples. Figure 1a shows the fungi concentration found in each CA and DCA condition studied. No significant difference was found among treatments applied in 'Fuji Mishima' apples. This result was expected because all samples are of the same severity scale (up to 25%).

Comparing only *Penicillium* spp. including *P. expansum* presence in samples, no differences were found in 'Fuji Mishima' apple from the lowest mean of contamination (1.8 log CFU/g in DCA-RQ1.3 + 2.0 kPa $CO_2$) to 3.5 log CFU/g in DCA-RQ1.3 + 1.2 kPa $CO_2$ (Figure 1b). This result shows that storage under CA or DCA-RQ1.3 with different $pCO_2$ did not influence the growth of these fungi.

The majority of fungi are aerobic; however, some fungi cause deterioration in an atmosphere containing only 0.1 to 0.2 kPa $O_2$, or in an atmosphere containing more than 80 kPa $CO_2$ (Taniwaki et al. 2009). In our study, even the extreme low oxygen reached by DCA-RQ 1.3 with four different treatments of $pCO_2$ during 8 months of storage plus 7 days of shelf-life, it was not possible to avoid the presence of these fungi.

A key factor involved in fungal infection is the length of the storage period and its environmental characteristics. The temperature, commonly in the range of −2 to 5 °C, select psychotropic fungi over those that cannot adapt to survive or grow at low temperatures (Patriarca 2019). In the present study, 'Fuji Mishima' apples were stored for 8 months at 0.5 °C where psychotropic fungi like *Penicillium* sp. can survive and grow.

Together with atmosphere and temperature, relative humidity and water activity are important factors in fungi growth. Most fungi require relative

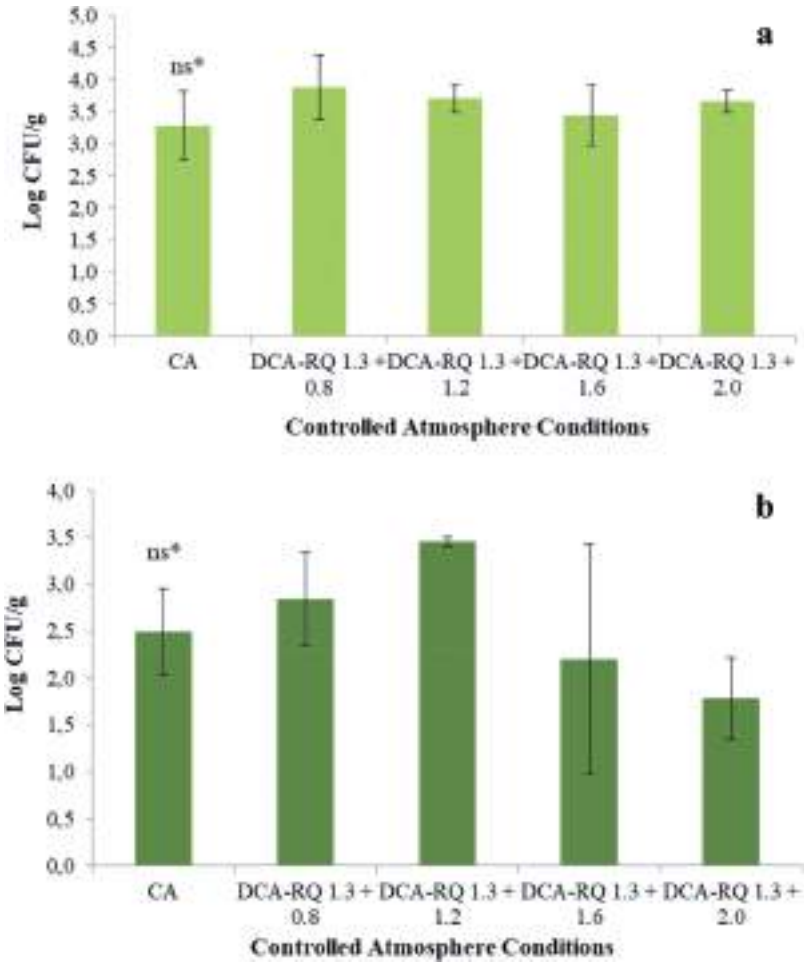



**Figure 1.** Fungal contamination (Log CFU/g) in 'Fuji Mishima' apples (a). *Penicillium* sp. including *P. expansum* contamination (Log CFU/g) in 'Fuji Mishima' apples. ns: no significant (b). CA: controlled atmosphere; DCA-RQ + kPa $CO_2$: dynamic controlled atmosphere with respiratory quotient.

humidity above 80% and minimum water activity (aw) to grow. In the present study, a fixed relative humidity of 94% was used for all treatments. *Penicillium* sp. grows in water activities (aw) between 0.83 and 0.99. Although *P. expansum* is cited as the main producer of patulin in apple juices, other species of *Penicillium* may be more toxigenic, and the industry should be alert to the possibility that these other species are also responsible for patulin occurrence (Welke et al. 2009).

According to Bruzone (2019), those fruits that do not meet quality standards for retailing therefore, be discarded for fresh consumption, are usually destined to processing. Patriarca (2019) indicates that the percentage of fruit destined for industrialisation varies among countries, and it depends on several factors, such as internal demand for fresh fruit, export trade and consumer preferences for different cultivars.

Prior to processing apples for juice production, the European Commission N°2003/598/EC recommends that all decayed fruit, even those with only small areas of rot, should be eliminated (EC 2003). To help reduce food waste, it would be useful to only discard the decay portion of the fruit and any unsafe surrounding areas (i.e. potentially containing mycotoxins). However, this cannot be done systematically in the industry, but trimming recommendations can be provided to the consumer (Coton et al. 2020).

This point is a very important, and according to our findings, even using modern storage technologies, the shelf-life can be decisive for growth fungal and, in this study, toxigenic species were found in apples from all treatments. Therefore, studies that report the mycotoxicological quality of these fruits are necessary in Brazil, because apple production and consumption of apple products is very relevant in the country.

### Patulin concentrations

None of the storage techniques studied was able to avoid the presence of patulin in 'Fuji Mishima' apples. In Supplementary Material 1, chromatograms of the quantifier (a) and qualifier (b) ions for patulin obtained from the matrix (apple) analytical curve are shown.

All decay apples (up to 25% of severity) contained patulin after 8 months of CA and DCA storage conditions plus 7 days shelf-life at 20 °C. According to Patriarca (2019) several pathogens, which have already colonised the interior of the apple, are capable of developing symptoms during the controlled atmosphere, and in some cases, even so, synthesise mycotoxins during storage. This is in agreement with our study because in 100% of the samples, it was possible to detect the presence of patulin. In addition, this fact is consistent with previous reports, when exposure to room temperature after cold storage leads to a rapid patulin formation in damaged tissue (evolution in decay severity) (Fallik et al. 2001).

For all storage conditions of 'Fuji Mishima' apples, patulin concentrations were above the maximum level allowed (50 μg $kg^{-1}$, ANVISA 2011) with the exception of DCA-RQ1.3 + 0.8 kPa $CO_2$. Mean values were 166, 45, 68, 97 and 62 μg $kg^{-1}$ for CA, DCA-RQ1.3 + 0.8 kPa $CO_2$, DCA-RQ1.3 + 1.2 kPa $CO_2$, DCA-RQ1.3 + 1.6 kPa $CO_2$ and DCA-RQ1.3 + 2.0 kPa $CO_2$, respectively (Figure 2). CA showed a higher patulin concentration as compared to all DCA conditions (166 μg $kg^{-1}$). Probably, this condition caused increased stress in fungi, which led to secondary metabolite production, including mycotoxins, which may explain the role of $O_2$ and $CO_2$ in apples.

Studies reporting the influence of CA and DCA on patulin production are recent (dos Santos et al. 2018). In 'Galaxy' apple, after 9 months stored under DCA-RQ1.3 + 1.2 kPa $CO_2$ plus 7 days shelf-life at 20 °C, patulin concentration was found below the maximum allowed concentration (26.6 μg $kg^{-1}$) (dos Santos et al. 2018).

dos Santos et al. (2018) determined patulin in 'Fuji Kiku' decayed apples in DCA-RQ1.3 + 1.2 kPa $CO_2$ condition, and patulin concentration of <LOQ (limit of quantification – 25 μg $kg^{-1}$) was found, thus, obtaining values lower than the result found for 'Fuji Mishima' apple (68 μg $kg^{-1}$) in the same storage condition at this study. These same authors did not find a significant difference in patulin accumulation after storage under CA, DCA-CF and DCA-RQ1.3.

In storage under low $pO_2$ and high $pCO_2$, beneficial effects include reduced respiration rate and ethylene production, thus preserving the physicchemical characteristics and inhibiting the occurrence of some physiological disorders (Weber et al. 2015). Baert et al. (2007) found a clear reduction in

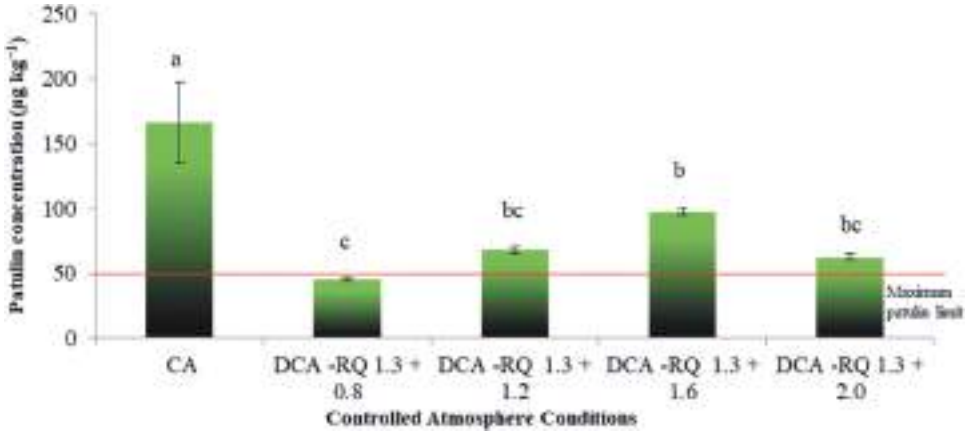



**Figure 2.** Patulin concentration of 'Fuji Mishima' apples after 8 months of controlled atmosphere storage conditions plus 7 days at 20 °C. *Means followed by different letters in the columns differ by Tukey' test, at 5% probability ($p < .05$); CA: controlled atmosphere; DCA-RQ + kPa $CO_2$: dynamic controlled atmosphere with respiratory quotient.

patulin content when $O_2$ decreased from 3% to 1%, independent of the strain tested of *P. expansum*. The authors concluded that temperature and $pO_2$ should be as low as possible to suppress patulin.

So far, there were no data in the literature relating DCA-RQ with different $CO_2$ concentrations on the accumulation of patulin in 'Fuji Mishima'. In this study, DCA-RQ1.3 with lower $CO_2$ concentration (0.8 kPa $CO_2$) was associated with least patulin production. In this sense, the low patulin concentration may be a response to the low stress of the fungus because $CO_2$ can control a variety of cellular processes including stress response and metabolism of fungi. According to Bahn and Muhlschlegel (2006), $CO_2$ is a key determinant involved in fundamental biological processes in fungi, including growth, morphology and also virulence. This variation in ambient $CO_2$ requires accurate adaptation as both low $CO_2$ amounts, which may cause a deficiency of substrates for carboxylating reactions (Aguilera et al. 2005), and high $CO_2$ concentrations, which may be toxic and could inhibit the growth of fungal cells (Jain and Agrawal 1994), are potentially hostile. Thus, it is necessary to continue exploring new techniques such as CA and DCA, as well as $O_2$ and $CO_2$ concentrations employed to better understand the effect of stress storage condition on the patulin accumulation.

Besides that, patulin accumulation is associated with genetic components and intrinsic chemical compositions of fruit that may limit patulin synthesis and/or stimulate patulin detoxification (Menniti et al. 2010), explaining the different patulin concentrations observed in 'Fuji Kiku' apples (dos Santos et al. 2018) and those in the present study in 'Fuji Mishima' apples (Figure 2). Neri et al. (2010) demonstrated that abundant ascorbic acid or thiols are naturally occurring in fruit reduced patulin production. The addition of ascorbic acid or phenolics to the juice resulted in partial degradation or limited production of patulin.

Due to the fact that Brazil is a country that exports fruits, the data found in this work are worrying in this aspect. Just like Brazil, European Commission, the United States Food and Drug Administration, the Ministry of Health of the People's Republic of China, and Health Canada established as the maximum limit of 50 µg $kg^{-1}$ is for patulin in several apple-based foods, such as apple juice, concentrated apple juice and cider (EC 2006). However, the European Commission established lower limits for solid apple products, including compote, and puree (25 µg $kg^{-1}$), and even more, strict regulations are applied for infant and baby food (10 µg $kg^{-1}$) (EC 2006).

Even though no information is available on the effects of long-term exposure in humans, patulin presence in food is limited in many countries as a preventive measure. Therefore, the concentration of 166 µg $kg^{-1}$ found in CA conditions of the present study calls attention. Children are more affected as their consumption of apple products is higher compared to adults and they are more susceptible to toxic compounds (Patriarca 2019).

Although certain process steps, like pasteurisation, for example, have been proved to reduce the toxin concentration (Welke et al. 2009), when heavily contaminated fruits are designed for industrialisation, detectable levels of toxin can be found in final products (Oteiza et al. 2017; Dias et al. 2019). According to Dias et al. (2019), the patulin concentration in apples and apple juices commercialised in Santa Maria city, Brazil, were generally above the legal limit. This fact must be an alert to the population, especially in those cases when these fruits or juices are used in children's diet, one of the most affected groups. Our findings call for attention from apple industries when processing deteriorated or spoilt fruit, even if it has a low decay incidence like apple analysed in this work (up to 25% of severity).

### Correlation between fungus and patulin

Considering fungal and patulin concentrations, there was no correlation between increased patulin and *Penicillium* spp. plus *P. expansum* counts for Fuji Mishima apples ($r^2 = 0.023$). In this sense, this lack of correlation suggests that even in samples with a low fungal presence, high concentrations of this mycotoxin can be detected.

There are many factors that can explain this non-correlation. First, in general, an important data is the fact that the absence of the fungus in food does not guarantee the absence of mycotoxins, since they are stable compounds. These metabolites can be found in food when the fungus is no longer present (Taniwaki et al. 2009). Authors report that patulin is a water-soluble mycotoxin and can migrate to other parts of the fruit up to 4 cm beyond a lesion (Baert et al. 2007). These results suggest that decayed apples contaminated with patulin, and stored in the same chambers, can contaminate other apples under the same treatment which may explain that in a certain sample *Penicillium* sp. and/or *P. expansum* may not be present, but patulin may be detected.

Second, so far, most of the work published regarding fungal contamination and mycotoxin production use inoculated strains. In this work, no inoculation was carried out, evidencing, therefore, the real population of those cultivars stored under CA and DCA conditions during8 months and 7 days of shelf-life at 20 °C. This is another point that may explain the appearance of *Penicillium* spp. in some samples and *P. expansum* in others.

Another point is that patulin can be produced by several species of *Penicillium*. Andersen et al. (2004) reported that almost all *P. expansum* strains are patulin producers, although patulin production varies among strains. Wilson and Nuovo (1973) isolated 60 strains of *P. expansum* producing patulin in apples with deterioration, kept under storage at 0 °C. Besides *P. expansum*, patulin could be produced by *P. carneum, P. clavigerum, P. concentricum, P. coprobium, P. dipodomyicols, P. glandicola, P. gladioli, P. griseofulvum, P. marinum, P. paneum, P. sclerotigennum and P. vulpinum* (Samson et al. 2004). Even then, *P. expansum* is the main species involved in patulin production in apple and other pomaceous fruits and was the only *Penicillium* potential producer of patulin recovered from the samples.

Patulin production depends on different factors, such as temperature, pH and other physiological parameters (Tannous et al. 2018). Ballester et al. (2015) reported that it is not yet clear when fungi secrete patulin. Inhibition of *P. expansum* sporulation might be another reason for patulin reduction at low temperatures. Secondary metabolites such as patulin are considered to be closely associated with sporulation rather than mycelial growth in *P. expansum*. Patulin synthesis and sporulation might share some common upstream signal transduction or regulating pathways (Zong et al. 2015).

Suitable growth temperatures for *P. expansum* range from 0 to 30 °C, with the optimum being 25 °C (Baert et al. 2007). Wang et al. (2017) evaluated the correlation between *P. expansum* growth and patulin production in the juices of eight kiwi cultivars. According to these authors, the optimum or minimum tolerated temperature for *P. expansum* growth and patulin production are different in some reports and this is mainly due to the differences in the strains and growth media. Based on their results and previous reports, it can be concluded that a temperature reduction reduces patulin production in comparison to higher temperature.

*P. expansum* infection has been kept under surveillance for many years in apples for industrialisation through quality control measures, usually by

visual inspection of fruits incorporated to the process. According to Ioi et al. (2017) the achievement of these requirements relies on an integrated management throughout the food processing chain; proper fruit selection and handling at harvest, combined with adequate storage conditions, which are key factors in patulin mitigation in final products. However, according to Copetti et al. (2014), once mycotoxins are formed, it will be difficult to manage their amounts, as they are stable in storage and very tolerant to physical and chemical treatments. Therefore, the best way to limit exposure to mycotoxins is to suppress their formation in the first place.

### *Volatile profile*

The fruit metabolism is mainly influenced by conditions under which they are stored. However, fungal growth can also contribute to changes in the composition of volatiles. High concentrations of benzaldehyde, ethanol and ethyl acetate (higher chromatographic peaks) were found in all treatments. However, these compounds were not quantified in the chromatographic runs due to the MS detector saturation.

Benzaldehyde might to be released from enzymatic hydrolysis of glycosidically bound volatiles, as described for kiwi juice (Garcia et al. 2012). Regarding ethanol and ethyl acetate, this result may be due to the induction of fermentative metabolism by the low $pO_2$ used in these treatments, as the pathway producing these compounds is favoured under anaerobic conditions. According to Wright et al. (2015) it is worth noting that high ethyl acetate levels are harmful to fruit tissues, as the compound is considered an off-flavour. At low concentrations, however, it can contribute to the quality of apple flavour. The higher emission of ethyl acetate in our experiments was probably due to the higher emission of its precursor, ethanol, which is the substrate for conversion by alcohol acyltransferase activity to the corresponding ester.

A total of 83 volatile compounds were identified, including the following chemical classes: alcohols (15), acids (16), esters (23), aldehyde (15), terpenes (5), ketones (6) and 'others' (3) (Supplementary material 2). However, aldehydes, alcohols and esters were the groups with compounds found in higher concentrations (Figure 3). The major analytes were hexanal, (E)-2-hexenal, 1-hexanol, 1-butanol 3-methyl, butanoic acid, ethyl ester, acetic acid, hexanoic acid, and 3-octanone.

In CA, DCA-RQ1.3 + 0.8 kPa $CO_2$ and DCA-RQ1.3 + 1.2 kPa $CO_2$ conditions aldehydes and alcohol were the classes presented in higher concentration, where (E)-2-hexenal, hexanal and 1-hexanol showed high concentrations. On the other hand, lower concentrations of these compounds were observed in DCA-RQ1.3 + 2.0 kPa $CO_2$. This result suggests that these treatments may be the best storage conditions since (E)-2-hexenal is an aldehyde marker of herbal notes in apples (Mehinagic et al. 2006) and, in this study, apples were harvested at the optimal harvest point for long-term storage. Thewes et al. (2017) reported for 'Fuji Suprema' apple the following compounds 2-methyl butyl acetate, ethyl 2-methyl propanoate, ethyl 2-methyl butanoate, and ethyl hexanoate as positively correlated to the characteristic aroma of this cultivar, also evidencing that infected apples provide a different volatile profile.

There are several studies about apple quality, including controlled atmosphere storage and volatile compounds determination; however, different from our study, these are related to healthy apples (Donadel et al. 2019; De Anese et al. 2020). For example, Thewes et al. (2017) evaluated the effect of CA, DCA-CF and DCA-RQ storage conditions in 'Fuji Suprema', and Both et al. (2017) evaluated 'Royal Gala' apple.

Besides this, when *P. expansum* infected fruit, the species found in samples of our study produce various enzymes during the infection process, such as glycosyl hydrolases and polygalacturonase (Ballester et al. 2015), which can contribute to the release of additional volatiles. Therefore, in addition to fruit metabolism, the volatiles profiles of all samples may have been influenced by different storage conditions after 8 months plus 7 days of shelf-life, presence of fungi, and a relationship with some compounds with patulin concentrations could be seen.

Studies involving fungal contamination, patulin presence and VOCs in apples are still limiting. However, studies in kiwi also showed the presence of volatile markers found in the apples of our study. Wang et al. (2017) studied growth and patulin production by *P. expansum* in kiwi juices. *P. expansum* infection caused a decrease of

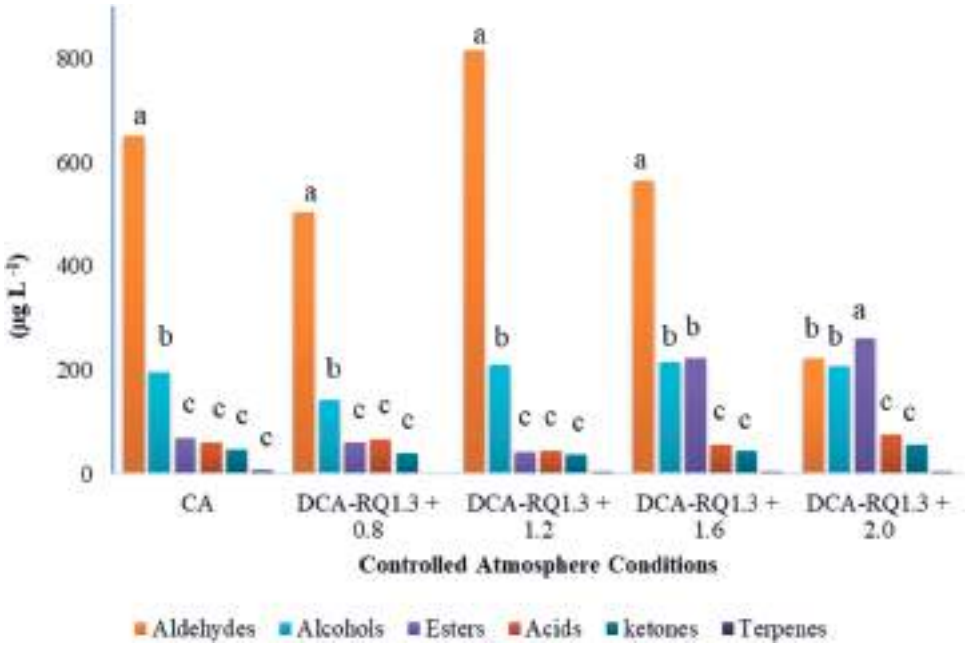



**Figure 3.** Classes of volatile compounds in 'Fuji Mishima' apples after 8 months of controlled atmosphere storage conditions plus 7 days at 20 °C. *Means followed by equal letters in the columns do not differ by Tukey' test, at 5% probability ($p > .05$); CA: controlled atmosphere; DCA-RQ + kPa $CO_2$: dynamic controlled atmosphere with respiratory quotient.

aldehydes in most kiwi cultivars, different from our results found in apples, where aldehydes were a major class. In this study, esters and terpenes were not related to fungal infection, but they were dependent on kiwi cultivars and strains to a greater extent than other classes of VOCs.

Although our study is with apples, major compounds found are similar to compounds found by Wang et al. (2017). Several aldehydes and alcohols such as (*E*)-2-hexenal, hexanal, (*Z*)-3-hexenol, (*E*)-2-hexenol and 1-hexanol were found (aroma compounds that characterise kiwi fruit) (Garcia et al. 2012). These compounds typically diminish after infection. Due the inoculation, it was possible to detect many VOCs that were secondary metabolites of *P. expansum*. The 3-methyl-1-butanol acetate, 3-hydroxy-hexanoic acid ethyl ester, (*E,E*)-2,4-hexadienoic acid ethyl ester, dodecanoic acid ethyl ester and hexadecanoic acid ethyl ester were possible candidates of contaminated biomarkers for *P. expansum* infection in kiwifruit.

A previous report has shown that styrene, 3-methyl-1-butanol and 3-methyl-1-butanol acetate were potential biomarkers obtained via GC/MS analysis for *P. expansum* infection in *Golden Delicious* apples (Karlshøj et al. 2007). In the present study, the relative content of 3-methyl-1-butanol ranged from nd to 51.1 µg $L^{-1}$ and in relation to styrene, its concentration ranged from nd to 3.2 µg $L^{-1}$. In our study, it was not possible to state that volatile compounds come only from *P. expansum*, because besides this fungus, there were other identified species.

In order to better visualise the correlation of patulin and VOCs, and find variables that may be markers of the patulin presence, Pearson correlation analysis was applied to the data (Figure 4). In this, correlation, variables (volatile compounds) that have a greater positive correlation with the dependent variable (patulin) were presented. It was possible to identify the high correlation of this analyte with styrene ($r^2$ = 0.75), n-decanoic acid ($r^2$ = 0.75), toluene ($r^2$ = 0.74), phenol ($r^2$ = 0.68) and alpha-farnesene ($r^2$ = 0.62). These compounds can be considered potential volatile markers. In studies with healthy apples, the presence of styrene has not been reported (Thewes et al. 2017; Donadel et al. 2019), suggesting that this compound may be a secondary metabolite of fungus and therefore, a volatile marker of patulin. Of the 24 volatile compounds that showed the most significant difference, 54% showed a correlation between 0.5 and 0.9, 20.8% showed a correlation between 0.1 and 0.4 and 25% presented a negative correlation. In addition, the results showed a negative correlation of

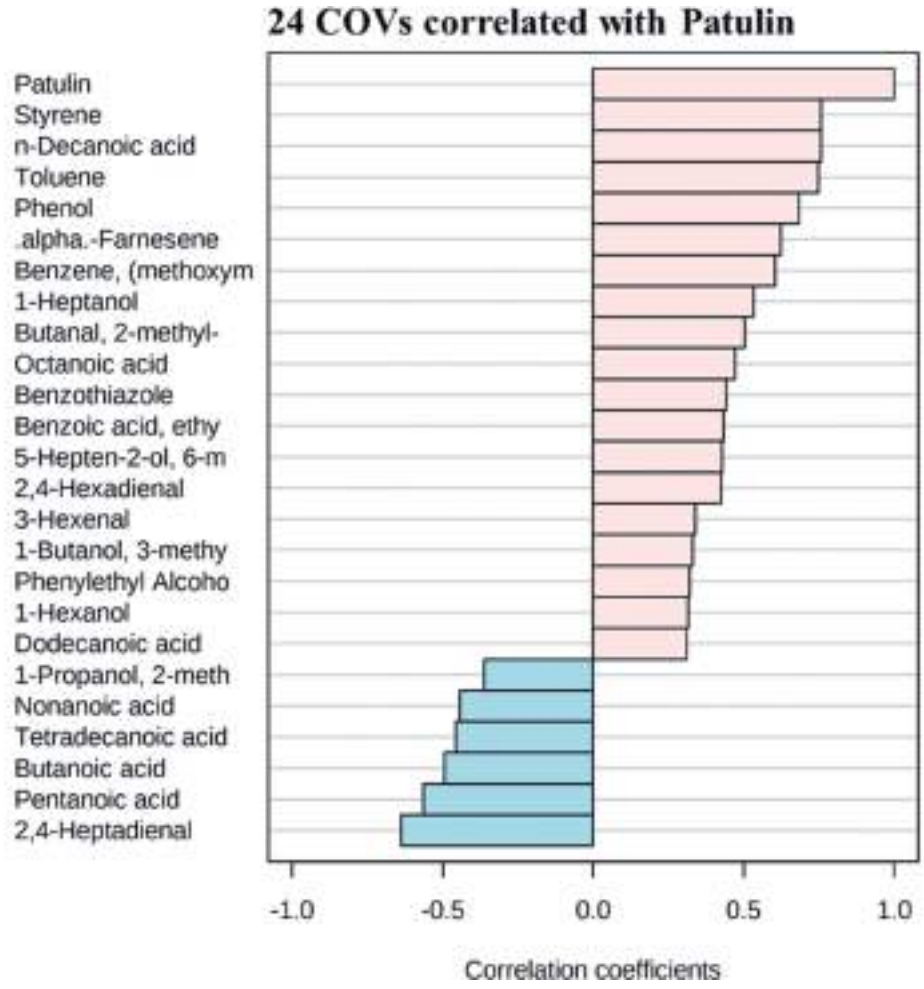



**Figure 4.** Pearson correlation for 24 volatile compounds correlated with patulin.

patulin with acids, for example, nonanoic acid, tetradecanoic acid, butanoic acid and pentanoic acid. This information suggests that in treatments with a higher patulin concentration, there were less acidic compounds.

VOCs production by microorganisms is highly dependent on both media composition and strain species (Sunesson et al. 1995). The study by Karlshøj et al. (2007) showed that two varieties of apples infected by the same *P. expansum* strain produced two different VOCs profiles. Intraspecific variability of *P. expansum* growth and metabolism under the same conditions was also confirmed by Garcia et al. (2012). Thus, the different VOCs produced by fungal infection in the two apple cultivars appear to be a common phenomenon.

Furthermore, VOCs production by fungi and yeasts could play a significant role in their antagonistic activities due to the small sizes of these molecules and their diffusion through the atmosphere and soil (Oro et al. 2018). In this study, yeasts were present in all treatments and may also have influenced the VOCs production.

## Conclusions

This is the first study to evaluate the effects of fungal growth, patulin accumulation and VOCs production of decayed apples of the cultivar Fuji Mishima under CA and DCA-RQ conditions plus 7 days of shelf-life at 20 °C. Even using modern storage technologies, toxigenic species and patulin were found in decayed apples from all treatments evidencing, therefore, that the risk of mycotoxigenic fungi and mycotoxin is particularly relevant to apple processing industries. *Penicillium expansum* was the most prevalent species. CA provides a higher patulin concentration (166 μg $kg^{-1}$), however, in the DCA-RQ1.3 + 0.8 kPa

$CO_2$ (lower concentration $CO_2$) lowest patulin concentration (45 µg $kg^{-1}$) was found. Only in this storage condition was the patulin concentration below the maximum patulin limit in Brazil, 50 µg $kg^{-1}$. Therefore, DCA storage conditions with lower $CO_2$ concentrations were associated with least patulin concentration consequently being a better storage condition for this cultivar. This is an important point that deserves further investigation. It was observed that fungal growth could also contribute to changes in the volatiles' composition. Styrene and 3-methyl-1-butanol were found, compounds considered in the literature to be fungal markers for *P. expansum* in some apple cultivars. Styrene, n-decanoic acid, toluene, phenol and alpha-farnesene were the compounds that showed the most positive correlation with patulin. In addition, in treatments with a higher patulin concentration, there were less acidic compounds. Interest in expanding Brazilian fruit exports is considerable, and, as post storage quality is crucial, future research in this area should be conducted to detect continuous VOCs changes before and after fungi and patulin production and to better understand fruit spoilage.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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# Chemical composition of chamomile essential oil cultivated with homeopathy

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**ABSTRACT**

The use of homeopathy in the cultivation of medicinal plants promotes fluctuations in the production of secondary metabolites, but there is no scientific reference to its effect on the productivity and chemical composition of the essential oil. In this context, the effect of Phosphorus dynamizations on chamomile essential oil was investigated. The essential oil was obtained by hydrodistillation to determine the content, productivity and chemical composition (GC-FID and GC/MS). The application of Phosphorus promoted an increase in the content and productivity, as well as changes in the percentage of concentration of the majority compounds of the chamomile essential oil. This is the first research that addresses the effect of homeopathy on the productivity and chemical composition of chamomile essential oil. Therefore, Phosphorus dynamizations alter the chemical composition and increase the productivity of essential oil in chamomile, demonstrating to be a sustainable practice for increasing the production of essential oil in medicinal species.

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**KEYWORDS**
*Chamomilla recutita*; ultra-diluted solutions; secondary metabolism; PCA

## 1. Introduction

Chamomile (*Chamomilla recutita* (L.) Rauschert) is an annual species in the Asteraceae family, well known in popular medicine. Its therapeutic properties are found only in fresh or dry inflorescences, which are used for the extraction of essential oil. Worldwide, it is the most consumed medicinal species in the form of tea, essential oil or by-products of the pharmaceutical, cosmetics and food industries (1–3). The benefits found in tea or chamomile infusions are related to the presence of flavonoids such as apigenin, luteolin, quercetin and their derivatives (4,5). Chamomile essential oil has as main compounds α-bisabolol and its α-bisabolol oxide A and B, and chamazulene (6), which is responsible for the blue coloration of the essential oil. The medicinal purpose of the essential oil is attributed to its anti-inflammatory, antimicrobial and spasmolytic properties (4,6,7). However, this species has low essential oil productivity, which leads many producers to use techniques to increase production, such as elevated chemical fertilization, which is a non-renewable resource and, in excess, accumulates and contaminates the ecosystem.

Essential oil is one of the products derived from the secondary metabolism of plants. Secondary metabolites are bioactive components, generally found in medicinal plants, and are the source of pharmaceutical products, food additives and flavors (8,9). Its properties stimulate the cultivation and consumption of medicinal plants, corroborate the growth of the economic market and encourage various researches that seek to portray its production in the plant and its performance as a medicine. Despite the existence of genetic control, the expression of secondary metabolites may undergo changes resulting from cultivation conditions such as temperature, humidity, light, availability of water and nutrients (10,11), as well as the use of homeopathy during the productive cycle of plants.

Homeopathy is a science and sustainable technique that does not contaminate the environment and in agriculture it is intended to control pests, diseases and alleviation of the abiotic effects that promote stress on the plant. The use of homeopathy in plant production is also recurrent, in order to increase production, independent of the cultivation system (conventional/organic/agroecological). In medicinal plants, generally, homeopathy has a direct influence on the production of essential oil, as highlighted for the species of picão preto (*Bidens pilosa*) (12), chambá (*Justicia pectoralis*) (13), carqueja (*Baccharis trimera*) (14) and mint (*Mentha arvensis*) (15). However, due to the large number of homeopathic medicines, only a few have been

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elucidated and evaluated as to their effects during the cultivation of plant species. Also, research focuses only on essential oil productivity and does not evaluate the effects on the chemical composition of the essential oil. In this context, it is worth mentioning the Phosphorus homeopathic medicine, which in agriculture is indicated for soils with low availability of the chemical element phosphorus (16), but does not present descriptions of its potential effect on the cultivation of medicinal plants in relation to the productivity and chemical composition of the essential oil.

Considering the medicinal importance of chamomile and the absence in the description of possible effects of homeopathy on the essential oil of this species, this study aimed to evaluate the effect of the application of Phosphorus dynamizations on the content, productivity and chemical composition of chamomile essential oil.

## 2. Material and methods

### 2.1. Obtaining plant material and experimental organization

The experiment was carried out at the Plant Science Department of the Federal University of Santa Maria (UFSM), Santa Maria – RS, Brazil. Sowing of the cultivar Mandirituba was carried out in 5 $dm^3$ pots containing substrate. The plants were cultivated in a protected environment to avoid the effect of some meteorological phenomena such as rain and wind. Each cultivation bench represented a treatment and after emergence, only one plant per pot was maintained. The plants received nutrient solution elaborated according to the recommendation for chamomile cultivation (17) and other experimental procedures were performed according to Ubessi et al. (18). Chamomile seeds for planting were obtained from Emater (Empresa de Assistência Técnica e Extensão Rural) of the State of Parana (PR), Brazil, and the botanical material corresponds to the voucher specimen deposited at the Herbarium of the Municipal Botanical Museum (MBM) of Curitiba (PR, Brazil), number MBM382674.

The chamomile plants received the application of Phosphorus, prepared in alcohol 70% (v $v^{-1}$), in four dynamizations: 3CH, 6CH, 12CH and 30CH (CH: Centesimal Hahnemannian). For application in plants, the dynamizations were diluted in distilled water in the proportion of 0.5 mL $L^{-1}$ and were applied from the emergence until the end of the experiment, totaling 135 days of cultivation. Each chamomile plant was sprayed with 100 mL of each treatment. The control treatments received only distilled water and alcohol 70% (v $v^{-1}$) (vehicle of homeopathic solutions) in the same amount and proportion of treatments with homeopathy.

### 2.2. Extraction of essential oil and determination of content and productivity

Chamomile flowers were harvested manually, followed by determination of the fresh mass of flowers per plant and, afterwards, stored in a refrigerator (approximately 4 °C) until the extraction of the essential oil (around 100 days of refrigerated storage). The extraction of the essential oil was carried out at the Plant Cytogenetics and Genotoxicity Laboratory (UFSM) by hydrodistillation in Clevenger apparatus for 2 hours and 30 minutes after the beginning of the boiling, using 50 grams of flowers. From extraction, the content and productivity of essential oil in each treatment was determined. The essential oil content was calculated by multiplying the amount of extracted oil (mL) by 100 and divided by the amount of fresh mass used for the extraction (50 grams), expressed in % $g^{-1}$ fresh mass. Oil productivity considers the production of fresh flowers per hectare, multiplied by the amount of oil extracted (mL) and divided by 1000 to express the value obtained in liters per hectare (L $ha^{-1}$). After extraction, the essential oil samples were stored in a refrigerator until the chromatographic analysis was performed.

### 2.3. Essential oil characterization

Chamomile essential oil was characterized by chromatographic analysis performed on a gas chromatograph (Varian Star 3400CX, CA, EUA) equipped with a flame ionization detector (GC-FID). 10 µL of the oil was diluted in 1 mL hexane and a 1 µL aliquot of the solution was injected into the chromatographic system injection port at a temperature of 270 °C, in split mode of 1:40 ratio. The compounds were separated on a non-polar Equity $^{TM}$-5 fused silica capillary column (60 m × 0.20 mm × 0.20 µm) (Supelco, USA). Hydrogen was used as a carrier gas at a constant pressure of 25 psi. The initial column temperature was adjusted to 40 °C and maintained for 1 min. Thereafter, the temperature was increased at a rate of 8 °C $min^{-1}$ to 160 °C, followed by an increase at 1 °C $min^{-1}$ to 180 °C, followed again by an increase at 20 °C $min^{-1}$ up to 300 °C and maintained under isothermal conditions for 10 minutes. The detector temperature was maintained at 280 °C. A series of homologous n-alkanes (C7 – C24) n-alkanes were analyzed under the same chromatographic conditions to calculate the linear retention index (IRL). Quantitation was performed by normalization of the peak chromatographic areas and expressed as a percentage.

Qualitative analysis of the compounds was performed by a gas chromatograph (Shimadzu QP2010 Ultra) coupled to a mass spectrometer (GC/MS, Shimadzu Corporation, Kyoto, Japan). For these analyzes, the same chromatographic conditions described above and helium as carrier gas were used. The detector was operated in electron impact ionization mode with an ionization energy of 70 eV and a mass scan range of 35–350 m/z. The compounds were identified based on the comparison with mass spectra available from the National Institute of Standards and Technology (NIST) and Adams library (19) and by comparing the calculated linear retention index with those available in the scientific literature (20,21). Each treatment was evaluated in triplicate.

### 2.4. Statistical analysis

The experiment was carried out according to a completely randomized design (double blind) with six treatments: four Phosphorus dynamizations (3CH, 6CH, 12CH and 30CH), distilled water and alcohol 70%. Each cultivation bench represented a treatment and was composed of 20 pots spaced 0.30 meters in two rows, totaling four replicates of five experimental units. The data referring to the concentration of the essential oil compounds were submitted to analysis of variance ($p < 0.05$) and to the mean comparison by Scott and Knott test (22) at 5% error probability level, with the aid of the Sisvar statistical program (23). The dataset referring to the content, productivity and chemical composition of the essential oil were subjected to multivariate principal component analysis (PCA) using Pirouette 3.11 software (InfoMetrix, Woodinville, USA). Before the PCA, the data matrix was self-scaled so that each variable assumed the same weight in the analysis.

## 3. Results and discussion

### 3.1. Essential oil content and productivity

The analysis of variance showed significant difference between the dynamizations for the content (Figure 1(a)) and productivity of essential oil (Figure 1(b)). For these variables, the 3CH dynamization expressed averages higher than the others, while the 6CH dynamization showed a decrease (Figure 1). It is important to highlight that the 3CH dynamization increased the productivity of essential oil by 1.35 L $ha^{-1}$ when compared to the value obtained for the distilled water control, an increase of 33.4% per hectare.

The content and productivity of chamomile essential oil demonstrated different responses regarding the evaluated Phosphorus dynamizations. The oil content values found for chamomile in the literature range from 0.30 to 1.50% (conventional cultivation) (24–26). The contents obtained for chamomile using Phosphorus meet the requirements of the Brazilian Pharmacopoeia (minimum content of 0.40%) only in the 3CH and 12CH dynamizations, which provided a content of 0.56 and 0.49%, respectively. Consequently, if there are changes in the essential oil content, these changes directly affect the oil productivity of the plant species.

The productivity of chamomile essential oil in conventional cultivation and without the application of homeopathy ranges from 1.54 L $ha^{-1}$ (27) to 2.40 L $ha^{-1}$ (28), however, with the application of Phosphorus in the 3CH dynamization, the oil productivity increased to 4.04 L $ha^{-1}$. This represents increases from around 40.59% (2.40 L $ha^{-1}$) to 61.88% (1.54 L $ha^{-1}$). With the exception of the 6CH dynamization, all the others increased oil productivity when compared to the distilled water control. The increase in productivity of chamomile essential oil with the use of Phosphorus coincides with the results obtained in other species such as *Aloysia gratissima* (29) and *Verbena gratissima* (30). This reaffirms homeopathy science as a promising and sustainable technique for increasing essential oil production in medicinal and aromatic plants.

### 3.2. Essential oil composition

The main compounds found in chamomile essential oil are presented in Table 1, while the chromatogram is demonstrated in Figure 2. 38 compounds present in the distilled water control were identified. The major compounds in all treatments were α-bisabolol oxide B (26.21%) and A (21.30%), cis-ene-yne-dicycloether (9.15%), bisabolone oxide (8.06%), β-trans-farnesene (7.25%), α-bisabolol (5.79%) and chamazulene (4.42%). To a lesser amount, the compounds epimanool (0.03%), α-terpineol (0.07%) and nerolidol isomer (0.09%) were observed. When comparing the dynamizations with the distilled water control treatment, three compounds were not found, such as trans-crysanthemol in 6CH dynamization, 4.8-dimethyl-nona-3.8-dien-2-one in 12CH dynamization and 1-naphthalene propanol (epimanool) in 70% alcohol and in 12CH and 30CH dynamizations (Table 1). The percentage of constituents identified in chamomile essential oil was greater than 92%. However, six compounds presented percentages in the essential oil, however the consulted library does not have such identifications. These compounds are named in Table 1 by the acronym NI (not identified).

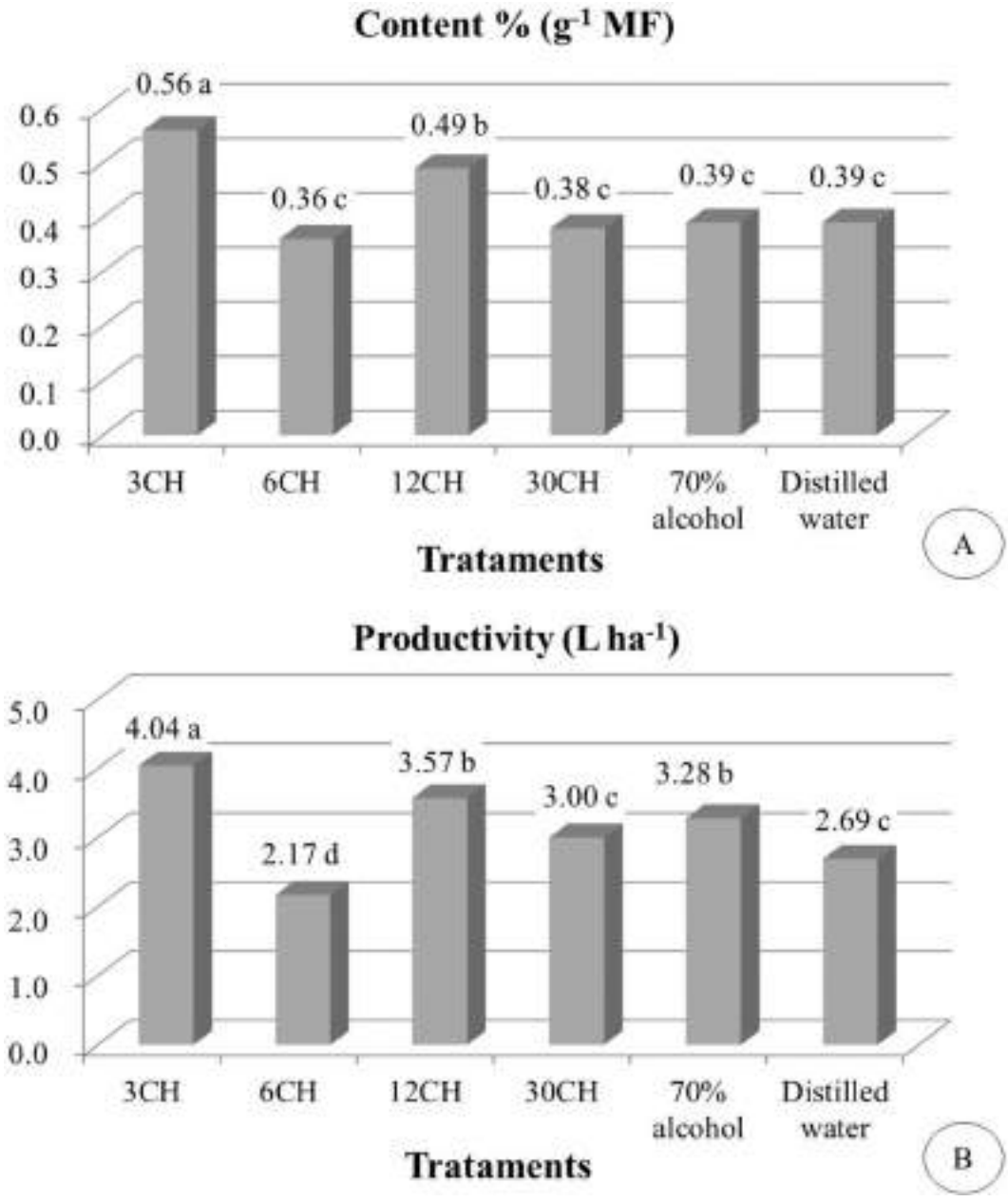



**Figure 1.** Content and productivity of chamomile essential oil submitted to Phosphorus dynamizations.

The Table 1 also reports the test of averages between treatments for the concentration of chemical compounds present in the essential oil. Compared to the distilled water (control treatment), it is possible to observe, for the aforementioned major compounds, an increase in the concentration with the application of the dynamizations. With the exception of chamazulene, which showed an inverse behavior, reducing the concentration with the increase of dynamizations. Each compound had a different response within each dynamization.

The dynamizations of the Phosphorus also promoted changes in the chemical composition of chamomile essential oil. Among the predominant compounds, α-bisabolol oxide A and B, cis-ene-yne-dicycloether, bisabolone oxide, β-trans-farnesene, α-bisabolol and chamazulene were the main components of the chamomile essential oil identified and corroborate with the results found by the authors Orav et al. (1), Srivastava et al. (4), Petronilho et al. (7), Amaral et al. (28), Raal et al. (3) and Mahdavi et al. (31). However, some dynamizations provided the absence of the components trans-crysanthemol, 4.8-dimethyl-nona -3.8-dien-2-one and epimanool (Table 1).

In the scientific framework there are few studies that evaluate the interaction between the chemical

**Table 1.** Chemical composition of chamomile essential oil submitted to Phosphorus dynamizations.

| | Compound | IRL Exp. | IRL Lit. | % Composition | | | | | | Average | CV(%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Water | 3CH | 6CH | 12CH | 30CH | Alcohol | | |
| 1 | 2-Isopropyltoluene | 1028 | 1022 | 0.21* C | 0.27 B | 0.33 A | 0.32 A | 0.14 D | 0.18 C | 0.24 | 7.78 |
| 2 | Eucalyptol | 1036 | 1026 | 0.09 D | 0.21 A | 0.17 B | 0.15 B | 0.13 C | 0.14 B | 0.14 | 9.16 |
| 3 | β-Ocimene | 1049 | 1032 | 0.33 B | 0.37 A | 0.32 B | 0.37 A | 0.20 C | 0.30 B | 0.31 | 8.01 |
| 4 | Artemesia Ketone | 1062 | 1056 | 1.00 A | 1.29 A | 1.18 A | 1.13 A | 1.20 A | 0.67 B | 1.08 | 8.73 |
| 5 | γ-Terpinene | 1068 | 1054 | 0.18 C | 0.24 B | 0.26 B | 0.34 A | 0.18 C | 0.27 B | 0.24 | 9.76 |
| 6 | Artemisia Alcohol | 1086 | 1080 | 0.25 A | 0.20 B | 0.23 A | 0.15 C | 0.17 C | 0.17 C | 0.19 | 8.13 |
| 7 | Trans-Chrysanthemol | 1163 | 1160 | 0.25 A | 0.25 A | 0.00 C | 0.14 B | 0.25 A | 0.16 B | 0.17 | 10.71 |
| 8 | Nonenal | 1167 | 1157 | 0.12 A | 0.09 B | 0.12 A | 0.08 B | 0.09 B | 0.12 A | 0.1 | 6.96 |
| 9 | Lavandulol | 1170 | 1165 | 0.15 A | 0.12 A | 0.14 A | 0.09 B | 0.14 A | 0.10 B | 0.12 | 8.44 |
| 10 | α-Terpineol | 1197 | 1186 | 0.08 B | 0.08 B | 0.11 A | 0.06 C | 0.05 C | 0.07 B | 0.07 | 17.34 |
| 11 | 4.8-Dimethyl-nona-3.8-dien-2-one | 1277 | 1275 | 0.09 C | 0.30 A | 0.16 B | 0.00 E | 0.04 D | 0.01 E | 0.1 | 11.61 |
| 12 | Ethyl Decanoate | 1396 | 1395 | 0.10 B | 0.16 A | 0.15 A | 0.04 C | 0.10 B | 0.09 B | 0.11 | 10.60 |
| 13 | β-trans-Farnesene | 1461 | 1440 | 7.42 B | 6.41 C | 8.35 A | 6.74 C | 8.61 A | 5.97 C | 7.25 | 5.51 |
| 14 | Dehydro-Sesquicineole | 1477 | 1469 | 3.24 C | 4.12 B | 4.01 B | 1.32 E | 4.73 A | 2.30 D | 3.29 | 5.95 |
| 15 | Germacrene D | 1495 | 1484 | 1.37 A | 0.92 C | 1.19 B | 0.85 C | 1.39 A | 0.92 C | 1.11 | 4.96 |
| 16 | α-Farnesene | 1502 | 1505 | 0.32 B | 0.25 C | 0.37 A | 0.23 C | 0.31 B | 0.30 B | 0.30 | 4.42 |
| 17 | Bicyclogermacrene | 1511 | 1500 | 1.06 C | 1.21 B | 1.05 C | 0.84 D | 1.46 A | 1.40 A | 1.17 | 6.35 |
| 18 | Delta-Cadinene | 1550 | 1522 | 0.29 A | 0.20 D | 0.26 B | 0.21 C | 0.27 B | 0.17 E | 0.23 | 2.03 |
| 19 | Nerolidol | 1569 | 1561 | 0.62 C | 0.51 D | 0.86 A | 0.44 E | 0.68 B | 0.29 F | 0.57 | 4.45 |
| 20 | Nerolidol Isomer | 1576 | 1561 | 0.08 A | 0.08 A | 0.10 A | 0.08 A | 0.12 A | 0.07 A | 0.09 | 31.56 |
| 21 | Spatulenol | 1595 | 1577 | 0.25 D | 0.24 D | 0.31 C | 0.32 C | 0.41 B | 0.50 A | 0.34 | 2.52 |
| 22 | NI1 | 1615 | - | 1.04 A | 0.38 D | 0.43 C | 0.34 E | 0.80 B | 0.20 F | 0.53 | 1.09 |
| 23 | NI2 | 1628 | - | 0.69 C | 0.82 B | 0.80 B | 0.31 E | 0.99 A | 0.48 D | 0.68 | 3.80 |
| 24 | NI3 | 1633 | - | 3.09 B | 1.96 D | 2.56 C | 1.76 E | 1.90 D | 3.38 A | 2.44 | 1.92 |
| 25 | NI4 | 1657 | - | 2.67 A | 0.96 C | 0.52 F | 0.56 E | 1.32 B | 0.86 D | 1.15 | 1.39 |
| 26 | α-Bisabolol Oxide B | 1670 | 1656 | 23.44 E | 27.08 D | 28.47 B | 33.43 A | 16.73 F | 28.10 C | 26.21 | 0.58 |
| 27 | NI5 | 1678 | - | 1.45 A | 1.28 B | 1.09 C | 0.73 D | 0.74 D | 1.48 A | 1.13 | 1.57 |
| 28 | NI6 | 1686 | - | 0.63 A | 0.59 B | 0.50 C | 0.01 E | 0.11 D | 0.04 E | 0.32 | 5.07 |
| 29 | Bisabolone Oxide | 1695 | 1684 | 7.37 D | 7.04 F | 8.56 C | 7.14 E | 8.99 B | 9.25 A | 8.06 | 0.68 |
| 30 | α-Bisabolol | 1699 | 1685 | 5.91 C | 6.03 B | 4.44 E | 7.82 A | 6.02 B | 4.50 D | 5.79 | 0.41 |
| 31 | Chamazulene | 1751 | 1730 | 5.59 A | 4.98 B | 4.84 C | 3.89 E | 4.38 D | 2.81 F | 4.42 | 1.03 |
| 32 | α-Bisabolol Oxide A | 1764 | 1748 | 19.03 C | 24.20 A | 17.89 D | 19.17 C | 22.55 B | 24.95 A | 21.30 | 2.16 |
| 33 | Cis-ene-yne-Dicycloether | 1895 | 1892 | 9.24 B | 5.97 D | 8.65 C | 9.66 B | 13.11 A | 8.29 C | 9.15 | 5.05 |
| 34 | NI7 | 1907 | - | 1.18 A | 0.23 D | 0.30 C | 0.28 C | 0.31 C | 0.44 B | 0.46 | 6.96 |
| 35 | Methyl Palmitate | 1969 | 1959 | 0.49 B | 0.36 D | 0.54 B | 0.46 C | 0.60 A | 0.45 C | 0.48 | 6.61 |
| 36 | 1-Naphthalene propanol (Epimanool) | 2085 | 2056 | 0.06 B | 0.06 B | 0.08 A | 0.00 C | 0.00 C | 0.00 C | 0.03 | 25.93 |
| 37 | Linolenic Acid | 2104 | 2116 | 0.20 B | 0.15 C | 0.27 A | 0.25 A | 0.31 A | 0.22 B | 0.23 | 10.40 |
| 38 | Linoleic Acid | 2164 | 2132 | 0.44 A | 0.44 A | 0.42 A | 0.29 B | 0.49 | 0.35 B | 0.40 | 8.36 |
| | Total identified (%) | | | 89.25 | 93.79 | 93.80 | 96.01 | 93.83 | 93.13 | - | - |

*Averages followed by the same letter on the line do not differ from each other by the Scott and Knott test at 5% error probability level. NI.: Not identified. IRL Exp.: Experimental linear retention index. IRL Lit.: Literature linear retention index by NIST (National Institute of Standards and Technology); Amiri and Sharafzadeh (2014); Stanojevic et al. (2016); Adams (2017). Water: distilled water. Alcohol: 70% alcohol. CV: Coefficient of Variation.

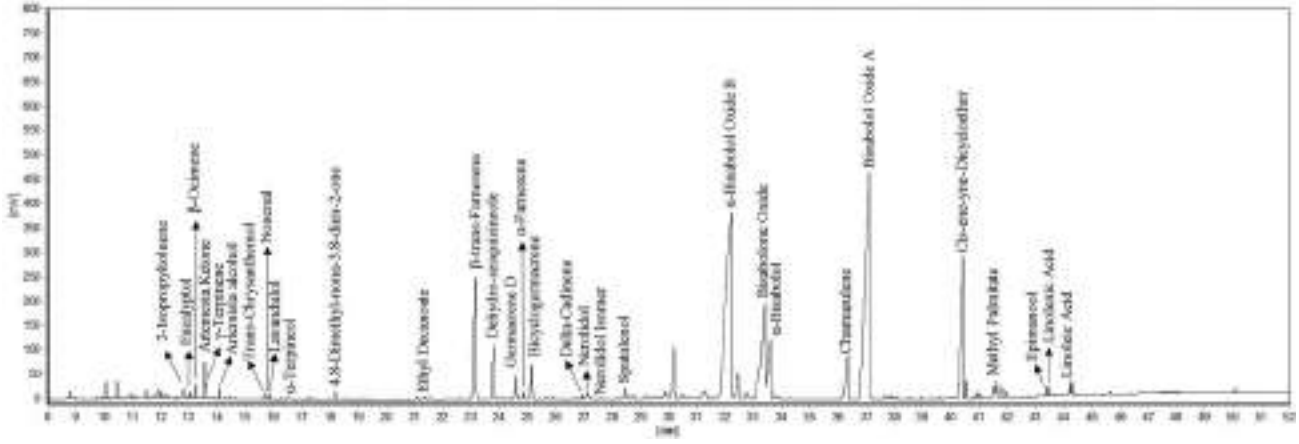


**Figure 2.** Chromatogram obtained by GC-FID analysis of chamomile essential oil submitted to Phosphorus dynamizations.

composition of the essential oil and the application of homeopathy in medicinal plants, the focus is restricted only to the content and productivity of essential oil. In this context, it is necessary to cite authors Santos et al. (29) who the evaluated the effect of Phosphorus on the composition of *Verbena gratissima* essential oil and, as observed in this research with chamomile, the dynamizations caused different responses to each compound, including the absence of some components in the 12CH and 30CH dynamizations. Homeopathy is capable of promoting changes in the secondary metabolism of medicinal plants, with variations from the content to the chemical composition of the essential oil, demonstrating positive correlations. Also, it is important to highlight that the application of homeopathy does not promote negative effects on the consumption of tea and essential oil by the population. This means that the application of homeopathy during plant cultivation does not alter the medicinal function of these products (32), even promoting variations in the concentration of essential oil compounds and apigenin content in aqueous extracts.

The cultivar Mandirituba, used in the evaluations, has 1.91% chamazulene, 16.20% bisabolol A oxide and 25.83% α-bisabolol oxide B (28,33). Comparing the results obtained with the above referential, we affirm that the 30CH dynamization reduced the percentage of α-bisabolol oxide B (16.73%), while the 12CH dynamization increased (33.43%). For the α-bisabolol oxide A compound, all dynamizations increased their concentration in relation to the data found by Côrrea Junior (33) and Amaral et al. (28). In comparison with distilled water, only 6CH dynamization reduced the percentage of α-bisabolol oxide A. In relation to chamazulene, when comparing the data found in the literature, all dynamizations increased the concentration of this compound, with a variation from 2.81 to 4.98%. However, statistically, the highest percentage of chamazulene was obtained in distilled water, again emphasizing the benefit of cultivating chamomile in a protected environment. In addition to these compounds, we can also mention the majority of cis-ene-yne-dicycloether, which presents corresponding percentages between 3.31 to 5.60% in chamomile essential oil obtained in conventional cultivation (31,34). However, the application of Phosphorus, in the 12CH and 30CH dynamizations, promoted an increase in the concentration of this compound, with values of 9.66 and 13.11%, respectively.

Thus, Phosphorus dynamizations can be used to increase the percentage of the main compounds found in chamomile essential oil, when compared to the results found in the literature, without the application of homeopathy. However, it is noteworthy that the environmental factors of the place of cultivation directly influence the composition and concentration of secondary metabolites (2,35), as well as the use of other homeopathic medicines, thus resulting in different responses. The application of homeopathy alters plant metabolism, which may increase or decrease the production of biomass and essential oil (36,37). That is, the homeopathic response in the plant is oscillatory within the group of dynamizations and the evaluated characters, which explains the results found in the content, productivity and composition of chamomile essential oil. And due to this oscillatory response, a range of Phosphorus dynamizations was used, contemplating a low (3CH, 6CH), intermediate (12CH) and a high power (30CH).

### 3.3. Principal component analysis

A multivariate exploratory analysis, Principal Component Analysis (PCA), was used to visualize the influence of Phosphorus dynamizations on the content, productivity and chemical composition of chamomile essential oil. In the first two main components it was possible to visualize the discrimination of the samples (Figure 3(a)) as a function of the correlation with the variables (Figure 3(b)), summing 56.86% of the total data variation.

Principal Component 1 (PC1) discriminated the 6CH, 30CH dynamizations and the distilled water control from the 3CH, 12CH dynamizations and alcohol 70% (Figure 3(a)). Compounds such as spatulenol, α-bisabolol oxide A, trans-chrysanthemol, productivity, α-bisabolol, γ-terpinene, content, α-bisabolol oxide B, eucalyptol, β-ocimene and 2-isopropyltoluene were correlated with the 3CH, 12CH dynamizations and 70% alcohol (Figure 3(b)). The concentration percentage of these compounds is positively influenced by these treatments. The 6CH, 30CH dynamizations and distilled water were associated with the intervention in the concentration percentage mainly of the cis-ene-yne-dicycloether, bisabolone oxide, linolenic acid, bicyclogermacrene, NI3, nerolidol isomer, linoleic acid, nonenal, α-farnesene, germacrene D, β-trans-farnesene, delta-cadinene, dehydrosesquicineole, nerolidol, lavandulol, artemesia ketone, chamazulene, artemisia alcohol, α-terpineol, ethyl decanoate, 1-naphthalene propanol (epimanool) and 4.8-dimethyl-nona-3.8-dien-2-one (Figure 3(b)).

In Principal Component 2 (CP2) there was the distinction of the smallest dynamizations, 3CH and 6CH, from the 12CH and 30CH dynamizations (Figure 3(a)). The 12CH and 30CH dynamizations were correlated with the highest percentage of spatulenol concentration,

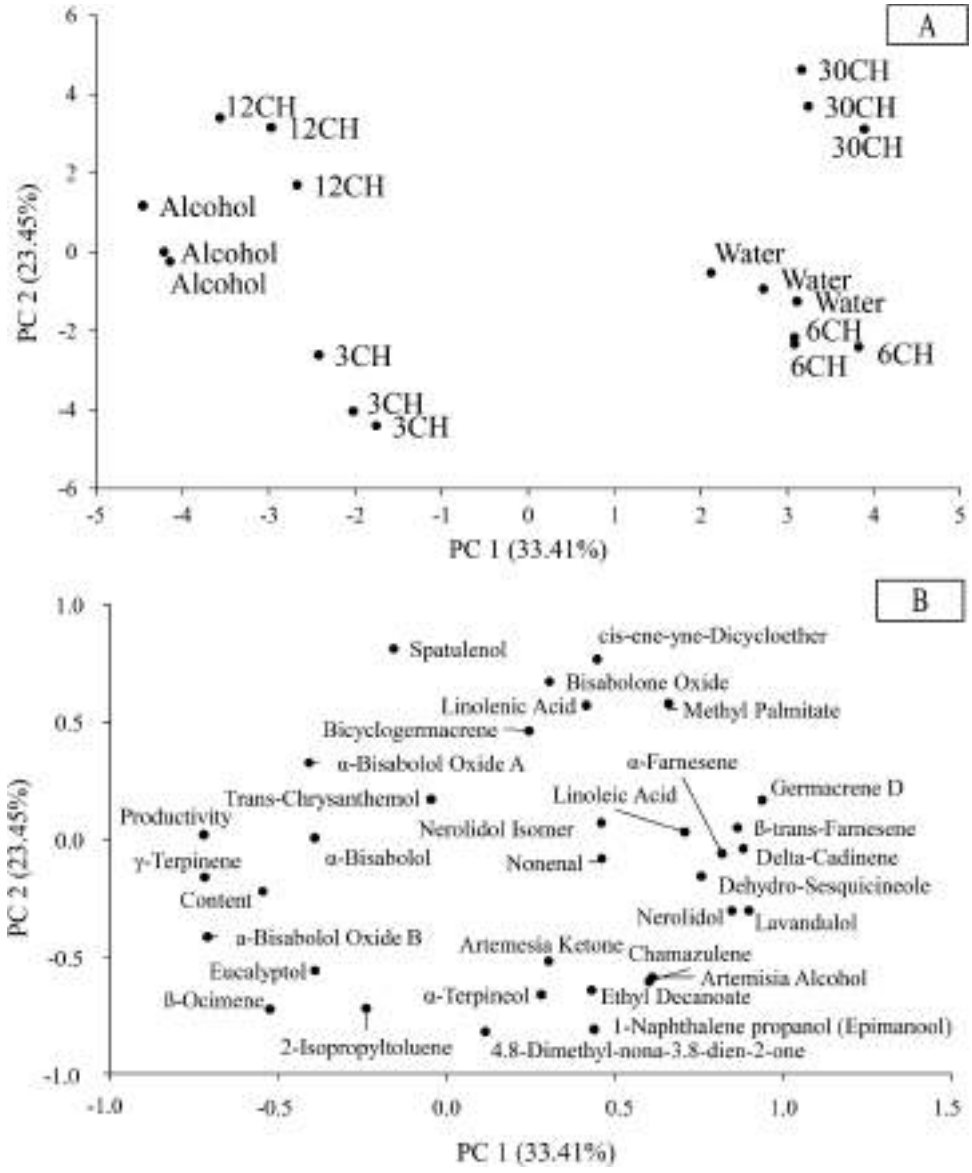



**Figure 3.** Principal Component Analysis applied to Phosphorus dynamizations in chamomile cultivation. (a) Representation of treatments. (b) Representation of the variables (compounds, content and productivity of chamomile essential oil).

while the lowest dynamizations were related to the concentration of the compounds 4.8-dimethyl-nona -3.8-dien-2-one, ethyl decanoate, epimanool and α-terpineol (Figure 3(b)). Thus, the principal component analysis clearly allows the visualization of the relationship between the evaluated treatments and their influence on the percentage of concentration of the compounds present in the chamomile essential oil.

In this unpublished research we confirmed the direct effect of homeopathy on secondary metabolism, with increased productivity and improved quality of the essential oil, the latter, provided by the increased concentrations of the major compounds found in chamomile essential oil. These results reaffirm the role and correlation of homeopathy with the secondary metabolism of medicinal plants, as a stimulating factor and a technique that can be used in agricultural crops. However, the response of the plant to the application of homeopathy is dependent on the species, dynamization and environmental conditions of the place of cultivation. However, scientific deepening is still necessary for the discovery and identification of the exact location or metabolic route of homeopathy in the vegetable.

## 4. Conclusion

The homeopathic medicine Phosphorus in the 3CH dynamization proved to be the most efficient treatment

in increasing the content and productivity of chamomile essential oil. Phosphorus dynamizations change the concentration percentage of the chemical compounds in the chamomile essential oil, specifically, with an increase in major compounds. Thus, homeopathy is configured as a cultivation technique capable of increasing productivity and improving the quality of essential oil in a sustainable manner, without environmental contamination and with low production cost.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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ORIGINAL ARTICLE

# Mixed culture microorganisms fermented soybean meal improves productive performance and intestinal health of Nile tilapia (*Oreochromis niloticus*) juveniles fed plant-based diets in a biofloc system

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## Abstract

This study aimed to evaluate the effect of plant-based diets containing different inclusion levels (7%, 14%, 21% and 28%) of mixed culture microorganisms fermented soybean meal (FSBM) on the zootechnical performance and intestinal health of juvenile Nile tilapia (*Oreochromis niloticus*) reared in a biofloc system. The FSBM diets were compared with a positive control diet with fish meal and a negative control diet with no animal protein. All diets were isoprotein (33% CP) and isoenergetic (4300 kcal GE/kg). The design was completely randomized with four replications and the experiment lasted 54 days. Juveniles (1.635 ± 0.198 g) were distributed in 24 tanks (70 L) at a density of seven animals per tank. Fermentation increased probiotic microorganisms count (lactic acid bacteria 4.75 ± 0.21 log CFU $g^{-1}$ and yeast 3.30 ± 0.54 log CFU $g^{-1}$) and improved nutritional characteristics of FSBM. In fish fed the diet containing 7% FSBM, growth performance did not differ from fish fed the fish meal diet. FSBM inclusion improved the food efficiency of plant-based diets. The inclusions above 21% of FSBM increased the intestinal villi height. In the inclusion of 28% of FSBM, the goblet cell was higher compared to that of the other plant-based diets. The inclusion of 7% FSBM allows a total replacement of fish meal without compromising the growth performance of the animals.

**KEYWORDS**
goblet cell, lactic acid bacteria, probiotic microorganism, villi height, yeast

## 1 | INTRODUCTION

Feed costs can reach up to 60% of operational expenses in intensive fish production systems (Barbosa et al., 2020). Fish meal is important in aquaculture diets as a protein source with high biological value. Its exclusion from the formulations can lead to poor performance and health (Glencross et al., 2011; Gomes et al., 1995; Turchini et al., 2019). Commercial availability of fish meal is decreasing (World Bank, 2020). The increase in the price of fish meal impacts the production costs of aquatic animals (El Basuini et al., 2016, 2017; Hossain et al., 2016). In this context, alternative biotechnological techniques should be considered for the development of plant-based diets that

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reduce costs and optimize production performance (Bulbul et al., 2015; Daniel, 2018; Zhang et al., 2016).

Soybean meal is widely used as a vegetable protein source due to its availability in the market, amino acid profile and affordable price (Daniel, 2018; Sørensen et al., 2009). However, the complete replacement of fish meal with soybean meal in fish diets is not always possible (Bonaldo et al., 2015; Goda et al., 2007; Hansen et al., 2011; Ferrara et al., 2015). Several factors can make its replacement unfeasible, such as poor palatability, high fibre content and deficiency of essential amino acids (Dunsford et al., 1989; El-Dahhar & El-Shazly, 1993). Strategies such as microbial fermentation of soybean meal can address these problems, boosting the performance and immune system of animals (Hong et al., 2004; Van Nguyen et al., 2018; Refstie et al., 2005; Sotoudeh et al., 2016).

Fermentation can result in useful bioconversions or metabolic decomposition. Complex substrates are transformed into simple compounds by the action of microorganisms (Balakrishnan & Pandey, 1996). It can be a preservation method that also improves the nutritional and functional properties of ingredients (Frias et al., 2008; Ross et al., 2002). During the fermentation process, there may be an increase in populations of probiotic microorganisms and secondary metabolites with prebiotic activity (Balakrishnan & Pandey, 1996; Dawood & Koshio, 2019; Mukherjee et al., 2015; Wang et al., 2019). Fermentation can be done with a single microorganism or with a mixed culture of microorganisms composed of bacteria and yeast. In mixed fermentation, there is a better use of the substrate, since these combined cultures have a wider range of enzymes and are capable of attacking a wide variety of compounds in the substrate (Dawood & Koshio, 2019; Fossi et al., 2014; Macfadyen, 1903).

It has been demonstrated that fermented soybean meal (FSBM) can partially replace fish meal in diets without compromising fish performance (Azarm & Lee, 2014; Barnes et al., 2015; Choi et al., 2019; He, Li, et al., 2020; He, Yu, et al., 2020; Ilham & Fotedar, 2016; Jiang et al., 2018; Lee et al., 2016; Liang et al., 2017; De Oliveira et al., 2022; Shiu et al., 2015; Sotoudeh et al., 2016; Wang et al., 2016, 2019; Wu et al., 2003; Yamamoto et al., 2010). In addition, FSBM can have beneficial effects on fish health, such as improvements in biochemical parameters (Novriadi et al., 2017), lipid metabolism, antioxidant response (Jiang et al., 2018; Li et al., 2020) and intestinal health (Li et al., 2019; Shiu et al., 2015; Sotoudeh et al., 2016; Wang et al., 2016, 2019; Yamamoto et al., 2010).

Nile tilapia production has increased in recent years (GOAL, 2019; FAO, 2020). Production in a biofloc technology (BFT) system is already being evaluated as an alternative to traditional semi-intensive production systems in ponds (Azim & Little, 2008; Lima et al., 2015, 2018; Luo et al., 2014; Luo et al., 2014). It is an intensive cultivation system, with reduced water use, less effluent emissions and increased productivity (Avnimelech, 2009). However, intensive systems pose challenges to animals, as they may suffer higher levels of stress than in traditional systems (Gabriel & Akinrotimi, 2011; Mohapatra et al., 2012). BFT production is done in small spaces and high population densities. When these factors are coupled with the constant fluctuations in water quality parameters and changes in management practices, they can easily affect the animal's growth and overall health (Fischer et al., 2020; Romano et al., 2020).

The use of soybean meal as a replacement for fish meal has been evaluated in diets for tilapia and total replacement is not always possible (Ajani et al., 2016; Al-Kenawy et al., 2008; El-Sayd & Gaber, 2002; Lin & Luo, 2011; Sharda Sharma & Saini, 2017). *Saccharomyces cerevisiae* fermented soybean meal allowed only a partial replacement of fishmeal in plant-based diets for Nile tilapia (Hassaan et al., 2015). Fermentation using mixed microbial cultures can provide better results and is a strategy that still needs to be evaluated as a tool for promoting the growth and health of Nile tilapia-fed plant-based diets. Furthermore, the use of fermented ingredients in a BFT system still needs to be evaluated. Therefore, the objective of this study was to evaluate the effect of plant-based diets containing different inclusion levels of a fermented soybean meal (FSBM) prepared with a mixed microbial culture on the zootechnical indices and intestinal health of juvenile Nile tilapia (*Oreochromis niloticus*) reared in a BFT system.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental design

The experiment was carried out at the Aquaculture Laboratory of the State University of Santa Catarina – UDESC, Campus Chapecó (Santa Catarina, Brazil) and was approved by the Ethics Committee on the Use of Animals (CEUA), under protocol number 3851120320. FSBM was evaluated at four inclusion levels in plant-based diets (7, 14, 21 and 28%) and compared with a positive control diet with fish meal and a negative control diet with no animal protein nor FSBM. The design was completely randomized (CRD) with six treatments and four replications, and it lasted for 54 days.

### 2.2 | FSBM production and characterization

The production of the fermented soybean was done according to an adaptation of the solid-state fermentation technique (Azarm & Lee, 2014). For this purpose, the soybean meal was autoclaved at a temperature of 100°C for 20 min and inoculated with Aquate Fertilizer® (Alltech™) in the proportion of 15 g $kg^{-1}$. The composition of the Aquate Fertilizer® is shown in Table 1. Distilled water was added in the proportion of 50% of the weight of the soybean meal and the mixture was placed in a polypropylene tray and kept in a forced air incubator at 36°C. Every 12 h, the material was weighed, and distilled water was added to keep the moisture level at 50%. After the fermentation process was completed (48 h), the fermented soybean meal (FSBM) was placed again in a forced air incubator at 36°C until it reached a constant weight. Subsequently, it was stored in a freezer at a temperature between −14°C and −25°C.

The proximal composition of soybean meal and FSBM (Table 2) was determined according to methods described in the Association

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**TABLE 1** Warranty levels and basic composition of Aquate Fertilizer®

| Product warranty levels | Quantity |
|---|---|
| *Saccharomyces cerevisiae* (min.) | $8 \times 10^7$ CFU $g^{-1}$ |
| Mannans (min.) | 26.3 g $kg^{-1}$ |
| Copper (min.) | 2133 mg $kg^{-1}$ |
| Zinc (min.) | 1249 mg $kg^{-1}$ |
| Selenium (min.) | 75 mg $kg^{-1}$ |
| **Basic product composition** | |
| Dry sugar cane yeast, hydrolyzed dehydrated yeast, dry brewery yeast, calcium carbonate, *Saccharomyces cerevisiae*, selenium-enriched yeast, copper proteinate, sodium and calcium aluminium silicate, dry fermentation product of *Aspergillus niger*, zinc proteinate, ascorbic acid, *Schizochytrium* sp. seaweed meal, *Enterococcus faecium*, *Lactobacillus acidophilus*, dry fermentation product of *Trichoderma longibrachiatum*, sodium chloride and *Chlorella vulgaris* seaweed meal | |

*Note:* Source: Alltech™, 2021.

**TABLE 2** Proximal composition, soluble protein, pH and microbiological count of fermented soybean meal (FSBM)

| Proximal composition | Soybean meal[a] | FSBM[a] |
|---|---|---|
| DM (%) | 88.35 | 90.82 |
| MM (%) | 7.42 | 7.34 |
| EE (%) | 5.23 | 5.05 |
| GE (kcal $kg^{-1}$) | 4259.09 | 4383.85 |
| CP (%) | 44.63 | 47.09 |
| SP (mg $g^{-1}$) | 19.90 | 40.80 |
| **Time** | | |
| **Parameters** | **0 (h)** | **48 (h)** |
| pH | 6.64 | 6.06 |
| LAB (log CFU $g^{-1}$) | 0 | 6.47 |
| Yeast (log CFU $g^{-1}$) | 0 | 7.43 |

Abbreviations: CFU, colony forming unit; CP, crude protein; DM, dry matter; EE, ether extract; FSBM, fermented soybean meal; g, grams; GE, gross energy; h, hours; LAB, lactic acid bacteria; MM, mineral matter; pH, hydrogenic potential; SP, soluble protein.

[a]Composition analyzed by the author.

of Official Analytical Chemists (AOAC, 2000). Lactic acid bacteria and yeast concentrations were determined on raw soybean meal and FSBM (Table 2). For this purpose, samples of one gram were serially diluted (1:10) in sterile saline solution at 0.65% and then seeded on MRS (Man Rogosa & Sharpe) agar, to count lactic acid bacteria, and in Sabouraud + chloramphenicol medium (0.5 g $L^{-1}$) to count yeasts. Inoculated Petri plates were incubated at a constant temperature of 37°C for 48 h and 25°C for 72 h, respectively (Abdel-Razek, 2019; Silva et al., 2013), for total colony forming unit (CFU) counts. Soluble protein concentration was determined by the Bradford method (Bradford, 1976) with bovine serum albumin (Sigma-Aldrich) as a standard. All samples were analyzed in triplicate (Table 2).

**TABLE 3** Amino acid composition of soybean meal and fermented soybean meal (FSBM) used for the formulation of experimental diets

| | Soybean meal** | FSBM* |
|---|---|---|
| Essential amino acids | | |
| Arginine | 3.43 | 3.09 |
| Histidine | 1.23 | 1.15 |
| Isoleucine | 2.18 | 2.14 |
| Leucine | 3.59 | 3.57 |
| Lysine | 2.87 | 2.89 |
| Methionine | 0.62 | 0.51 |
| Phenylalanine | 2.39 | 2.50 |
| Threonine | 1.82 | 1.75 |
| Tryptophan | 0.66 | *** |
| Valine | 2.27 | 2.28 |
| Nonessential amino acids | | |
| Alanine | 2.08 | 2.13 |
| Aspartic acid | 3.30 | 5.06 |
| Glutamic acid | 4.55 | 8.15 |
| Cystine | 0.69 | 0.64 |
| Glycine | 2.02 | 1.94 |
| Hydroxyproline | **** | 0.08 |
| Proline | 2.37 | 2.34 |
| Serine | 2.52 | 2.30 |
| Tyrosine | 1.70 | 1.57 |
| Sum of amino acids | 40.29 | 44.09 |

*Note:* Source: *The authors; **Rostagno et al. (2017); ***The tryptophan amino acid analysis for FSBM was not performed. ****No hydroxyproline values for soybean meals were reported in Rostagno et al. (2017).

Abbreviation: FSBM, fermented soybean meal.

The pH analysis was also performed according to the potentiometric method (AOAC, 2000). 10 g of FSBM and 100 ml of distilled water at 25°C were mixed (electronic shaker, brand DiagTech, model DT3110H) in an Erlenmeyer (250 ml) for 30 min. This mixture was transferred to a 500 ml beaker, where it remained at rest for 10 min and, subsequently, measurements were carried out with the aid of a bench-top microprocessed pH metre (Alfakit™, AT-355) (Table 2). Finally, the FSBM amino acid composition was determined according to White et al. (1986), and for soybean meal, data from Rostagno et al. (2017) were used (Table 3).

## 2.3 | Experimental diets

The experimental diets were isoprotein (33% crude protein) and isoenergetic (4300 kcal of gross energy $kg^{-1}$) and were formulated (Table 4) in order to meet the nutritional requirements of protein (El-Sayd & Gaber, 2002) and amino acids for tilapia (Santiago &

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**TABLE 4** Formulation, chemical composition and microorganism count of experimental diets (g $kg^{-1}$) based on dry matter

| | Treatments | | | | | |
|---|---|---|---|---|---|---|
| | C+ | C− | 7% | 14% | 21% | 28% |
| Ingredients (%) | | | | | | |
| Soybean meal | 42.00 | 65.00 | 57.00 | 50.00 | 42.00 | 35.00 |
| FSBM | – | – | 7.00 | 14.00 | 21.00 | 28.00 |
| Fish meal | 20.00 | – | – | – | – | – |
| Corn | 21.70 | 19.50 | 21.00 | 21.50 | 22.50 | 22.50 |
| Wheat flour | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 | 13.00 |
| Soy oil | 2.00 | 1.00 | 0.50 | – | – | – |
| Premix | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| DL-Methionine | 0.30 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Total (%) | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| Analyzed bromatological composition and microorganism count | | | | | | |
| DM (%) | 85.94 ± 4.34 | 86.49 ± 4.11 | 84.77 ± 0.46 | 85.24 ± 0.52 | 83.90 ± 0.69 | 83.97 ± 1.81 |
| CP (%) | 32.93 ± 0.27 | 32.85 ± 0.42 | 32.96 ± 0.06 | 31.95 ± 0.66 | 33.23 ± 0.40 | 34.54 ± 0.38 |
| EE | 6.01 ± 0.17 | 3.80 ± 0.06 | 3.17 ± 0.09 | 3.23 ± 0.04 | 3.10 ± 0.09 | 3.61 ± 0.09 |
| MM (% (%)) | 10.78 ± 0.06 | 4.84 ± 0.03 | 4.87 ± 0.02 | 4.84 ± 0.04 | 4.79 ± 0.01 | 5.29 ± 0.02 |
| GE (kcal $kg^{-1}$) | 4,200.53 | 4397.47 | 4,416.35 | 4,261.477 | 4,338.91 | 4,411.57 |
| LAB (log CFU $g^{-1}$) | NG | NG | 4.73 ± 0.33 | 4.90 ± 0.37 | 5.26 ± 0.11 | 4.11 ± 0.62 |
| Yeast (log CFU $g^{-1}$) | 2.45 ± 0.21 | 2.00 ± 0.00 | 3.32 ± 0.35 | 3.25 ± 0.59 | 3.35 ± 0.33 | 3.28 ± 0.91 |

*Note:* Premix: folic acid—2,400 mg, nicotinic acid—48 g, pantothenic acid—24 g, biotin—96 mg, vit. A—2,400,000 IU, vit. D3—400,000 IU, vit. E—24,000 IU, vit. B1—9,600 mg, vit. B2—9,600 mg, vit. B6—9,600 mg, vit. B12—9,600 mg, vit K3—4,800 mg, vit. C—96 g, iron—100 g, manganese—40 g, zinc—6,000 mg, cobalt—20 mg, iodine—200 mg, selenium—200 mg, antioxidant—19.6 g.

Abbreviations: 14%, plant-based diet with 14% inclusion of FSBM; 21%, plant-based diet with 21% inclusion of FSBM; 28%, plant-based diet including 28% of FSBM; 7%, plant-based diet including 7% FSBM; C−, negative control (plant-based diet without fish meal); C+, positive control (fish meal diet); CFU, colony forming unit; CP, crude protein; DM, dry matter; FSBM, fermented soybean meal; GE, *gross energy; LAB, lactic acid bacteria; MM, mineral matter; NG, no growth in colony forming units was observed.

Lovell, 1988). The amino acid compositions of the diets are shown in Table 5. Inclusion levels of fermented soybean meal were determined based on similar studies (Hassaan et al., 2015; De Oliveira et al., 2022; Wang et al., 2016). In addition to FSBM, the formulated diets contained fish meal, soybean meal as protein sources, and wheat flour and soy oil as energy sources. The fish meal was purchased from Agroforte™, and the other ingredients were purchased from local suppliers. All ingredients were ground in an industrial processor and sieved in 0.71 mm mesh. Subsequently, they were mixed with the addition of water (50%), pelletized in a 1 mm "spaghetti" matrix (Malta®, model 1036, no. 8 mouthpiece) and placed in an incubator (Cienlab®, model CE-220/150) at 55°C for 48 h. The diets were stored in plastic containers and placed in refrigerators (4°C) until use.

The counting of yeasts and lactic acid bacteria was also performed in the experimental diets (Table 4). For this purpose, one-gram samples were serially diluted (1:10) in sterile saline solution at 0.65% and then seeded on MRS (Man Rogosa & Sharpe) agar for the counting of lactic acid bacteria and on Sabouraud + chloramphenicol agar (0.5 g $L^{-1}$) to count yeasts (*Saccharomyces cerevisiae*). After sowing in Petri dishes, the plates with MRS were incubated at a constant temperature of 35°C for 48 h (Silva et al., 2013), and the plates with Sabouraud + chloramphenicol were incubated at 25°C for 72 h (Abdel-Razek, 2019). At the end of the respective periods, the total counts of colony forming units (CFUs) were performed, as shown in Table 4.

## 2.4 | Animals and facilities

The fish were acquired from Acqua Sul Piscicultura, located in the city of Ilhota, Santa Catarina State and previously acclimated to the experimental conditions. A total of 168 Nile tilapia juveniles with an average initial weight of 1.635 ± 0.198 g were distributed in 24 circular polyethylene tanks (useful volume of 70 L) connected to a recirculating aquaculture system (RAS). The tanks were equipped with an individual aeration system using a porous stone (1 × 2 cm) coupled through a radial air compressor and distributed over two benches. These benches were arranged and connected to two circular tanks (useful volume: 850 L) called "macrocosms" that contained a peripheral water pump (Intech®, model BP500) responsible for the distribution and recirculation of water in the system. Each macrocosm was

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**TABLE 5** Required and calculated amino acid composition of experimental diets.

| Composition | C+ | C− | 7% | 14% | 21% | 28% | Requirement[a] |
|---|---|---|---|---|---|---|---|
| Arginine | 2.11 | 2.33 | 2.31 | 2.31 | 2.28 | 2.28 | 1.38 |
| Histidine | 0.89 | 0.84 | 0.83 | 0.83 | 0.82 | 0.82 | 0.57 |
| Isoleucine | 1.79 | 1.55 | 1.54 | 1.54 | 1.52 | 1.52 | 1.03 |
| Leucine | 2.93 | 2.73 | 2.72 | 2.72 | 2.70 | 2.70 | 1.12 |
| Lysine | 2.49 | 2.10 | 2.08 | 2.08 | 2.05 | 2.05 | 1.69 |
| Methionine | 0.93 | 0.88 | 0.88 | 0.88 | 0.88 | 0.88 | 0.88 |
| Phenylalanine | 1.56 | 1.48 | 1.47 | 1.47 | 1.46 | 1.46 | 1.24 |
| Threonine | 1.49 | 1.40 | 1.39 | 1.39 | 1.37 | 1.37 | 1.27 |
| Tryptophan | 0.48 | 0.57 | 0.57 | 0.57 | 0.56 | 0.56 | 0.33 |
| Valine | 1.98 | 1.77 | 1.76 | 1.76 | 1.74 | 1.74 | 0.92 |
| Sum | 16.65 | 15.65 | 15.55 | 15.55 | 15.38 | 15.38 | 10.43 |

Abbreviations: 14%, plant-based diet with 14% inclusion of FSBM; 21%, plant-based diet with 21% inclusion of FSBM; 28%, plant-based diet including 28% of FSBM; 7%, plant-based diet including 7% FSBM; C−, negative control (plant-based diet without fish meal); C+, positive control (fish meal diet).
[a]Santiago & Lovell, 1988.

equipped with a heater resistor (Nobre Brasil®, 3000 W), connected to a digital temperature controller with a sensor (XH-W3001®, 12V, China).

Each experimental unit received an initial biofloc inoculum (10 L) from previous Nile tilapia cultures in the BFT system and non-chlorine water until completing the useful volume of 70 L. For the formation and maintenance of the microbial community and suitable conditions of the culture medium, a C:N ratio of 10–15:1 was maintained (Pérez-Fuentes et al., 2016) with sugarcane molasses being used as an external source of carbon. Thus, a biomass of 2.25 kg $m^{-3}$ of tilapia was also maintained in each macrocosm, fed with commercial feed (32% CP supplied one time per day at 12 p.m.).

The animals were fed once a day (12 p.m.) with an initial feeding rate of 10% of the biomass/day until they reached twice their initial weight (Lopez-Betancur et al., 2020). Subsequently, a rate of 5% of biomass/day was maintained until the end of the experimental period (Lopez-Betancur et al., 2020). The monitoring of the animals' biomass was carried out every 18 days through biometrics of all experimental units.

Water temperature, dissolved oxygen (Alfakit™ oximeter, model AT170) and pH (Alfakit™ pH metre, model AT315) were measured daily as indicators of water quality. Analyses of nitrogen compounds (ammonia, nitrite, nitrate), orthophosphate and alkalinity of the culture medium were performed weekly using a photocolorimeter (Alfakit™, model AT100P). Total sedimentable solids (TSS) were measured with an Imhoff cone. Mean values were found for temperature, dissolved oxygen and pH, of 25.15 ± 2.20°C, 9.03 ± 0.60 mg/$L^{-1}$ and 6.92 ± 0.25 respectively. Ammonia, nitrite, nitrate, orthophosphate, alkalinity and total sedimentable solids, total values of 0.17 ± 0.09 mg $L^{-1}$, 0.22 ± 0.07 mg $L^{-1}$, 10.70 ± 9.11 mg $L^{-1}$, 13.67 ± 1.20 mg $L^{-1}$ $PO_4$, 11.49 ± 4.64 mg $L^{-1}$ $CaCO_3$ and 5.99 ± 3.28 mg $L^{-1}$ were observed respectively. The parameters remained within the recommended range for tilapia throughout the study (El-Sayed, 2006).

## 2.5 | Zootechnical performance

After 53 days, the animals were fasted for 24 h and were individually weighed (0.01 g precision scale, Marte™, model ML 600) and measured to assess the zootechnical parameters: final weight (FW); mean weight gain (MWG) = final weight (g)−initial weight (g); specific growth rate (SGR) = [ln final weight (g)−ln initial weight (g)]/experimental period × 100; (L); Fulton's condition factor (K) = weight/length × 100; feed conversion rate (FCR) = feed intake/weight gain; survival; feed consumption (FC) = food consumed in the trial period (g) and survival (S) = number of dead animals/total fish × 100.

## 2.6 | Intestinal histomorphometry

For the histomorphometric analysis, intestine fractions (proximal) were collected from the same animals used in the organometric indices (12 animals/treatment). The histological processing of these samples was performed at the Histology Laboratory at UDESC, Campus Lages (Santa Catarina, Brazil), according to routine methods for preparing histological slides (Nunes & Cinsa, 2016). The intestinal fragments were placed in sterile plastic containers with a common formalin solution (100 ml of 37–40% formalin; 900 ml of common water and 9.0 g of sodium chloride), identified and after being fixed for 12–16 h, preserved in 70% alcohol until the evaluation of the morphohistological characteristics of its mucosa. For this purpose, these intestinal fractions were sectioned into slices of approximately 0.3 cm using the technique of histology, diaphanization and embedding in histological paraffin. Three μm-thick sections were prepared on a semi-automatic microtome according to the methodology adapted from Mello et al. (2013). Longitudinal sections were prepared, semiserialized and stained using the Schiff histochemical method (Periodic Acid-Schiff - PAS) (de Tolosa et al., 2003).

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Three slides were made from each sample and photographed using a digital camera coupled to a microscope (10× objective). The images were digitized and the measures were made using the TCapture software (Laborana® brand). After the digitization was completed, ten villi per animal were selected by integrity criterion (Picoli et al., 2019) for the measurements of the intestinal villi, where: (i) villi height (VH), that is, distance from the apex of the villi to the beginning of the muscle layer; (ii) total villi height (TVH), that is, height from the apex of the villi to the end of the serosa; (iii) villi width (VW); and (iv) villi thickness (VT), according to the methodology by Mello et al. (2013). In the same intestinal villi, goblet cells (GC) count was performed. Each goblet cell was considered as a unit (Picoli et al., 2019). All these measurements were performed using ImageJ software (National Institute for Health).

### 2.7 | Statistical analysis

Normality (Shapiro–Wilk test) and homogeneity of variances (Levene test) were verified and the data were submitted to analysis of variance (ANOVA). When significant, the means were compared using the Tukey test at a 5% probability level. In the case of differences between plant-based diets, regression analysis was performed, and $R^2$ values above 0.8 were presented in the captions of the respective tables.

## 3 | RESULTS

### 3.1 | Zootechnical parameters

In fish fed the diet containing 7% FSBM, the average weight gain did not differ ($p > 0.05$) from that in fish fed the diet containing fish meal (Figure 1). However, the results of this treatment did not differ from the negative control. Similar results ($p > 0.05$) were obtained for final weight, specific growth rate and condition factor (Table 6). In all diets containing FSBM, feed conversion did not differ ($p > 0.05$) from the positive control. In this case, there was also no ($p < 0.05$) difference in relation to the plant-based diet.

### 3.2 | Intestinal histomorphometry

The villi of the different treatments can be seen in Figure 2. In the animals fed with 21% and 28% FSBM in diets, greater ($p < 0.0001$) villi height (Figure 3) and total villi height was observed (Table 7) compared with other treatments. Villi width and thickness (Table 7) were not affected ($p > 0.05$) by the experimental diets. In the inclusion of 28% of FSBM, the goblet cell count (Figure 4) did not differ ($p > 0.05$) from the positive control but was higher compared to that of the other treatments ($p < 0.05$).

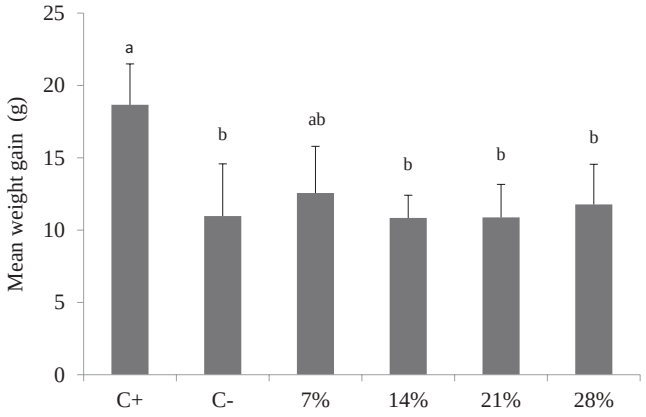



**FIGURE 1** Mean weight gain (mean ± standard deviation) of Nile tilapia juveniles cultivated in a biofloc system after 54 days of feeding with fermented soybean meal. C+, positive control (fish meal diet); C−, negative control (plant-based diet without fish meal); 7%, plant-based diet including 7% FSBM; 14%, plant-based diet with 14% inclusion of FSBM; 21%, plant-based diet with 21% inclusion of FSBM; 28%, plant-based diet including 28% of FSBM. Means accompanied by different letters differ from each other by the Tukey test ($p < 0.05$). Regression analysis was performed between plant-based diets, but due to the low $R^2$ value, the equation was not presented

## 4 | DISCUSSION

The water quality parameters remained within the expected range for the cultivation of tilapia in a BFT system throughout the experiment (Azim & Little, 2008; Lima et al., 2015, 2018; Luo et al., 2014). At the beginning of the experiment, the tanks were inoculated with water from previously matured BFT systems. This is a strategy that can be adopted to prevent the occurrence of unwanted variations in water quality parameters (Krummenauer et al., 2014; Martins et al., 2020). In addition, the culture macrocosm tanks were connected to a recirculation system, which ensured homogeneity between treatments. Therefore, it is possible to infer that the results were obtained due to diet and supplementation strategy and were not influenced by the conditions in the BFT system.

During soybean meal fermentation, there were greater lactic acid bacteria and yeast counts. There was also a reduction in pH, confirming the production of organic acids by microorganisms (Alexander, 1961; Dai et al., 2020; Imelda et al., 2008). These results confirm that fermentation occurred as expected (Balakrishnan & Pandey, 1996; Dawood & Koshio, 2019; Sanjukta & Rai, 2016). In FSBM, the growth of microorganism colonies resulted in greater microbial protein synthesis and greater crude protein. Similarly, a higher crude protein level was observed in soybean meal fermented with *Bacillus subtilis* (Shiu et al., 2015), *Aspergillus awamori* (Li et al., 2019), *Enteroccocus faecium* (Li et al., 2020) and a mixed culture of microorganisms composed of *B. subtilis*, *Lactobacillus* and *S. cerevisiae* (He, Yu, et al., 2020). Furthermore, soluble protein and the sum of amino acids in FSBM were also greater.

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**TABLE 6** Zootechnical parameters (mean ± standard deviation) of Nile tilapia juveniles cultivated in a biofloc system after 54 days of feeding with fermented soybean meal

| | Treatment | | | | | | |
|---|---|---|---|---|---|---|---|
| **Parameters** | **C+** | **C–** | **7%** | **14%** | **21%** | **28%** | ***p* value** |
| IW (g) | 1.63 ± 0.19 | 1.63 ± 0.21 | 1.63 ± 0.20 | 1.65 ± 0.22 | 1.63 ± 0.20 | 1.64 ± 0.19 | 0.9995NS |
| FW (g) | 20.29 ± 2.81 a | 12.60 ± 3.61 b | 14.22 ± 3.22 ab | 12.49 ± 1.56 b | 12.52 ± 2.26 b | 13.41 ± 2.77 b | 0.0061** |
| SGR (% day$^{-1}$) | 2.03 ± 0.28 a | 1.26 ± 0.36 b | 1.42 ± 0.32 ab | 1.24 ± 0.16 b | 1.25 ± 0.23 b | 1.34 ± 0.28 b | 0.0061** |
| K | 2.01 ± 0.15 a | 1.46 ± 0.26 b | 1.56 ± 0.25 ab | 1.44 ± 0.12 b | 1.46 ± 0.18 b | 1.51 ± 0.21 b | 0.0071** |
| FCR | 0.92 ± 0.02 b | 1.44 ± 0.27 a | 1.27 ± 0.24 ab | 1.29 ± 0.13 ab | 1.40 ± 0.18 ab | 1.29 ± 0.31 ab | 0.05* |
| FC (g animal$^{-1}$) | 17.19 ± 3.12 | 15.25 ± 3.26 | 15.63 ± 2.89 | 13.89 ± 0.84 | 15.02 ± 2.31 | 14.58 ± 1.64 | 0.5264NS |
| S (%) | 92.85 ± 14.28 | 89.28 ± 13.67 | 89.28 ± 7.14 | 96.42 ± 7.14 | 82.14 ± 13.67 | 85.71 ± 11.66 | 0.5606NS |

*Note:* Means followed by distinct letters in the rows differ from each other by the Tukey test ($p < 0.05$).

Abbreviations: 14%, plant-based diet with 14% inclusion of FSBM; 21%, plant-based diet with 21% inclusion of FSBM; 28%, plant-based diet including 28% of FSBM; 7%, plant-based diet including 7% FSBM; C–, negative control (plant-based diet without fish meal); C+, positive control (fish meal diet); FC, feed consumption; FCR, feed conversion rate; FW, final weight; g, gram; g/day, gram per day; IW, initial weight; K, Fulton's condition factor; kg/m$^3$, kilogram per cubic metre; NS, not significant; S, survival; SGR, specific growth rate.

*$p < 0.05$; **$p < 0.01$.

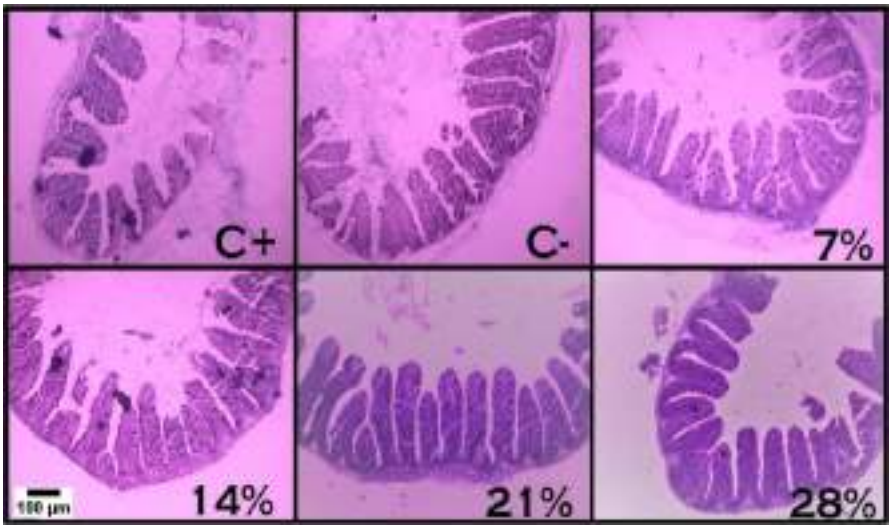



**FIGURE 2** Intestinal villi of Nile tilapia juveniles after 54 days of feeding with fermented soybean meal (FSBM) in a biofloc system. Coloration: periodic acid-schiff (PAS). Objective: 10×. C+, positive control (fish meal diet); C–, negative control (plant-based diet without fish meal); 7%, plant-based diet including 7% FSBM; 14%, plant-based diet with 14% inclusion of FSBM; 21%, plant-based diet with 21% inclusion of FSBM; 28%, plant-based diet including 28% of FSBM

There was considerably higher soluble protein present in FSBM (40.80 mg g$^{-1}$) compared to that of the unfermented soybean meal (19.90 mg g$^{-1}$). This is consistent with greater availability of low molecular weight peptides (Runho, 2001; Sanjukta & Rai, 2016; Teng et al., 2012) and demonstrates that the nutritional characteristics of the soybean meal were positively affected in the fermentation process.

The growth and body condition of fish fed on plant-based diets containing 7% FSBM did not differ from the diet containing fishmeal. These results are unprecedented and demonstrate the feasibility of using low levels of fermentation products as a strategy to optimize the use of plant-based diets. The use of soybean meal fermented with *S. cerevisiae* had previously been evaluated for Nile tilapia, but only a partial replacement of fish meal was viable (Hassaan et al., 2015). In the present study, the utilization of a mixed culture of microorganisms may have favoured the positive results due to the synergistic interaction commonly observed in fermentations between lactic acid bacteria and yeasts (Sieuwerts et al., 2018). Lactic acid produced by lactic acid bacteria encourages the growth of yeasts, which also use part of the excreted glucose as a carbon source (Gobbetti & Corsetti, 1997; Sieuwerts et al., 2018; De Vuyst & Neysens, 2005). In contrast, the $CO_2$ produced by yeasts stimulates the production of these bacteria. Other authors also found favourable results when using a mixed culture of microorganisms composed of *Lactobacillus plantarum*, *B. subtilis* and *S. cerevisiae* as probiotics for Nile tilapia (Essa et al., 2010).

The FSBM inclusion levels of over 14% compromised the growth performance of tilapia cultivated in BFT. For other species, such as the orange-spotted grouper (*Epinephelus coioides*), rockfish (*Sebastes schlegeli*), Japanese sea bass (*Lateolabrax japonicus*) and rainbow trout (*Oncorhynchus mykiss*), there was also a reduction in performance with the inclusion of high levels of FSBM (Choi et al., 2019; Lee et al., 2016; Liang et al., 2017; Shiu et al., 2015). Fermented products are rich in low molecular weight peptides (Jiang et al., 2018). At high levels of inclusion, these peptides may contribute to the saturation of intestinal absorption sites and transport mechanisms can occur,

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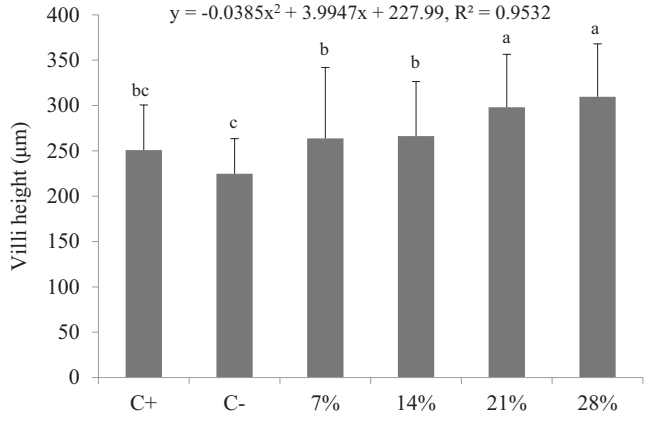



**FIGURE 3** Villi height of Nile tilapia juveniles cultivated in a biofloc system after 54 days of feeding with fermented soybean meal. C+, positive control (fish meal diet); C−, negative control (plant-based diet without fish meal); 7%, plant-based diet including 7% FSBM; 14%, plant-based diet with 14% inclusion of FSBM; 21%, plant-based diet with 21% inclusion of FSBM; 28%, plant-based diet including 28% of FSBM. Means accompanied by different letters differ from each other by the Tukey test ($p < 0.05$). Regression analysis was performed between plant-based diets

compromising nutrient absorption and feed utilization (Khosravi et al., 2015; Sanjukta & Rai, 2016; Siddik et al., 2018; Wei et al., 2016). Furthermore, an excessive quantity of free peptides from the fermented ingredient can increase amino acid oxidation and reduce dietary protein retention (Chai et al., 2020; Wei et al., 2019).

Fermentation improved the feed efficiency of plant-based diets for Nile tilapia in a BFT system. In all treatments with FSBM, the feed conversion did not differ from the diet containing fish meal. This result is in agreement with the literature, in which the inclusion of 15% to 45% FSBM in fish meal replacement did not affect the feed conversion of Japanese sea bass (*Lateolabrax japonicus*), yellow croaker (*Larimichthys crocea*) and black bass (*Micropterus salmoides*) (He, Li, et al., 2020; He, Yu, et al., 2020; Liang et al., 2017; Wang et al., 2019). The improvement in feed conversion can be explained by the higher soluble protein in FSBM. Peptides are more easily absorbed by enterocytes and assimilated at higher rates than intact proteins (Ganapathy et al., 1994; Runho, 2001). Furthermore, the improvement in feed conversion can also be attributed to the presence of probiotic microorganisms (Welker & Lim, 2011). The microorganisms inoculated in the FSBM remained active (lactic acid bacteria and yeasts) and their beneficial effects on feed conversion have already been demonstrated (Ghazalah et al., 2010; Lara-Flores et al., 2003, 2010; Welker & Lim, 2011).

The productive performance results showed that the inclusion of FSBM can enable the use of plant-based diets for tilapia juveniles reared in a BFT system. However, it is important to highlight that even with evidence of the possibility of replacing fishmeal, the results with the use of FSBM also did not differ from the plant-based negative control diet. Other factors may be involved and it is still necessary to confirm the results obtained. Long-term studies can highlight the differences between treatments. In addition, the

**TABLE 7** Intestinal histomorphometry (mean ± standard deviation) of Nile tilapia juveniles ($n = 12$/treatment) cultivated in a biofloc system after 54 days of feeding with fermented soybean meal (FSBM)

| Parameters | Treatment | | | | | | *p* value |
|---|---|---|---|---|---|---|---|
| | C+ | C− | 7% | 14% | 21% | 28% | |
| TVH (µm) | 269.10 ± 45.60 bc | 248.72 ± 38.15 c | 289.50 ± 73.89 b | 290.89 ± 65.28 b | 320.38 ± 59.72 a | 337.27 ± 61.73 a | <0.0001*** |
| VW (µm) | 90.27 ± 13.37 | 99.18 ± 7.04 | 86.69 ± 14.63 | 92.34 ± 17.37 | 96.12 ± 15.37 | 97.90 ± 8.36 | 0.4736NS |
| VT (µm) | 53.65 ± 4.29 | 52.04 ± 4.84 | 45.63 ± 6.87 | 48.72 ± 7.53 | 49.96 ± 8.79 | 51.27 ± 4.32 | 0.4765NS |

*Note*: Means followed by different letters in the lines differ from each other by the Tukey test ($p < 0.05$). Regression analysis was performed between plant-based diets.
Abbreviations: 14%, plant-based diet with 14% inclusion of FSBM; 21%, plant-based diet with 21% inclusion of FSBM; 28%, plant-based diet including 28% of FSBM; 7%, plant-based diet including 7% FSBM; C−, negative control (plant-based diet without fish meal); C+, positive control (fish meal diet); NS, not significant; TVH, total villi height ($y = -0.028x^2 + 3.7622x + 253.03$, $R^2 = 0.9469$); VT, villi epithelium thickness; VW, villi width.
***$p < 0.0001$.

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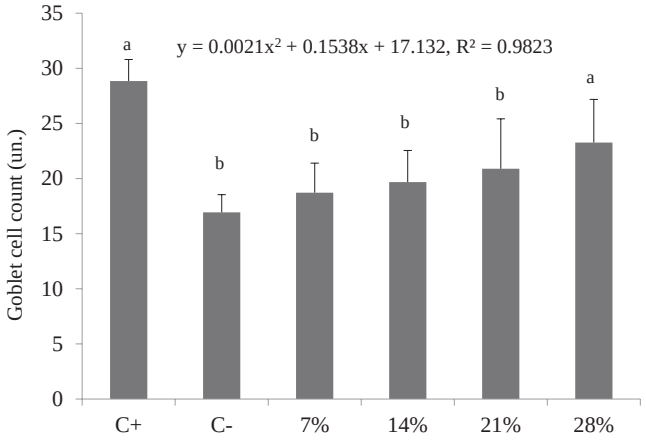



**FIGURE 4** Goblet cell count of Nile tilapia juveniles cultivated in a biofloc system after 54 days of feeding with fermented soybean meal. C+, positive control (fish meal diet); C–, negative control (plant-based diet without fish meal); 7%, plant-based diet including 7% FSBM; 14%, plant-based diet with 14% inclusion of FSBM; 21%, plant-based diet with 21% inclusion of FSBM; 28%, plant-based diet including 28% of FSBM. Means accompanied by different letters differ from each other by the Tukey test ($p < 0.05$). Regression analysis was performed between plant-based diets

BFT system may have positive effects on fish performance (Hisano et al., 2019; Da Silva et al., 2018) and may have masked the effects of treatments. The relationship between fermented products and production systems still needs to be better studied and understood. More studies are still needed to optimize the use of fermented foods in plant-based fish diets.

The villi height was greater in fish fed with diets containing levels above 21% of FSBM compared with the positive control diet containing fish meal. This result of greater villi height is unprecedented and demonstrates the potential of including fermented soybean meal in feeds to promote fish intestinal health. More intact mucosa with larger villi has a better nutrient absorptive capacity (Clements et al., 2014; Merrifield & Ringo, 2014), which may reflect on fish performance and health (Jobling, 1995; Silva et al., 2010). It is noteworthy that at the lowest levels of FSBM inclusion, 7% and 14%, the villi did not differ from the positive control and were greater when compared to the negative control diet. These results demonstrate that fermentation was effective in protecting against epithelial damage that may occur with unfermented soybean meal supplementation (Lilleeng et al., 2009; Marjara et al., 2012). As noted in the negative control, diets with a high plant protein level affect the integrity of the intestinal epithelium and can lead to inflammation.

At the highest level of FSBM inclusion (28%), a higher count of goblet cells was observed in relation to the other plant-based diets, and the values did not differ from the positive control. This result is also unprecedented, and it indicates greater intestinal immunity, since the primary function of these cells is the production of mucus and, consequently, protection and lubrication of the intestinal lining (Clements et al., 2014; Merrifield & Ringo, 2014). Furthermore, they are closely related to good quality local microbiota (Schwarz et al., 2010). The probiotic microorganisms and the bioactive metabolites present in FSBM may have favourably supported these cells and mitigated possible inflammation caused by the greater inclusion of soybean meal in plant-based diets (Merrifield et al., 2011; Urán et al., 2009). This result is confirmed by the improvement in the characteristics of the intestinal epithelium and demonstrates the positive effects of FSBM on intestinal health.

## 5 | CONCLUSION

The utilization of a mixed microbial culture increased the probiotic microorganisms count and improved the nutritional characteristics of FSBM. The inclusion of 7% FSBM in the diets allows a total replacement of fish meals without compromising the growth performance of the animals. At all levels of FSBM inclusion, there was an improvement in the food efficiency of plant-based diets. In the inclusions above 21% of FSBM, there was also evidence of improvement in the intestinal health of Nile tilapia juveniles.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### AUTHOR CONTRIBUTIONS

Fernanda Picoli conceived and designed the analysis, collected the data, contributed to the data of analysis tools, performed the analysis and wrote the paper; Suelyn de Oliveira Marques collected the data; Alana Duarte de Oliveira collected the data; Cristina Gradin Nunes collected the data; Suélen Serafini contributed to the data of analysis tools and wrote the paper; Bruna Klein contributed to the data of analysis tools and performed the analysis; Nandara Soares de Oliveira collected the data; Nataly Neves Oliveira dos Santos contributed to the data of analysis tools and wrote the paper; Aline Zampar conceived and designed the analysis, contributed to the data of analysis tools and performed the analysis; Diogo Luiz de Alcantara Lopes conceived and designed the analysis, collected the data, contributed to the data of analysis tools, performed the analysis and wrote the paper; Thiago El Hadi Perez Fabregat conceived and designed the analysis, collected the data, contributed to the data of analysis tools, performed the analysis and wrote the paper.

### ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the Ethics Committee on the Use of Animals (CEUA - UDESC) approval has been received (CEUA protocol number 3851120320).

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## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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# Effects of controlled atmosphere and storage temperature on the quality of shelled 'Barton' pecan nuts during long-term storage

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ABSTRACT

This study aimed to evaluate the effects of controlled atmosphere (CA) storage at a low oxygen partial pressure ($pO_2$; 2 kPa of $O_2$) and low $pO_2$ associated with high $pCO_2$ (2 kPa $O_2$ + 15 kPa $CO_2$) in relation to ambient atmospheric conditions (control), at different temperatures, on shelled 'Barton' pecan nuts quality after storage. Color, respiration rates, moisture content (MC), and oxidation markers, such as peroxide value (PV), acidity value (AV), and volatile compounds (VC), were evaluated. During six months of storage, the MC decreased in all CA treatments, and treatments at 10 °C had the lowest AVs and PVs. However, the treatment with high $pCO_2$ levels also guaranteed lower AVs at 20 °C. The color parameter b* (yellow), which is related to the golden appearance of pecans and is a highly desirable visual attribute in the commercialization of nuts, was maintained high in treatments at 10 °C with CA treatments until six months of storage. The VCs, characteristic of lipid oxidation (aldehydes, acids, alcohols, and ketones), increase in all treatments with prolonging storage. When $pCO_2$ was associated to 10 °C at six month of storage, it showed a larger area in the acids and unsaturated aldehyde classes, while it was relevant for alcohols, lactones, and esters at 20 °C, with sweet characteristics, including ethyl ethanoate, ethyl hexanoate, and butyrolactone. Thus, this study shows another advance in the storage techniques of shelled pecans, pointing to alternatives for reducing energy costs in the cooling chain.

## 1. Introduction

The pecan (*Carya illinoinensis* (Wangenh.) K. Koch) is among the most consumed nuts worldwide and recognized for its sensory and nutritional attributes as well as commercial value (INC, Internacional, 2018). In 2019, 132 thousand tons of pecans were produced in the USA alone (the world's largest pecan producer). In that same year, Brazilian pecan production reached 3.5 thousand tons, raising the potential to produce this nut. According to the Brazilian Association of Nuts and Dried Fruits (ABNC), Brazil is the fourth-largest producer globally, with Rio Grande do Sul State leading by roughly 50% of Brazilian production (Lesina, 2020).

The pecan is an important source of lipids (50–70%) and is composed mainly of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (Ribeiro, Klein et al., 2020), which can contribute to reducing cholesterol levels and the risk of heart disease (Mozaffarian, 2016). Pecans' main fatty acids (FA) are oleic and linoleic acids, contributing around 60 and 20%, respectively, of total lipid fraction (Ribeiro, Klein et al., 2020). Nonetheless, this high lipid value with unsaturated fatty acids (UFA) directly influences pecan quality attributes during storage (Ribeiro, Ribeiro et al., 2020).

In this sense, avoiding lipid oxidation is the biggest challenge for nut producers and traders (Buthelezi, Magwaza, & Tesfay, 2019). The most common reactions involve hydrolytic rancidity, with increasing acidity values (AV), and oxidation (autoxidation), which generates several typical volatile compounds (rancid odors) and color changes (browning) via lipid peroxidation (Pannico et al., 2015; Ribeiro, Ribeiro et al., 2020; Thewes, Both, Thewes, Brackmann, Schultz et al., 2021). Several techniques to maintain nut quality have been evaluated, such as roasting (Buthelezi, Magwaza, & Tesfay, 2019), irradiation (Sajilata, & Singhal, 2006), modified atmosphere (Pannico et al., 2015), controlled atmosphere (CA) (Ribeiro, Ribeiro et al., 2020), vacuum packaging (Ribeiro et al., 2021; Maskan & Karataş, 1999), packaging with $N_2$, and use of low temperatures (Tsantili, Konstantinidis, Christopoulos, & Roussos, 2011;

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Ribeiro, Ribeiro et al., 2020). However, the CA technique associated with high $CO_2$ levels is still little explored in storing pecans (mostly without shell), and other nuts.

Pistachios stored for six months with 98 kPa $CO_2$ presented lower peroxide formation than those stored with air (20 kPa $O_2$), regardless of the temperatures tested (10, 20, and 30 °C) (Maskan, & Karataş, 1999). In another study, pistachios were stored in vacuum packaging and modified atmosphere (70 kPa $CO_2$) for 30 days and showed lower peroxide values (PV) and free FAs compared to the control packaging (20 kPa $O_2$) (Ozturk et al., 2016). In addition, the flavor and color of peanuts and pecans stored in plastic containers at a high $CO_2$ atmosphere were better preserved than refrigerated treatments and air systems (20 kPa $O_2$) (Holaday, Pearson, & Slay, 1979).

The nuts' shell is an efficient physical barrier against spoilage (Thompson, & Kader, 2012), although nuts become more commercially attractive when shelled. After shelling and the lack of demand, kernels may be exposed to ambient conditions in warehouses and markets for long periods, consequently affecting their color and texture and leading to rancidity. According to Thewes, Both, Thewes, Brackmann, Wagner et al. (2021), more significant amount of unwanted aldehyde and alcohols were detected in shelled pecan kernels after six months. However, these authors just evaluate 1-methylcyclopropene application, storing the pecans at ambient conditions (17 ± 5 °C). Therefore, there is a need to evaluate the time and storage conditions that maintain the quality attributes of shelled pecans. Hence, CA conditions with reduced $pO_2$ and increased $pCO_2$ can efficiently improve the maintenance of food quality for long-term storage, such as soybeans (Makino et al., 2020), Brazil nuts (Scussel et al., 2011), and pistachios (Maskan, & Karataş, 1999). Given the above, this study aimed to evaluate the effects of low $pO_2$ isolated or in combination with high $pCO_2$ under different temperatures on shelled 'Barton' pecan nut quality maintenance during storage.

## 2. Material and methods

### 2.1. Sample

Pecans were harvested in 2017 in a commercial orchard in Cachoeira do Sul (30°02′21″S, 52°53′38″W), Rio Grande do Sul State, southern Brazil. The nuts were then collected and transported to the Post-Harvest Research Center at the Federal University of Santa Maria. For the drying process, the fruits were placed on thin layers of perforated plastic packaging under ambient conditions (10–20 °C and 60% relative humidity) for twenty-five days. The pecans were then selected for experimental sample formation by considering the absence of insect attack, fungal growth, or poorly filled kernels. The pecans were shelled using a manual nutcracker with a lever system to obtain the whole kernels.

### 2.2. Storage conditions

The pecans were stored according to the method of Ribeiro, Ribeiro et al. (2020), with modifications. About 4.2 kg of shelled pecans were placed in airtight 20-L containers adapted to allow air circulation through an external gas analyzer. The different $O_2$ and/or $CO_2$ conditions were handled for each container. The containers of each atmospheric condition and the packages were placed inside cold rooms at different temperatures (20 and 10 °C). A total of six storage conditions were installed (Table 1).

During the entire period of the experiment, temperature control in the cold rooms was carried out by electronic thermostats and checked daily with a mercury thermometer located inside the chambers. The $pO_2$ inside containers was reduced by flushing with $N_2$, which was produced by a generator using pressure swing adsorption principle. The condition with high $pCO_2$ levels was obtained in the experimental container by flushing them with $CO_2$ from high-pressure cylinders (99.9%; White Martins, Brazil). A set of hoses connected the experimental containers to a gas analyzer (Siemens, Ultramat 23, Germany) to monitor and correct the gas composition. Determinations were made twice a week. The storage was carried out for six months, and the analyses were performed at the initial time (after drying, time zero), two, four, and six months. For each sampling point, three replicates of approximately 460 g per treatment were take for analyses, totaling 1400 g of nuts for each treatment in each evaluation point.

**Table 1**
The experimental design applied to the variable's temperature and partial pressure of $O_2$ and high $CO_2$ in shelled 'Barton' pecan nut storage.

| Treatment | Atmosphere | Temperature (°C) |
|---|---|---|
| AMB-20 | 20 kPa of $O_2$ * | 20 |
| AMB-10 | 20 kPa of $O_2$ | 10 |
| $pO_2$-20 | 2 kPa of $O_2$ | 20 |
| $pO_2$-10 | 2 kPa of $O_2$ | 10 |
| $pCO_2$-20 | 2 kPa of $O_2$ + 15 kPa of $CO_2$ | 20 |
| $pCO_2$-10 | 2 kPa of $O_2$ + 15 kPa of $CO_2$ | 10 |

*20 kPa of $O_2$ is considered the ambient atmosphere condition; AMB – ambient atmosphere condition, $pO_2$ – 2 kPa of $O_2$, $pCO_2$ – 2 kPa of O2 + 15 kPa of $CO_2$.

### 2.3. Quality analyses

#### 2.3.1. Color analysis

Color evaluation of the pecan nuts was carried out using a colorimeter (Konica Minolta CR-310, Osaka, Japan), obtaining values of the coordinates L* (measurable in terms of white to black intensity), a* (measurable in terms of intensity from red to green), b* (measurable in terms of yellow and blue intensity), C (chroma, saturation), and hue angle. Calibration was performed with a standard white tile. Two half-shelled pecans were placed side by side in a black-bottomed container where the color reading was performed. Fifteen determinations were performed per replicate, with three replicates per treatment.

#### 2.3.2. Moisture content and water activity

The oven method (AOAC 925.10, 2012) was used with forced air circulation at 105 ± 1 °C until reaching constant weight to determine pecan nut MC. Water activity (aw) was determined directly (2.0 ± 0.1 g each, ground sample) in a water activity meter (Aqualab Series 4 TEV, USA). The readings were performed in triplicate at 25 ± 1 °C.

#### 2.3.3. Respiration rate analyses

Approximately 200 g of shelled nuts were placed in hermetically sealed 625-mL glass containers, at 20 °C. Due to the low respiratory rate of the product (Thewes, Both, Thewes, Brackmann, Schultz et al., 2021), the containers were closed for seven days to accumulate $CO_2$ from respiration. Then, the gas from each vessel was circulated by a gas analyzer (Isolcell Oxycarb 6, Laives, Italy) using small hoses with needles at the tips, which were inserted into rubber seals at the container lids. Three flasks were evaluated for each treatment. From the pecan mass, the air volume inside each flask, the closure time (hours), and $CO_2$ percentage, determined with the analyzer, the respiration rate was calculated and the results were expressed in mL $CO_2$ $kg^{-1}h^{-1}$.

#### 2.3.4. Sample preparation for chemical analyses

Before each analysis, the shelled pecans were ground in a knife mill (Marconi 630/1, São Paulo, Brazil). The oil was obtained according to Hara and Radin (1978), with modifications according to Ribeiro, Ribeiro et al. (2020).

#### 2.3.5. Acidity values

The acidity values were determined using the "Cd 3d-63" method of AOCS (2009) with modifications according to Ribeiro, Ribeiro et al. (2020) using oil (1.0 g). The results were expressed in g of oleic acid 100 $g^{-1}$ oil.

### 2.3.6. Peroxide values

Peroxide values were determined using the International Dairy Federation (IDF) method 74A:1991 (Shanta & Decker, 1994). The results were expressed in meq of $O_2$ $kg^{-1}$ oil.

### 2.3.7. Volatile compounds analyses

Volatile compounds extraction was performed by headspace solid-phase microextraction (HS-SPME) of vials containing samples; analyses were carried out in gas chromatography coupled to a mass spectrometer (GC/MS Shimadzu QP2010 Plus; Shimadzu Corporation, Tokyo, JP) according to Ribeiro, Ribeiro et al. (2020).

### 2.3.8. Statistical analyses

Statistical analyses were performed in the Sisvar Software (Version

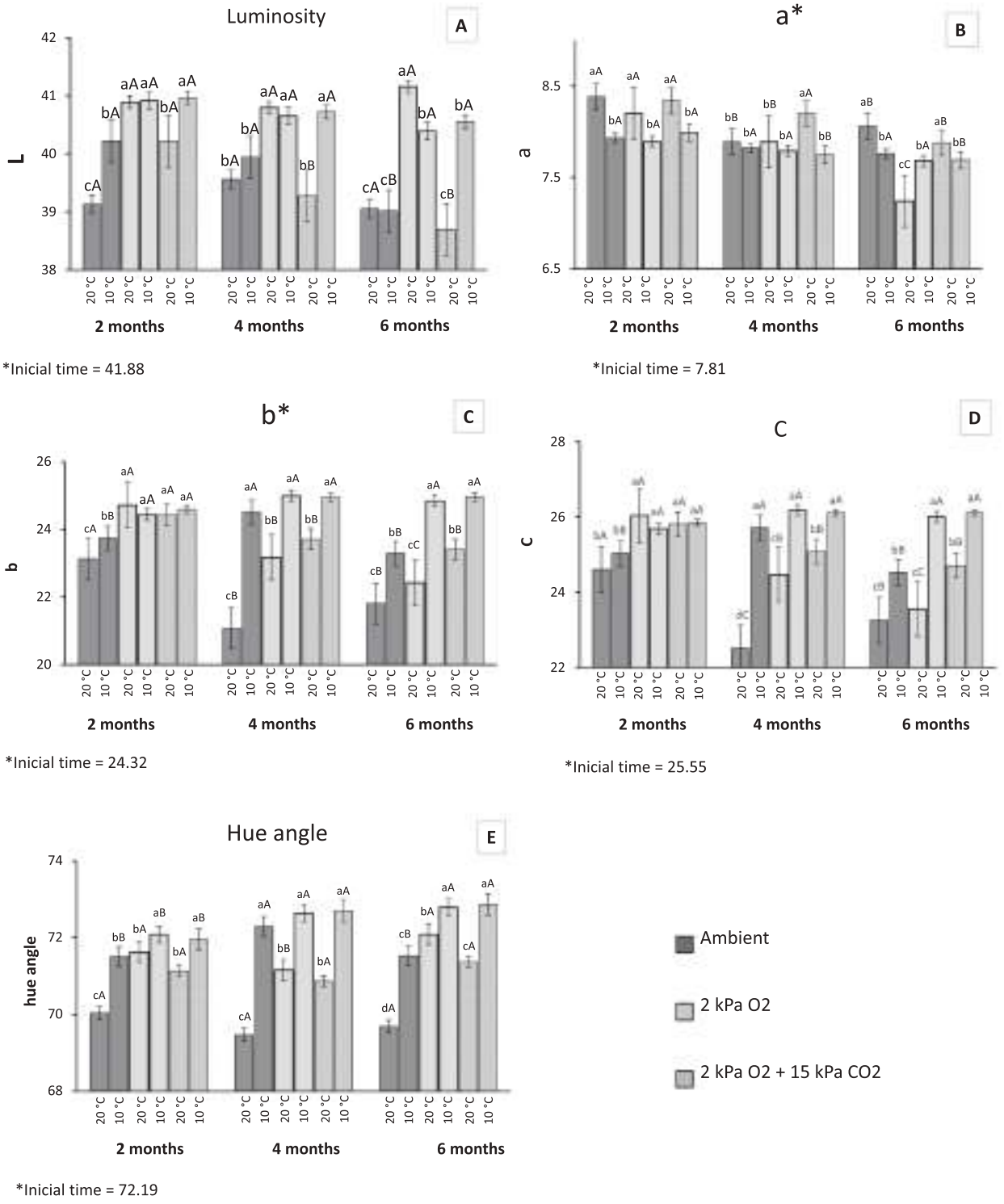



**Fig. 1. A, B, C, D and E.** Color parameters of the shelled Barton pecan nut during six months of storage with different $pO_2$, $pCO_2$, and temperature. The mean values followed by the same lowercase letter within each assessed period, and upper-case letter at columns of the same storage condition, did not differ according to the Scott Knott test ($p < 0.05$). Initial sample values: **(A)** L = 41.88; **(B)** parameter a = 7.81, **(C)** parameter b = 24.32, **(D)** parameter c = 25. 5 and **(E)** parameter hue = 72.19.

5.6, Build 86); data were submitted to analysis of variance (ANOVA). The means adjusted by the least squares method were compared by the Scott Knott test with a significance level of 5%. In addition, multivariate correlation analysis and principal component analysis (PCA) were performed to evaluate groupings between samples and variables using the statistical program Pirouette 3.11 (Infometrix, Woodinville, USA). The data matrix was auto-scaled for each variable for pre-processing (Ribeiro et al., 2000).

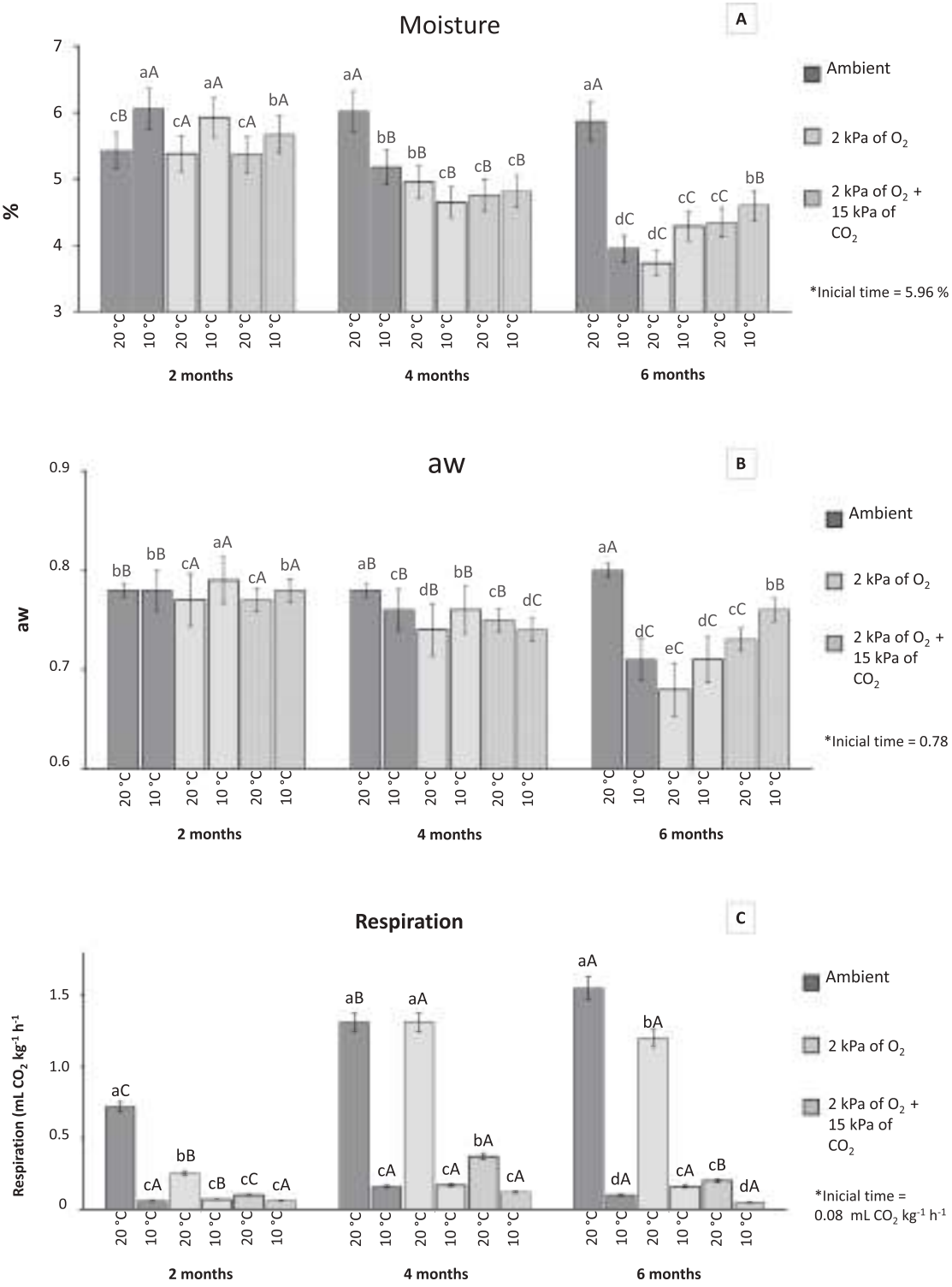



**Fig. 2. A, B and C – (A)** Moisture of the shelled Barton pecan nut during six months of storage with different $pO_2$, $pCO_2$, and temperature. Initial moisture value was 5.96%. **(B)** Water activity of the shelled Barton pecan nut after six months of storage with different $pO_2$, $pCO_2$, and temperature. Initial aw was 0.78. **(C)** Respiration rate of the shelled Barton pecan nut after six months of storage with different $pO_2$, $pCO_2$, and temperature. The mean values followed by the same lowercase letter within each assessed period, and upper-case letter at columns of the same storage condition, did not differ according to the Scott Knott test ($p < 0.05$). Initial respiration value was 0.08 mL $CO_2$ $kg^{-1}h^{-1}$.

## 3. Results

### 3.1. Color

The luminosity (L*) of pecan kernels at harvest (initial time) was 41.88 (Fig. 1A), and at the second month of storage, the lowest L* value was in AMB-20, indicating a darker color. After the fourth month, the AMB-10 and $pCO_2$-20 treatments did not differ ($p > 0.05$) from AMB-20. The L* parameter was better preserved in the treatments with low $pO_2$ (regardless of temperature) and $pCO_2$-10. Nonetheless, the color parameter $a^*$ (red) (Fig. 1B) in the second month showed higher values in samples submitted to 20 °C, irrespective of storage conditions. In the fourth month of storage, the nuts in $pCO_2$-20 showed a more intense red color. In the sixth month, the a* parameter remained higher in AMB-20 and $pCO_2$-20.

A significant reduction in b* value (yellow color) was found from the second to the fourth month of storage under all conditions at 20 °C, although this behavior was not observed when pecans were stored at 10 °C. Thereafter, in the fourth and sixth months, b* values remained higher in treatments at 10 °C. In addition, the $pCO_2$-20 treatment did not differ ($p > 0.05$) from AMB-10 in the sixth month. From the fourth to the sixth month, there was a further reduction in b* value in $pO_2$-20, albeit this color parameter did not change during storage in $pO_2$-10 and $pCO_2$-10 (Fig. 1C). Thus, the parameter b* tends to reduce in samples stored at AMB temperature, while treatments at 10 °C and CA conditions maintained this parameter at constant values. The color parameter C (saturation) (Fig. 1D) and hue angle (Fig. 1E) showed the same trend as b*, which were higher in samples submitted to 10 °C; $pCO_2$-20 did not differ ($p > 0.05$) from AMB-10 at six months. The highest hue angle was observed on pecans stored at 10 °C, associated to CA, with little changes from the harvest up to six months (Fig. 1E). The hue angle is related to red and yellow color, and lower values are linked to kernels with more red and lower yellow color, and were found in pecan stored with shell at higher $pO_2$ (ambient) (Thewes, Both, Thewes, Brackmann, Schultz et al., 2021), corroborating to our findings.

### 3.2. Moisture content and water activity

Before storage, all nuts were dried until reaching 5.96% of MC. In the second month, the MC ranged from 5.38 to 6.06%, being higher in AMB-10 and $pO_2$-10 treatments (Fig. 2A). In the fourth month, treatments under CA conditions at 20 °C and AMB-10 showed more significant moisture losses, with values reaching 4.65%. The AMB-20 presented a moisture content near 6.0% at the sixth month, and all other treatments had significantly lower ($p < 0.05$) MC except for $pCO_2$-10. Lower MC was observed in AMB-10 and $pO_2$-20 (<4.0%). The aw values ranged from 0.68 to 0.80 and decreased with storage time except for AMB-20 (Fig. 2B). The higher aw values in AMB-20 remained constant and increased at the sixth month, making it easy to observe visible fungal contamination (mold). Lower aw values were observed under storage conditions with low oxygen even at higher temperatures ($pO_2$-20 treatment), evidencing the effects of low $O_2$.

### 3.3. Respiration rates

The nut respiration rate before storage was 0.08 mL $CO_2$ $kg^{-1}h^{-1}$. After the second month, the respiration rate increased in the AMB and with low $pO_2$ treatments at 20 °C (0.73 and 0.25 mL $CO_2$ $kg^{-1}h^{-1}$, respectively) (Fig. 2C). This trend continued in the periods analyzed later, in which the respiration rates of AMB-20 and $pO_2$-20 significantly increased ($p < 0.05$) in the fourth and sixth months and were significantly higher than the other storage conditions. Pecan kernels stored in $pCO_2$-20 and stored at 10 °C, guaranteed lower respiration rates regardless of the atmospheric condition.

### 3.4. Acidity values

The AV of the pecans at harvest was 0.29 g of oleic acid 100 $g^{-1}$ oil. During storage, acidity increased in the treatments submitted to 20 °C, especially in the AMB and under $pO_2$ conditions (Fig. 3A). Nevertheless, at this same temperature, in the treatment with 15 kPa $pCO_2$, the AV values showed the lowest increment compared to AMB and $pO_2$ treatments. The AV remained lower in treatments at 10 °C, regardless of gas composition. In addition, in the fourth month, $pCO_2$-20 showed no difference ($p > 0.05$) compared to CA treatments at 10 °C. In the sixth month, CA treatments (at 10 °C) guaranteed lower AV than AMB-10.

### 3.5. Peroxide values

Before storage, the PV was 1.12 meq of $O_2$ $kg^{-1}$ oil. In the second month, the values increased up to 2 meq of $O_2$ $kg^{-1}$ oil, and the AMB-10 treatment showed the highest PVs among the treatments (Fig. 3B). In the fourth month, the PVs increased significantly ($p < 0.05$) in all treatments, showing values of up to 4 meq of $O_2$ $kg^{-1}$ oil, although AMB-20 had the highest value. The use of CA in treatments at 20 °C guaranteed lower PV than AMB-20. In addition, the PVs under CA at 20 °C did not differ ($p < 0.05$) from the treatments subjected to 10 °C. In the sixth month, PVs increased significantly under CA conditions at 20 °C compared to the fourth month. In this evaluation point, all treatments at 20 °C showed higher PVs, and less peroxide formation occurred in treatments at 10 °C, which showed no difference ($p > 0.05$) among them.

### 3.6. Volatile compounds

The results for the volatile compounds are listed in Table 2; 95 chromatographic peaks were detected and classified into distinct chemical classes: alcohols (25), esters (15), acids (15), aldehydes (14), terpenes (8), ketones (8), lactones (7), aromatic hydrocarbons (2), and furan (1).

*Acids*. Acetic acid showed the highest amount in $pCO_2$-20 until the fourth month. In the sixth month, it significantly increased in the $pCO_2$-10 treatment; C4 - C10 aliphatic chain acids were detected in all samples, mainly in treatments at 10 °C, sharply increasing in almost all treatments during storage. Undecanoic acid and benzoic acid were not detected in nuts at 20 °C. Moreover, we noticed that acid peak areas increased during storage and detected branched acids (e.g., 4-methyl 3-pentenoic acid and 2-ethyl hexanoic acid), and the aliphatic acids tetradecanoic acid and undecanoic acid were detected only in the sixth month. Therefore, overall, there was an increase in the peak area of acids during storage.

*Alcohols*. C2-C6 aliphatic alcohols showed the largest peak areas in $pCO_2$-20, followed by AMB-10. Ethanol showed the highest amount in AMB-10, followed by CA treatments at 10 °C and $pCO_2$-20 until the fourth month. Branched alcohols (2-methyl 1-butanol and 3-methyl 1-butanol) also had the largest peak areas until the fourth month, thereafter decreasing or disappearing in the sixth month. In addition, ethanol, propanol, and 2-propanol showed larger areas in treatments at 10 °C and in $pCO_2$-20. Nevertheless, 2-methyl propanol, 2-pentanol, and butanol showed larger areas in $pCO_2$-20. In general, C5 to C9 alcohols increased after six months of storage. 1-Octen-3-ol was more abundant in AMB-20 treatment and increased during storage; it showed lower values in the area in treatments at 10 °C. Dodecanol and tridecanol were detected with the highest amounts in treatments at 20 °C. The aromatic alcohols benzenemethanol and benzeneethanol presented higher concentrations in treatments at 10 °C, followed by $pCO_2$-20.

*Aldehydes*. In the second month of storage, ethanal showed the largest amount in treatments at 10 °C, decreasing in the following periods. The branched aldehydes, 2-methyl butanal, and 3-methyl butanal, as well as the aliphatic aldehyde hexanal, were more abundant in treatments at 10 °C, especially in AMB-10. In addition, hexanal presented a tendency of increasing in the storage periods, except to AMB-

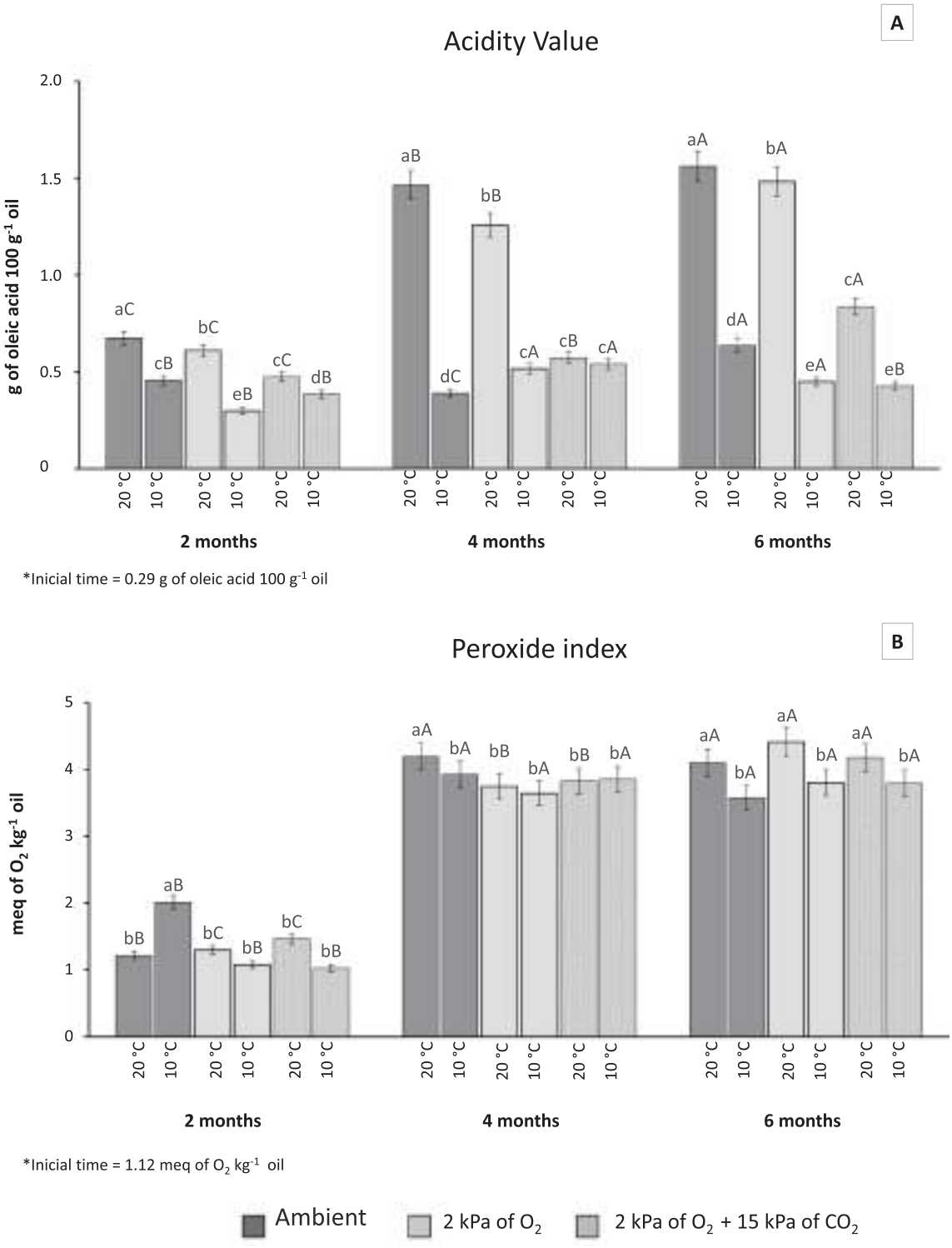



**Fig. 3. A and B. (A)** Acidity value of the shelled Barton pecan nut during six months of storage with different $pO_2$, $pCO_2$, and temperature. Initial value of the AI of sample (harvest) 0.29 g oleic acid 100 $g^{-1}$ oil. **(B)** Peroxide value of the of the shelled Barton pecan nut during six months of storage with different $pO_2$, $pCO_2$, and temperature. The mean values followed by the same lowercase letter within each assessed period, and upper-case letter at columns of the same storage condition, did not differ according to the Scott Knott test ($p < 0.05$). Initial value of sample (harvest) 1.12 meq $O_2$ $kg^{-1}$ oil.

20, that it decreased until 6th months. Octanal and nonanal were detected with smaller peak areas at $pCO_2$-10. However, in the sixth month, octanal increased significantly in $pCO_2$-20, $pCO_2$-10, and $pO_2$-10. Unsaturated aldehydes such as (E)-2-octenal, (E)-2-nonenal, and 2-decenal showed the highest amounts in the sixth month of storage. Benzaldehyde was detected only after the fourth month for all treatments. Furthermore, (E)-2-octenal, (E)-2-nonenal, 2-decenal, and benzaldehyde showed larger areas in the $pCO_2$-10 treatment.

*Ketones*. The ketone class showed a higher amount in treatments submitted to 20 °C than at 10 °C. Except for 6-methyl 5-hepten-2-one and 2-propanone, most compounds in this class showed greater abundance in the first months of storage, decreasing throughout the six

**Table 2**
Volatile compounds (peak area value × $10^6$) of 'Barton' pecan at harvest and after storage without shell, in different controlled atmosphere conditions and temperatures, during 2, 4, and 6 months.

| Compound | Month | AMB-20 | $pO_2$-20 | $pCO_2$-20 | AMB-10 | $pO_2$-10 | $pCO_2$-10 |
|---|---|---|---|---|---|---|---|
| Acetic acid | 2 | $25.16 \pm 3.78^{cA**}$ | $40.3 \pm 4.59^{bA}$ | $67.78 \pm 4.82^{aA}$ | $37.35 \pm 3.74^{bA}$ | $33.54 \pm 4.69^{bA}$ | $35.12 \pm 6.33^{bB}$ |
| (4.8 ± 1.44*) | 4 | $1.89 \pm 1.54^{dB}$ | $4.84 \pm 0.93^{dB}$ | $74.63 \pm 7.98^{aA}$ | $40.18 \pm 5.29^{bA}$ | $23.77 \pm 18.17^{cB}$ | $32.35 \pm 7.43^{bB}$ |
| | 6 | $5.08 \pm 1.66^{dB}$ | $6.79 \pm 1.28^{dB}$ | $17.59 \pm 3.15^{cB}$ | $41.22 \pm 3.31^{bA}$ | $42.91 \pm 3.44^{bA}$ | $62.08 \pm 4.9^{aA}$ |
| 4-Methyl 3-pentenoic acid | 2 | $3.1 \pm 0.42^{aA}$ | $3.27 \pm 0.19^{aA}$ | $3.69 \pm 0.35^{aA}$ | $2.03 \pm 0.15^{bA}$ | $2.42 \pm 0.41^{bA}$ | $2.99 \pm 0.1^{aA}$ |
| (0.87 ± 0.26) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | $0.21 \pm 0.01^{dB}$ | $1.33 \pm 0.05^{cB}$ | $4.41 \pm 0.16^{aA}$ | $2.49 \pm 0.23^{bA}$ | $2.59 \pm 0.05^{bA}$ | $2.5 \pm 0.28^{bA}$ |
| Butanoic acid | 2 | nd | nd | $0.13 \pm 0.05^{aB}$ | nd | nd | $0.14 \pm 0.04^{aB}$ |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | $0.16 \pm 0.01^{cA}$ | $0.24 \pm 0.09^{cA}$ | $0.7 \pm 0.12^{bA}$ | $0.82 \pm 0.1^{aA}$ | $0.85 \pm 0.05^{aA}$ | $0.66 \pm 0.1^{bA}$ |
| Pentanoic acid | 2 | $0.14 \pm 0.02^{aA}$ | $0.15 \pm 0.02^{aA}$ | $0.27 \pm 0.17^{aA}$ | $0.16 \pm 0.05^{aB}$ | $0.11 \pm 0.01^{aB}$ | $0.25 \pm 0.05^{aB}$ |
| (nd) | 4 | $0.11 \pm 0.03^{aA}$ | $0.28 \pm 0.13^{aA}$ | $0.14 \pm 0.02^{aA}$ | $0.26 \pm 0.07^{aB}$ | $0.45 \pm 0.22^{aA}$ | $0.26 \pm 0.08^{aB}$ |
| | 6 | $0.18 \pm 0.02^{cA}$ | $0.30 \pm 0.16^{bA}$ | $0.37 \pm 0.09^{bA}$ | $0.52 \pm 0.07^{aA}$ | $0.39 \pm 0.03^{bA}$ | $0.74 \pm 0.8^{aA}$ |
| Hexanoic acid | 2 | $0.86 \pm 0.21^{aA}$ | $0.69 \pm 0.12^{aA}$ | $1.37 \pm 0.58^{aA}$ | $0.79 \pm 0.04^{aB}$ | $0.65 \pm 0.36^{aA}$ | $2.27 \pm 0.3^{aB}$ |
| (nd) | 4 | $1.1 \pm 0.35^{aA}$ | $1.9 \pm 0.5^{aA}$ | $0.93 \pm 0.02^{aA}$ | $1.96 \pm 0.7^{aB}$ | $2.73 \pm 0.73^{aA}$ | $2.53 \pm 0.49^{aB}$ |
| | 6 | $1.24 \pm 0.36^{bA}$ | $2.12 \pm 0.28^{bA}$ | $3.13 \pm 0.8^{bA}$ | $4.61 \pm 0.8^{aA}$ | $2.9 \pm 0.31^{bA}$ | $6.7 \pm 7.84^{aA}$ |
| 2-Ethyl hexanoic acid | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.07 \pm 0.01^{cB}$ | $0.11 \pm 0.03^{cB}$ | $0.09 \pm 0.03^{cA}$ | $0.14 \pm 0.06^{bA}$ | $0.15 \pm 0.05^{bA}$ | $0.2 \pm 0.05^{aA}$ |
| | 6 | $0.15 \pm 0.01^{bA}$ | $0.22 \pm 0.04^{aA}$ | $0.10 \pm 0.05^{bA}$ | $0.16 \pm 0.06^{bA}$ | $0.15 \pm 0.04^{bA}$ | nd |
| Heptanoic acid | 2 | nd | nd | $0.91 \pm 0.78^{aA}$ | nd | nd | $0.84 \pm 0.43^{aA}$ |
| (nd) | 4 | $0.71 \pm 0.09^{aA}$ | $0.94 \pm 0.3^{aA}$ | nd | $0.66 \pm 0.23^{aA}$ | $0.65 \pm 0.15^{aA}$ | $0.67 \pm 0.12^{aA}$ |
| | 6 | $0.56 \pm 0.17^{bA}$ | $0.63 \pm 0.29^{bA}$ | $1.29 \pm 0.31^{aA}$ | $1.18 \pm 0.22^{aA}$ | $1.11 \pm 0.14^{aA}$ | $0.91 \pm 0.78^{aA}$ |
| Octanoic acid | 2 | nd | nd | $2.19 \pm 0.73^{aB}$ | nd | nd | $1.74 \pm 0.54^{aC}$ |
| (nd) | 4 | $1.92 \pm 0.15^{bA}$ | $1.97 \pm 0.51^{bA}$ | $1.53 \pm 0.11^{bB}$ | $1.39 \pm 0.68^{bB}$ | $4.19 \pm 1.89^{aA}$ | $5.21 \pm 1.16^{aA}$ |
| | 6 | $0.87 \pm 0.41^{bB}$ | $1.62 \pm 0.08^{bA}$ | $3.58 \pm 0.23^{aA}$ | $2.91 \pm 0.51^{aA}$ | $2.56 \pm 0.21^{aB}$ | $3.58 \pm 1.73^{aB}$ |
| Nonanoic acid | 2 | $0.83 \pm 0.36^{aA}$ | $0.87 \pm 0.28^{aA}$ | $1.67 \pm 1.25^{aA}$ | $0.5 \pm 0.04^{aB}$ | $0.46 \pm 0.16^{aB}$ | $2.26 \pm 1.18^{aB}$ |
| (nd) | 4 | $0.67 \pm 0.06^{aA}$ | $1.45 \pm 0.65^{aA}$ | $0.76 \pm 0.16^{aB}$ | $0.95 \pm 0.13^{aB}$ | $1.09 \pm 0.2^{aB}$ | $1.78 \pm 0.5^{aB}$ |
| | 6 | $0.96 \pm 0.4^{bA}$ | $1.5 \pm 0.15^{bA}$ | $1.72 \pm 0.34^{bA}$ | $2.52 \pm 1.28^{aA}$ | $1.99 \pm 0.64^{bA}$ | $3.05 \pm 2.77^{aA}$ |
| Decanoic acid | 2 | $0.35 \pm 0.10^{aA}$ | $0.58 \pm 0.51^{aA}$ | $0.99 \pm 0.92^{aA}$ | $0.29 \pm 0.02^{aA}$ | $0.6 \pm 0.26^{aB}$ | $0.88 \pm 0.15^{aC}$ |
| (nd) | 4 | $0.52 \pm 0.26^{bA}$ | $1.11 \pm 0.71^{bA}$ | $0.72 \pm 0.42^{bA}$ | $0.21 \pm 0.05^{bA}$ | $3.04 \pm 1.42^{aA}$ | $4.35 \pm 1.77^{aA}$ |
| | 6 | nd | nd | $1.04 \pm 0.41^{bA}$ | $1.65 \pm 0.59^{aA}$ | $2.05 \pm 1.06^{aA}$ | $2.62 \pm 2.13^{aB}$ |
| Undecanoic acid | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | nd | nd | $9.35 \pm 5.59^{aA}$ | $13.46 \pm 11.23^{aA}$ | $12.63 \pm 7.87^{aA}$ |
| Benzoic acid | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | $0.28 \pm 0.09^{bA}$ | $0.65 \pm 0.28^{aA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| Dodecanoic acid | 2 | $0.3 \pm 0.09^{aB}$ | $0.2 \pm 0.05^{aB}$ | $0.49 \pm 0.17^{aA}$ | $0.34 \pm 0.03^{aA}$ | $0.32 \pm 0.27^{aB}$ | $0.37 \pm 0.07^{aB}$ |
| (2.13 ± 0.12) | 4 | $0.85 \pm 0.16^{aA}$ | $0.83 \pm 0.28^{aA}$ | $0.8 \pm 0.14^{aA}$ | $0.52 \pm 0.13^{aA}$ | $0.85 \pm 0.32^{aA}$ | $0.83 \pm 0.23^{aA}$ |
| | 6 | nd | nd | $0.68 \pm 0.14^{aA}$ | $0.54 \pm 0.07^{aA}$ | $0.64 \pm 0.10^{aA}$ | $0.58 \pm 0.25^{aB}$ |
| Tetradecanoic acid | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | $1.15 \pm 0.34^{bA}$ | $0.78 \pm 0.13^{bA}$ | $0.88 \pm 0.12^{bA}$ | $1.4 \pm 0.33^{bA}$ | $1.11 \pm 0.14^{bA}$ | $2.43 \pm 2.32^{aA}$ |
| 2-Propanol | 2 | $3.1 \pm 0.02^{dB}$ | $2.71 \pm 0.06^{dC}$ | $7.57 \pm 0.42^{cB}$ | $15.53 \pm 0.66^{aA}$ | $2.66 \pm 0.5^{dC}$ | $11.28 \pm 0.21^{bA}$ |
| (1.82 ± 0.42) | 4 | $5.27 \pm 0.81^{bA}$ | $5.27 \pm 1.29^{bB}$ | $5.85 \pm 0.94^{bC}$ | $6.08 \pm 0.16^{bC}$ | $6.92 \pm 0.47^{aB}$ | $5.46 \pm 0.19^{bC}$ |
| | 6 | $1.61 \pm 0.08^{cC}$ | $16.45 \pm 0.10^{aA}$ | $8.71 \pm 0.30^{bA}$ | $9.6 \pm 0.46^{bB}$ | $9.11 \pm 0.54^{bA}$ | $8.54 \pm 0.66^{bB}$ |
| Ethanol | 2 | $20.71 \pm 0.15^{cA}$ | $33.14 \pm 0.06^{bA}$ | $36.74 \pm 2.15^{bB}$ | $54.04 \pm 0.39^{aA}$ | $47.99 \pm 22.7^{aA}$ | $47.42 \pm 0.46^{aA}$ |
| (10.93 ± 2.22) | 4 | $10.5 \pm 2.09^{bB}$ | $15.13 \pm 3.04^{bB}$ | $46.76 \pm 12.69^{aA}$ | $54.63 \pm 3.72^{aA}$ | $58.42 \pm 5.70^{aA}$ | $52.28 \pm 0.98^{aA}$ |
| | 6 | $7.32 \pm 0.95^{bB}$ | $26.3 \pm 2.35^{aA}$ | $29.05 \pm 1.10^{aB}$ | $26.69 \pm 0.33^{aB}$ | $32.42 \pm 1.07^{aB}$ | $25.16 \pm 0.93^{aB}$ |
| Propanol | 2 | nd | $0.31 \pm 0.02^{aA}$ | $0.42 \pm 0.03^{aB}$ | $0.32 \pm 0.02^{aB}$ | nd | $0.28 \pm 0.04^{aB}$ |
| (nd) | 4 | nd | $0.4 \pm 0.03^{cA}$ | $1.07 \pm 0.11^{aA}$ | $0.89 \pm 0.01^{bA}$ | $1.23 \pm 0.41^{aA}$ | $0.85 \pm 0.01^{bA}$ |
| | 6 | nd | nd | $0.37 \pm 0.06^{aB}$ | $0.42 \pm 0.11^{aB}$ | $0.18 \pm 0.06^{bB}$ | nd |
| 2-Methyl 1-propanol | 2 | $1.3 \pm 0.07^{aA}$ | $1.06 \pm 0.01^{bA}$ | $1.04 \pm 0.02^{bB}$ | $1.26 \pm 0.10^{aA}$ | $1.13 \pm 0.18^{bA}$ | $1.14 \pm 0.06^{bA}$ |
| (nd) | 4 | nd | nd | $1.44 \pm 0.29^{aA}$ | $0.87 \pm 0.10^{bB}$ | nd | nd |
| | 6 | $0.25 \pm 0.03^{cB}$ | $0.82 \pm 0.09^{bB}$ | $1.13 \pm 0.04^{aB}$ | $0.73 \pm 0.08^{bB}$ | $0.73 \pm 0.01^{bB}$ | $0.85 \pm 0.28^{bB}$ |
| 2-Pentanol | 2 | $2.92 \pm 0.22^{bA}$ | $3.22 \pm 0.21^{aA}$ | $2.8 \pm 0.12^{bC}$ | $1.3 \pm 0.06^{cA}$ | $1.51 \pm 0.28^{cA}$ | $1.61 \pm 0.08^{cA}$ |
| (nd) | 4 | $0.38 \pm 0.13^{dB}$ | $1.94 \pm 0.22^{bB}$ | $3.66 \pm 0.10^{aA}$ | $1.43 \pm 0.16^{cA}$ | $1.58 \pm 0.38^{cA}$ | $1.42 \pm 0.24^{cA}$ |
| | 6 | nd | $0.95 \pm 0.08^{cC}$ | $3.23 \pm 0.03^{aB}$ | $1.2 \pm 0.03^{cA}$ | $1.55 \pm 0.02^{bA}$ | $1.5 \pm 0.16^{bA}$ |
| 1-Butanol | 2 | $0.51 \pm 0.07^{cA}$ | $0.81 \pm 0.05^{bA}$ | $0.99 \pm 0.02^{aA}$ | $0.42 \pm 0.09^{cB}$ | $0.35 \pm 0.02^{cB}$ | $0.38 \pm 0.08^{cB}$ |
| (nd) | 4 | nd | nd | $1.01 \pm 0.10^{aA}$ | $0.9 \pm 0.13^{aA}$ | $0.72 \pm 0.08^{bA}$ | $0.45 \pm 0.19^{cB}$ |
| | 6 | $0.16 \pm 0.01^{cB}$ | $0.66 \pm 0.07^{bB}$ | $0.7 \pm 0.03^{bB}$ | $0.82 \pm 0.010^{aA}$ | $0.71 \pm 0.01^{bA}$ | $0.58 \pm 0.09^{bA}$ |
| 3-Methyl 2-pentanol | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.4 \pm 0.11^{aA}$ | nd | nd | nd | nd | nd |
| | 6 | $0.27 \pm 0.05^{bB}$ | $1.93 \pm 0.23^{aA}$ | nd | nd | nd | nd |
| 2-Methyl 1-butanol | 2 | $2.79 \pm 0.38^{aA}$ | $2.02 \pm 0.09^{aA}$ | $2.09 \pm 0.11^{aA}$ | $2.47 \pm 0.24^{aA}$ | $2.6 \pm 0.05^{aB}$ | $2.14 \pm 0.16^{aA}$ |
| (0.52 ± 0.04) | 4 | $1.48 \pm 0.2^{bB}$ | $1.75 \pm 0.36^{bA}$ | $2.32 \pm 0.48^{bA}$ | $2.02 \pm 0.40^{bA}$ | $3.81 \pm 2.02^{aA}$ | $1.82 \pm 0.34^{bA}$ |
| | 6 | $0.84 \pm 0.08^{bB}$ | nd | nd | $1.45 \pm 0.13^{aA}$ | nd | nd |
| 3-Methyl 1-butanol | 2 | $4.35 \pm 0.35^{aA}$ | $3.78 \pm 0.21^{aA}$ | $4.05 \pm 0.09^{aA}$ | $2.06 \pm 0.15^{bA}$ | $2.66 \pm 0.17^{bB}$ | $2.25 \pm 0.26^{bA}$ |
| (0.3 ± 0.03) | 4 | $0.82 \pm 0.2^{cB}$ | $2.49 \pm 0.57^{bA}$ | $5.16 \pm 0.61^{bA}$ | $2.21 \pm 0.2^{bA}$ | $10.07 \pm 8.93^{aA}$ | $2.58 \pm 0.35^{bA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| Pentanol | 2 | $3.66 \pm 0.59^{bA}$ | $3.83 \pm 0.33^{bA}$ | $5.04 \pm 0.13^{aC}$ | $2.78 \pm 0.34^{cC}$ | $1.69 \pm 0.23^{cC}$ | $1.67 \pm 1.18^{cC}$ |
| (0.23 ± 0.14) | 4 | $1.28 \pm 0.7^{cB}$ | $3.78 \pm 0.43^{bA}$ | $9.83 \pm 0.55^{aA}$ | $9.65 \pm 2.31^{aB}$ | $4.05 \pm 0.25^{bB}$ | $4.3 \pm 0.23^{bB}$ |
| | 6 | $0.6 \pm 0.09^{cB}$ | nd | $8.37 \pm 0.40^{bB}$ | $14.09 \pm 0.77^{aA}$ | $9.41 \pm 0.08^{bA}$ | $8.91 \pm 1.09^{bA}$ |
| | 2 | $18.09 \pm 3.22^{bA}$ | $18.19 \pm 1.59^{bA}$ | $28.29 \pm 1.56^{aC}$ | $8.43 \pm 1.10^{cC}$ | $8.11 \pm 1.46^{cC}$ | $7.08 \pm 3.74^{cC}$ |

(*continued on next page*)

**Table 2** (*continued*)

| Compound | Month | AMB-20 | $pO_2$-20 | $pCO_2$-20 | AMB-10 | $pO_2$-10 | $pCO_2$-10 |
|---|---|---|---|---|---|---|---|
| Hexanol | 4 | $3.47 \pm 0.56^{cB}$ | $19.06 \pm 0.68^{bA}$ | $33.05 \pm 2.49^{aB}$ | $32.54 \pm 6.47^{aB}$ | $16.95 \pm 1.74^{bB}$ | $16.5 \pm 1.03^{bB}$ |
| (0.48 ± 0.21) | 6 | nd | $6.67 \pm 0.48^{dB}$ | $64.42 \pm 3.01^{aA}$ | $60.12 \pm 4.48^{aA}$ | $48.29 \pm 0.92^{bA}$ | $31.43 \pm 3.27^{cA}$ |
| 3-Octanol | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.67 \pm 0.23^{aA}$ | nd | $0.69 \pm 0.28^{aA}$ | $0.37 \pm 0.01^{bA}$ | $0.7 \pm 0.33^{aA}$ | $0.55 \pm 0.26^{aA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| 1-Octen-3-ol | 2 | $1.52 \pm 0.22^{aC}$ | $0.49 \pm 0.06^{bC}$ | $0.24 \pm 0.06^{bB}$ | nd | nd | nd |
| (nd) | 4 | $3.43 \pm 0.27^{aB}$ | $1.56 \pm 0.09^{bB}$ | $0.41 \pm 0.05^{dB}$ | $0.34 \pm 0.07^{dA}$ | $0.81 \pm 0.8^{cA}$ | nd |
| | 6 | $7.85 \pm 0.81^{aA}$ | $2.12 \pm 0.08^{bA}$ | $1.14 \pm 0.07^{cA}$ | $0.37 \pm 0.09^{dA}$ | $0.4 \pm 0.03^{dB}$ | $0.43 \pm 0.27^{dA}$ |
| Heptanol | 2 | $2.3 \pm 0.61^{aA}$ | $1.97 \pm 0.3^{aB}$ | $3.26 \pm 0.52^{aB}$ | $0.86 \pm 0.11^{bC}$ | $0.6 \pm 0.16^{bC}$ | $0.61 \pm 0.41^{bC}$ |
| (0.09 ± 0.08) | 4 | $2.66 \pm 0.59^{cA}$ | $4.93 \pm 0.23^{bA}$ | $2.83 \pm 0.64^{cB}$ | $6.82 \pm 3.19^{aB}$ | $2.26 \pm 0.55^{cB}$ | $2.31 \pm 0.18^{cB}$ |
| | 6 | nd | $2.36 \pm 0.52^{eB}$ | $15.48 \pm 0.84^{aA}$ | $10.49 \pm 1.27^{bA}$ | $8.03 \pm 0.38^{cA}$ | $4.58 \pm 1.28^{dA}$ |
| Octanol | 2 | $1.58 \pm 0.46^{bB}$ | $1.59 \pm 0.23^{bB}$ | $2.62 \pm 0.49^{aB}$ | $0.46 \pm 0.07^{cC}$ | $0.37 \pm 0.08^{cC}$ | $0.37 \pm 0.26^{cC}$ |
| (nd) | 4 | $2.29 \pm 0.86^{bA}$ | $3.39 \pm 0.04^{aA}$ | $1.9 \pm 0.22^{bB}$ | $3.00 \pm 1.27^{aB}$ | $1.85 \pm 0.70^{bB}$ | $1.43 \pm 0.25^{cB}$ |
| | 6 | $0.28 \pm 0.21^{eB}$ | $2.27 \pm 0.55^{dB}$ | $12.04 \pm 0.80^{aA}$ | $5.69 \pm 0.84^{bA}$ | $4.49 \pm 0.28^{cA}$ | $3.08 \pm 1.38^{dA}$ |
| (Z)-2-Octen-1-ol | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | $0.2 \pm 0.04^{aA}$ | nd | nd | nd | nd | nd |
| Nonanol | 2 | nd | nd | $0.45 \pm 0.26^{aB}$ | nd | nd | nd |
| (nd) | 4 | $0.7 \pm 0.58^{aA}$ | $0.14 \pm 0.05^{bB}$ | $0.63 \pm 0.43^{aB}$ | $0.17 \pm 0.12^{bB}$ | $0.82 \pm 0.44^{aA}$ | $0.34 \pm 0.33^{bA}$ |
| | 6 | nd | $0.69 \pm 0.08^{cA}$ | $1.87 \pm 0.06^{aA}$ | $1.27 \pm 0.47^{bA}$ | nd | nd |
| Decanol | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.16 \pm 0.06^{bA}$ | nd | nd | nd | $0.35 \pm 0.11^{aA}$ | $0.2 \pm 0.12^{bA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| Benzenemethanol | 2 | $0.36 \pm 0.05^{aA}$ | $0.26 \pm 0.06^{aA}$ | $0.39 \pm 0.02^{aA}$ | $0.32 \pm 0.01^{aB}$ | $0.21 \pm 0.04^{aA}$ | $0.26 \pm 0.04^{aB}$ |
| (nd) | 4 | $0.16 \pm 0.05^{aA}$ | nd | $0.44 \pm 0.26^{aA}$ | $0.65 \pm 0.12^{aA}$ | $0.45 \pm 0.20^{aA}$ | nd |
| | 6 | $0.20 \pm 0.15^{cA}$ | $0.30 \pm 0.04^{cA}$ | $0.23 \pm 0.01^{cA}$ | $1.12 \pm 0.44^{bA}$ | $0.46 \pm 0.02^{cA}$ | $2.79 \pm 0.13^{aA}$ |
| Benzeneethanol | 2 | $0.68 \pm 0.1^{aA}$ | $0.51 \pm 0.04^{aA}$ | $0.8 \pm 0.15^{aB}$ | $0.39 \pm 0.05^{aB}$ | $0.34 \pm 0.01^{aB}$ | $0.31 \pm 0.04^{aC}$ |
| (nd) | 4 | $0.10 \pm 0.03^{cB}$ | $0.25 \pm 0.02^{cA}$ | $0.74 \pm 0.01^{cB}$ | $0.57 \pm 0.08^{cB}$ | $2.55 \pm 1.67^{aA}$ | $1.55 \pm 0.28^{bA}$ |
| | 6 | nd | $0.22 \pm 0.1^{bA}$ | $1.32 \pm 0.12^{aA}$ | $1.03 \pm 0.15^{aA}$ | $0.75 \pm 0.12^{aB}$ | $0.97 \pm 0.23^{aB}$ |
| Dodecanol | 2 | $0.93 \pm 0.09^{aA}$ | $1.46 \pm 1.22^{aA}$ | $0.77 \pm 0.73^{bA}$ | $1.3 \pm 0.52^{aA}$ | $0.54 \pm 0.16^{bA}$ | nd |
| (nd) | 4 | $1.23 \pm 0.46^{aA}$ | $0.64 \pm 0.07^{aA}$ | nd | nd | nd | nd |
| | 6 | $0.48 \pm 0.12^{aA}$ | $0.82 \pm 0.84^{aA}$ | $0.31 \pm 0.28^{aA}$ | $0.46 \pm 0.27^{aB}$ | $0.73 \pm 0.52^{aA}$ | nd |
| Tridecanol | 2 | $0.53 \pm 0.13^{aA}$ | $0.69 \pm 0.40^{aA}$ | $0.42 \pm 0.18^{aA}$ | nd | $0.28 \pm 0.10^{bA}$ | nd |
| (nd) | 4 | $0.49 \pm 0.13^{aA}$ | $0.17 \pm 0.13^{bB}$ | $0.32 \pm 0.05^{bA}$ | $0.31 \pm 0.11^{bA}$ | $0.27 \pm 0.05^{bA}$ | $0.55 \pm 0.12^{aA}$ |
| | 6 | $0.23 \pm 0.04^{aB}$ | $0.32 \pm 0.19^{aB}$ | $0.21 \pm 0.15^{aA}$ | $0.25 \pm 0.16^{aA}$ | nd | nd |
| Tetradecanol | 2 | $0.15 \pm 0.09^{bA}$ | $0.21 \pm 0.05^{bA}$ | $0.55 \pm 0.58^{aA}$ | nd | nd | nd |
| (nd) | 4 | $0.33 \pm 0.23^{bA}$ | $0.3 \pm 0.19^{bA}$ | nd | nd | nd | $1.00 \pm 0.73^{aA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| Hexadecanol | 2 | nd | nd | nd | $0.22 \pm 0.07^{aB}$ | nd | $0.28 \pm 0.08^{aA}$ |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | $2.17 \pm 1.38^{aA}$ | nd | nd | $1.29 \pm 1.29^{bA}$ | 2.73 ± 2.83aA | nd |
| Octadecanol | 2 | nd | nd | $0.66 \pm 0.23^{aA}$ | nd | nd | $0.39 \pm 0.20^{bB}$ |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | nd | nd | $0.42 \pm 0.15^{bA}$ | nd | $1.31 \pm 0^{aA}$ |
| Ethanal | 2 | $11.2 \pm 1.81^{bA}$ | $13.16 \pm 0.9^{bA}$ | $12.47 \pm 0.53^{bA}$ | $26.73 \pm 0.63^{aA}$ | $14.71 \pm 1.39^{bA}$ | $25.55 \pm 1.52^{aA}$ |
| (nd) | 4 | nd | nd | nd | $8.69 \pm 0.89^{aB}$ | $9.62 \pm 0.81^{aB}$ | nd |
| | 6 | nd | nd | nd | nd | nd | nd |
| Butanal | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | nd | nd | $0.30 \pm 0.03^{bA}$ | nd | nd |
| 2-Methyl butanal | 2 | $0.18 \pm 0.03^{bA}$ | nd | nd | $0.47 \pm 0.04^{aA}$ | $0.37 \pm 0.01^{aA}$ | $0.18 \pm 0.01^{bA}$ |
| (0.27 ± 0.05) | 4 | nd | nd | nd | $0.17 \pm 0.02^{aB}$ | nd | $0.10 \pm 0.01^{aA}$ |
| | 6 | $0.19 \pm 0.02^{aA}$ | nd | nd | $0.11 \pm 0.01^{aB}$ | nd | nd |
| 3-Methyl butanal | 2 | $0.38 \pm 0.04^{bA}$ | $0.14 \pm 0.01^{cB}$ | $0.13 \pm 0.01^{cA}$ | $0.73 \pm 0.05^{aA}$ | $0.53 \pm 0.01^{bA}$ | $0.40 \pm 0.01^{bA}$ |
| (nd) | 4 | $0.16 \pm 0.03^{bB}$ | $0.22 \pm 0.06^{aA}$ | nd | $0.21 \pm 0.02^{aB}$ | $0.17 \pm 0.01^{bB}$ | $0.12 \pm 0.01^{cB}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| Hexanal | 2 | $12.72 \pm 2.7^{aA}$ | $4.19 \pm 0.27^{bC}$ | $5.77 \pm 0.51^{bB}$ | $12.66 \pm 2.21^{aC}$ | $3.95 \pm 0.49^{bB}$ | $6.54 \pm 3.2^{bC}$ |
| (1.38 ± 0.98) | 4 | $8.28 \pm 0.85^{cA}$ | $22.7 \pm 0.94^{bA}$ | $7.48 \pm 0.19^{cA}$ | $40.64 \pm 7.55^{aB}$ | $11.68 \pm 0.61^{cA}$ | $13.9 \pm 2.34^{cB}$ |
| | 6 | $2.73 \pm 1.61^{dB}$ | $11.17 \pm 0.98^{cB}$ | $9.72 \pm 0.74^{cA}$ | $47.37 \pm 4.61^{aA}$ | $14.46 \pm 0.52^{cA}$ | $32.62 \pm 17.51^{bA}$ |
| Octanal | 2 | $0.67 \pm 0.22^{aB}$ | $0.37 \pm 0.08^{aB}$ | $0.57 \pm 0.16^{aB}$ | nd | $0.31 \pm 0.07^{aB}$ | $0.21 \pm 0.06^{aC}$ |
| (nd) | 4 | $3.53 \pm 1.02^{aA}$ | $4.17 \pm 1.07^{aA}$ | $1.25 \pm 1.08^{bB}$ | $4.71 \pm 2.74^{aA}$ | $1.16 \pm 0.35^{bB}$ | $1.45 \pm 0.36^{bB}$ |
| | 6 | $0.56 \pm 0.04^{dB}$ | nd | $9.24 \pm 1.08^{aA}$ | $5.29 \pm 0.89^{bA}$ | $5.52 \pm 0.3^{bA}$ | $3.15 \pm 1.43^{cA}$ |
| Nonanal | 2 | $0.89 \pm 0.13^{bA}$ | $1.05 \pm 0.26^{bB}$ | $1.55 \pm 0.79^{aA}$ | $0.45 \pm 0.09^{cB}$ | nd | $0.27 \pm 0.04^{cB}$ |
| (0.3 ± 0.08) | 4 | $0.94 \pm 0.18^{bA}$ | $1.67 \pm 0.58^{aA}$ | $0.43 \pm 0.15^{cB}$ | $1.12 \pm 0.30^{bA}$ | $1.00 \pm 0.99^{bA}$ | $0.73 \pm 0.19^{cA}$ |
| | 6 | nd | $0.61 \pm 0.13^{aB}$ | $0.65 \pm 0.09^{aB}$ | nd | $0.24 \pm 0.06^{bB}$ | nd |
| (E)-2-Octenal | 2 | $0.11 \pm 0.01^{aB}$ | $0.26 \pm 0.06^{aB}$ | $0.22 \pm 0.07^{aB}$ | nd | nd | nd |
| (nd) | 4 | $0.48 \pm 0.51^{aA}$ | $0.42 \pm 0.05^{aA}$ | $0.30 \pm 0.01^{aB}$ | $0.41 \pm 0.16^{aB}$ | nd | $0.62 \pm 0.68^{aB}$ |
| | 6 | nd | nd | $2.09 \pm 0.20^{bA}$ | $1.75 \pm 0.47^{bA}$ | $1.55 \pm 0.08^{bA}$ | $2.98 \pm 2.83^{aA}$ |
| Benzaldehyde | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.34 \pm 0.07^{aB}$ | $0.32 \pm 0.06^{aA}$ | $0.35 \pm 0.04^{aA}$ | $0.44 \pm 0.11^{aA}$ | $0.40 \pm 0.13^{aA}$ | $0.53 \pm 0.08^{aA}$ |
| | 6 | $0.78 \pm 0.02^{aA}$ | $0.43 \pm 0.03^{bA}$ | $0.47 \pm 0.05^{bA}$ | $0.38 \pm 0.18^{bA}$ | $0.23 \pm 0.04^{cB}$ | $0.69 \pm 0.35^{aA}$ |
| (E)-2-Nonenal | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | $0.46 \pm 0.11^{bA}$ | $0.65 \pm 0.02^{bA}$ | $0.70 \pm 0.18^{bA}$ | $0.55 \pm 0.05^{bA}$ | $1.16 \pm 1.27^{aA}$ |
| 2-Decen-1-al | 2 | $0.15 \pm 0.03^{aA}$ | $0.2 \pm 0.03^{aB}$ | $0.21 \pm 0.08^{aB}$ | nd | nd | nd |
| (nd) | 4 | $0.35 \pm 0.11^{aA}$ | $0.51 \pm 0.03^{aB}$ | $0.19 \pm 0.03^{aB}$ | $0.29 \pm 0.08^{aB}$ | $0.71 \pm 0.46^{aA}$ | $0.48 \pm 0.13^{aB}$ |

(*continued on next page*)

**Table 2** (*continued*)

| Compound | Month | AMB-20 | $pO_2$-20 | $pCO_2$-20 | AMB-10 | $pO_2$-10 | $pCO_2$-10 |
|---|---|---|---|---|---|---|---|
| | 6 | $0.77 \pm 0.75^{bA}$ | $1.23 \pm 0.3^{bA}$ | $1.04 \pm 0.05^{bA}$ | $1.54 \pm 0.85^{bA}$ | $0.81 \pm 0.12^{bA}$ | $6.15 \pm 9.76^{aA}$ |
| 2-Undecen-1-al | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.12 \pm 0.02^{aB}$ | $0.14 \pm 0.02^{aB}$ | nd | nd | $0.16 \pm 0.01^{aA}$ | nd |
| | 6 | $0.57 \pm 0.60^{bA}$ | $0.61 \pm 0.29^{bA}$ | $0.45 \pm 0.02^{bA}$ | $1.08 \pm 0.71^{bA}$ | $0.30 \pm 0.03^{bA}$ | $6.34 \pm 10.47^{aA}$ |
| (E.Z)-2, 4-Decadienal | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | nd | nd | $0.32 \pm 0.2^{aA}$ | nd | nd |
| (E.E)-2, 4-Decadienal | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | $0.55 \pm 0.69^{aA}$ | nd | nd | $0.58 \pm 0.54^{aA}$ | nd | nd |
| m-Xylene | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $2.24 \pm 0.12^{aA}$ | $1.27 \pm 0.06^{cA}$ | $1.43 \pm 0.19^{cA}$ | $2.15 \pm 0.37^{aA}$ | $1.78 \pm 0.37^{bA}$ | $1.42 \pm 0.21^{cA}$ |
| | 6 | $1.04 \pm 0.06^{bB}$ | $1.32 \pm 0.16^{aA}$ | $1.08 \pm 0.07^{bB}$ | $0.41 \pm 0.04^{dB}$ | $0.66 \pm 0.05^{cB}$ | $0.37 \pm 0.10^{dB}$ |
| p-Xylene | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.47 \pm 0.05^{aA}$ | $0.33 \pm 0.02^{bA}$ | $0.34 \pm 0.03^{bA}$ | $0.35 \pm 0.06^{bA}$ | $0.43 \pm 0.03^{aA}$ | $0.26 \pm 0.14^{cA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| 2-Propanone | 2 | $6.91 \pm 1.4^{bB}$ | $6.76 \pm 1.43^{bB}$ | $7.34 \pm 0.30^{bA}$ | $10.23 \pm 0.28^{aB}$ | $5.20 \pm 1.4^{bA}$ | $9.44 \pm 0.78^{aA}$ |
| ($1.88 \pm 0.61$) | 4 | $7.19 \pm 3.17^{aB}$ | $5.35 \pm 0.74^{bB}$ | $4.14 \pm 0.28^{bB}$ | $5.38 \pm 0.38^{bC}$ | $5.18 \pm 0.21^{bA}$ | $5.05 \pm 0.21^{bB}$ |
| | 6 | $17.98 \pm 1.35^{aA}$ | $11.89 \pm 1.02^{bA}$ | $7.78 \pm 0.08^{cA}$ | $14.09 \pm 1.22^{bA}$ | $5.80 \pm 0.08^{cA}$ | $4.21 \pm 0.61^{cB}$ |
| 2-Butanone | 2 | $0.45 \pm 0.03^{bA}$ | $1.26 \pm 0.06^{aA}$ | $1.26 \pm 0.03^{aA}$ | $0.98 \pm 0.1^{aA}$ | $1.06 \pm 0.28^{aA}$ | $0.83 \pm 0.04^{aA}$ |
| (nd) | 4 | $0.42 \pm 0.11^{aA}$ | $0.41 \pm 0.04^{aB}$ | $0.57 \pm 0.20^{aB}$ | $0.73 \pm 0.15^{aA}$ | $0.90 \pm 0.12^{aA}$ | $0.79 \pm 0.05^{aA}$ |
| | 6 | $0.20 \pm 0.05^{aB}$ | $0.36 \pm 0.02^{aB}$ | $0.18 \pm 0.06^{aB}$ | $0.26 \pm 0.03^{aB}$ | nd | $0.23 \pm 0.13^{aB}$ |
| 3-Methyl 2-butanone | 2 | $0.30 \pm 0.02^{aA}$ | $0.19 \pm 0.01^{aA}$ | $0.26 \pm 0.02^{aA}$ | $0.26 \pm 0.01^{aA}$ | $0.30 \pm 0.11^{aA}$ | $0.17 \pm 0.01^{aA}$ |
| ($0.12 \pm 0.05$) | 4 | nd | nd | $0.12 \pm 0.07^{aB}$ | $0.13 \pm 0.01^{aB}$ | $0.10 \pm 0.03^{aB}$ | $0.11 \pm 0.02^{aA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| 2-Pentanone | 2 | $4.84 \pm 0.7^{aA}$ | $5.08 \pm 0.38^{aA}$ | $4.80 \pm 0.09^{aA}$ | $4.70 \pm 0.35^{aA}$ | $3.98 \pm 0.11^{aA}$ | $3.60 \pm 0.23^{aA}$ |
| ($2.06 \pm 0.32$) | 4 | $0.63 \pm 0.06^{cB}$ | $2.33 \pm 0.29^{bB}$ | $3.24 \pm 0.16^{bA}$ | $5.16 \pm 0.32^{aA}$ | $3.32 \pm 0.04^{bA}$ | $3.30 \pm 0.22^{bA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| 4-Methyl 2-pentanone | 2 | nd | nd | Nd | nd | nd | nd |
| (nd) | 4 | nd | nd | $0.60 \pm 0.12^{aA}$ | $0.65 \pm 0.06^{aA}$ | $0.37 \pm 0.27^{bA}$ | $0.21 \pm 0.02^{bA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| 2-Heptanone | 2 | $1.85 \pm 0.32^{aA}$ | $1.8 \pm 0.16^{aA}$ | $2.12 \pm 0.09^{aA}$ | $0.54 \pm 0.03^{bB}$ | nd | $0.71 \pm 0.25^{bB}$ |
| (nd) | 4 | $0.89 \pm 0.09^{bB}$ | $2.69 \pm 1.91^{bA}$ | $1.80 \pm 0.40^{bA}$ | $7.54 \pm 3.16^{aA}$ | $0.95 \pm 0.18^{bA}$ | $1.64 \pm 0.59^{bA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| 6-Methyl 5-hepten-2-one | 2 | $0.33 \pm 0.02^{aA}$ | $0.36 \pm 0.02^{aA}$ | $0.36 \pm 0.03^{aB}$ | $0.23 \pm 0.05^{aB}$ | nd | nd |
| (nd) | 4 | $0.26 \pm 0.03^{aA}$ | nd | $0.47 \pm 0.18^{aB}$ | $0.35 \pm 0.06^{aB}$ | nd | $0.35 \pm 0.06^{aB}$ |
| | 6 | $0.41 \pm 0.08^{dA}$ | nd | $1.13 \pm 0.25^{aA}$ | $0.81 \pm 0.11^{bA}$ | nd | $0.77 \pm 0.41^{bA}$ |
| 4-Oxoisophorone | 2 | $0.3 \pm 0.09^{aA}$ | $0.28 \pm 0.02^{aA}$ | $0.25 \pm 0.03^{aA}$ | $0.37 \pm 0.03^{aA}$ | $0.28 \pm 0.05^{aA}$ | $0.15 \pm 0.02^{bA}$ |
| (nd) | 4 | nd | $0.16 \pm 0.05^{bA}$ | $0.28 \pm 0.02^{aA}$ | $0.32 \pm 0.01^{aA}$ | $0.12 \pm 0.04^{bA}$ | $0.23 \pm 0.01^{aA}$ |
| | 6 | nd | nd | $0.22 \pm 0.01^{aA}$ | $0.31 \pm 0.05^{aA}$ | $0.20 \pm 0.03^{aA}$ | $0.23 \pm 0.06^{aA}$ |
| Methyl ethanoate | 2 | $1.66 \pm 0.68^{bA}$ | $3.30 \pm 0.74^{aA}$ | $3.02 \pm 0.14^{aA}$ | $1.64 \pm 0.11^{bA}$ | $2.01 \pm 1.41^{bA}$ | $1.48 \pm 0.19^{bA}$ |
| (nd) | 4 | $1.30 \pm 0.39^{bA}$ | $0.69 \pm 0.47^{cB}$ | $2.41 \pm 0.44^{aA}$ | $1.83 \pm 0.31^{bA}$ | $1.63 \pm 0.11^{bA}$ | $1.74 \pm 0.05^{bA}$ |
| | 6 | nd | $2.71 \pm 1.07^{aA}$ | $0.89 \pm 0.03^{bB}$ | $0.63 \pm 0.67^{bB}$ | $0.85 \pm 0.01^{bB}$ | $1.06 \pm 0.08^{bA}$ |
| Ethyl ethanoate | 2 | $1.15 \pm 0.15^{eA}$ | $2.2 \pm 0.15^{eA}$ | $9.11 \pm 0.41^{bA}$ | $3.93 \pm 0.36^{dA}$ | $5.05 \pm 1.62^{cA}$ | $3.31 \pm 0.19^{dB}$ |
| ($1.62 \pm 0.21$) | 4 | $1.23 \pm 0.15^{eA}$ | $1.22 \pm 0.15^{eA}$ | $8.87 \pm 0.92^{bA}$ | $0.19 \pm 0.05^{eB}$ | $6.34 \pm 0.34^{cA}$ | $4.97 \pm 0.31^{cA}$ |
| | 6 | $0.61 \pm 0.08^{cA}$ | $1.56 \pm 0.04^{bA}$ | $1.60 \pm 0.05^{bB}$ | $2.39 \pm 0.07^{aA}$ | $2.84 \pm 0.04^{aB}$ | $2.59 \pm 0.25^{aB}$ |
| Methyl butanoate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.19 \pm 0.02^{bB}$ | $0.25 \pm 0.10^{bB}$ | $0.48 \pm 0.06^{bB}$ | $0.68 \pm 0.09^{aB}$ | $0.72 \pm 0.05^{aB}$ | $0.35 \pm 0.02^{bB}$ |
| | 6 | $0.92 \pm 0.25^{dA}$ | $0.93 \pm 0.05^{dA}$ | $1.78 \pm 0.02^{cA}$ | $4.72 \pm 0.23^{aA}$ | $2.7 \pm 0.06^{cA}$ | $3.18 \pm 1.33^{bA}$ |
| Ethyl butanoate | 2 | $0.54 \pm 0.10^{aC}$ | $0.52 \pm 0.05^{aB}$ | $0.73 \pm 0.09^{aB}$ | $0.61 \pm 0.07^{aB}$ | $0.33 \pm 0.05^{aB}$ | $0.37 \pm 0.01^{aC}$ |
| (nd) | 4 | $2.17 \pm 0.01^{aB}$ | $1.43 \pm 0.09^{bB}$ | $2.35 \pm 0.3^{aA}$ | $3.03 \pm 0.4^{aA}$ | $3.37 \pm 0.42^{aA}$ | $2.48 \pm 0.20^{aB}$ |
| | 6 | $9.17 \pm 1.13^{aA}$ | $4.15 \pm 0.51^{bA}$ | $3.5 \pm 0.11^{bA}$ | $2.62 \pm 0.27^{cA}$ | $2.44 \pm 0.15^{cA}$ | $3.86 \pm 3.36^{bA}$ |
| 2-Methyl ethyl butanoate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | $0.27 \pm 0.07^{cA}$ | $0.44 \pm 0.02^{bA}$ | $0.43 \pm 0.06^{bA}$ | $0.21 \pm 0.02^{dA}$ | $0.69 \pm 0.04^{aA}$ | $0.47 \pm 0.11^{bA}$ |
| Butyl ethanoate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.33 \pm 0.07^{bA}$ | $0.23 \pm 0.06^{bA}$ | $0.29 \pm 0.05^{bA}$ | $0.25 \pm 0.05^{bA}$ | $0.53 \pm 0.24^{aA}$ | $0.25 \pm 0.02^{bA}$ |
| | 6 | nd | $0.13 \pm 0.01^{cA}$ | $0.31 \pm 0.07^{aA}$ | $0.26 \pm 0.03^{aA}$ | $0.28 \pm 0.02^{aB}$ | $0.31 \pm 0.01^{aA}$ |
| 2-Methyl 1-butyl ethanoate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | $0.72 \pm 0.05^{aB}$ | nd | $0.52 \pm 0.14^{aB}$ | $0.21 \pm 0.06^{bB}$ |
| | 6 | $0.33 \pm 0.04^{cA}$ | $1.71 \pm 0.07^{bA}$ | $2.82 \pm 0.09^{aA}$ | $1.72 \pm 0.15^{bA}$ | $1.86 \pm 0.08^{bA}$ | $1.86 \pm 0.18^{bA}$ |
| Ethyl hexanoate | 2 | $1.95 \pm 0.34^{aB}$ | $1.30 \pm 0.15^{bB}$ | $2.82 \pm 0.19^{aC}$ | $1.48 \pm 0.19^{bB}$ | $0.76 \pm 0.17^{bB}$ | $1.09 \pm 0.10^{bB}$ |
| ($1.23 \pm 0.12$) | 4 | $4.54 \pm 0.31^{bA}$ | $2.65 \pm 0.22^{cA}$ | $5.41 \pm 1.19^{bB}$ | $8.23 \pm 1.30^{aA}$ | $5.26 \pm 1.17^{bA}$ | $6.76 \pm 0.88^{bA}$ |
| | 6 | $0.72 \pm 0.17^{dC}$ | $3.85 \pm 0.36^{cA}$ | $10.1 \pm 0.83^{aA}$ | $6.13 \pm 0.58^{bA}$ | $6.70 \pm 0.38^{bA}$ | $6.72 \pm 1.02^{bA}$ |
| Hexyl ethanoate | 2 | nd | nd | $0.27 \pm 0.04^{aC}$ | nd | nd | nd |
| (nd) | 4 | nd | nd | $1.48 \pm 1.25^{aB}$ | nd | $0.2 \pm 0.03^{bB}$ | nd |
| | 6 | nd | nd | $2.28 \pm 0.37^{aA}$ | $0.57 \pm 0.05^{bA}$ | $1.04 \pm 0.05^{bA}$ | $0.71 \pm 0.29^{bA}$ |
| Butyl hexanoate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | $0.19 \pm 0.02^{cA}$ | $0.64 \pm 0.06^{aA}$ | $0.42 \pm 0.08^{bA}$ | $0.29 \pm 0.01^{bA}$ | $0.34 \pm 0.08^{bA}$ |
| Ethyl octanoate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | nd | $0.4 \pm 0.05^{aA}$ | nd | nd | nd |
| Methyl nonanoate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | $0.23 \pm 0.05^{cA}$ | $0.99 \pm 0.07^{aA}$ | $0.48 \pm 0.09^{bA}$ | $0.35 \pm 0.02^{bA}$ | $0.43 \pm 0.07^{bA}$ |

(*continued on next page*)

**Table 2** (*continued*)

| Compound | Month | AMB-20 | $pO_2$-20 | $pCO_2$-20 | AMB-10 | $pO_2$-10 | $pCO_2$-10 |
|---|---|---|---|---|---|---|---|
| Ethyl decanoate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | $4.03 \pm 3.09^{aA}$ | $1.45 \pm 0.94^{bA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| Diethyl succinate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | $1.34 \pm 0.87^{aA}$ | $0.29 \pm 0.11^{bA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| Ethyl dodecanoate | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | $1.24 \pm 0.43^{aA}$ | $1.64 \pm 0.54^{aA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| Triacetin | 2 | $0.41 \pm 0.23^{aA}$ | $0.43 \pm 0.21^{aA}$ | $0.47 \pm 0.22^{aA}$ | $0.28 \pm 0.05^{aA}$ | $0.26 \pm 0.11^{aA}$ | $0.22 \pm 0.12^{aA}$ |
| (nd) | 4 | $0.15 \pm 0.03^{aB}$ | $0.28 \pm 0.11^{aB}$ | $0.3 \pm 0.08^{aA}$ | $0.39 \pm 0.26^{aA}$ | $0.21 \pm 0.06^{aA}$ | $0.43 \pm 0.14^{aA}$ |
| | 6 | $0.2 \pm 0.02^{bB}$ | $0.54 \pm 0.14^{aA}$ | nd | $0.5 \pm 0.15^{aA}$ | $0.33 \pm 0.09^{aA}$ | $0.35 \pm 0.12^{aA}$ |
| 2-Pentyl furan | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.4 \pm 0.05^{aA}$ | nd | nd | nd | nd | nd |
| | 6 | $0.42 \pm 0.11^{cA}$ | $2.51 \pm 0.19^{aA}$ | $1.83 \pm 0.17^{bA}$ | $0.39 \pm 0.23^{cA}$ | $0.43 \pm 0.08^{cA}$ | $1.43 \pm 1.42^{bA}$ |
| γ-Lactone | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.66 \pm 0.32^{cA}$ | $2.28 \pm 0.42^{bA}$ | $3.78 \pm 0.05^{aA}$ | $1.99 \pm 0.05^{bA}$ | $3.35 \pm 1.10^{aA}$ | $2.30 \pm 0.65^{bA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| 5,5-Dimethyl-2(5H)-furanone | 2 | $0.43 \pm 0.08^{aA}$ | $0.51 \pm 0.03^{aA}$ | $0.45 \pm 0.03^{aA}$ | $0.25 \pm 0.01^{bA}$ | $0.26 \pm 0.04^{bA}$ | $0.25 \pm 0.01^{bA}$ |
| (nd) | 4 | nd | $0.19 \pm 0.01^{bB}$ | $0.44 \pm 0.02^{aA}$ | $0.22 \pm 0.01^{bA}$ | $0.15 \pm 0.08^{bA}$ | $0.16 \pm 0.01^{bB}$ |
| | 6 | $0.20 \pm 0.02^{cB}$ | nd | $0.47 \pm 0.03^{aA}$ | $0.24 \pm 0.06^{cA}$ | $0.19 \pm 0.02^{cA}$ | $0.32 \pm 0.16^{bA}$ |
| Butyrolactone | 2 | $1.07 \pm 0.15^{bA}$ | $1.41 \pm 0.09^{bA}$ | $2.54 \pm 0.11^{aB}$ | $0.56 \pm 0.01^{cB}$ | $0.62 \pm 0.33^{cB}$ | $0.71 \pm 0.01^{cB}$ |
| (nd) | 4 | nd | nd | $4.34 \pm 0.03^{aA}$ | $0.85 \pm 0.03^{bB}$ | $0.8 \pm 0.25^{bB}$ | $0.64 \pm 0.01^{bB}$ |
| | 6 | $0.16 \pm 0.01^{cB}$ | $0.27 \pm 0.03^{cB}$ | $2.9 \pm 0.05^{aB}$ | $1.16 \pm 0.10^{bA}$ | $1.46 \pm 0.02^{bA}$ | $1.08 \pm 0.13^{bA}$ |
| γ-Hexalactone | 2 | $0.12 \pm 0.02^{aA}$ | nd | $0.14 \pm 0.02^{aB}$ | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | nd | $0.31 \pm 0.03^{aA}$ | $0.34 \pm 0.05^{aA}$ | nd | nd |
| γ-Octalactone | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.09 \pm 0.06^{aA}$ | $0.07 \pm 0.01^{aA}$ | nd | nd | nd | nd |
| | 6 | nd | nd | $0.32 \pm 0.01^{aA}$ | $0.19 \pm 0.05^{bA}$ | $0.13 \pm 0.01^{b/A}$ | nd |
| δ-Nonalactone | 2 | $0.25 \pm 0.02^{aA}$ | $0.30 \pm 0.08^{aA}$ | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | $0.27 \pm 0.03^{aA}$ | nd | nd | nd |
| | 6 | nd | nd | nd | nd | nd | nd |
| Pantolactone | 2 | $0.15 \pm 0.03^{aA}$ | $0.12 \pm 0.04^{aA}$ | $0.11 \pm 0.01^{aB}$ | nd | $0.12 \pm 0.06^{aB}$ | $0.13 \pm 0.05^{aA}$ |
| (nd) | 4 | $0.22 \pm 0.05^{aA}$ | nd | $0.25 \pm 0.01^{aA}$ | nd | $0.66 \pm 0.05^{aA}$ | nd |
| | 6 | nd | nd | nd | nd | nd | nd |
| Sabinene | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $0.64 \pm 0.17^{bA}$ | $0.8 \pm 0.28^{bA}$ | nd | $1.2 \pm 0.49^{aA}$ | nd | $0.95 \pm 0.49^{aA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| α-Phellandrene | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $1.10 \pm 0.26^{bA}$ | nd | nd | $1.68 \pm 0.23^{aA}$ | $1.01 \pm 0.13^{bA}$ | nd |
| | 6 | nd | nd | nd | nd | nd | nd |
| α-Myrcene | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | $1.06 \pm 0.11^{bA}$ | $0.88 \pm 0.24^{cA}$ | $1.10 \pm 0.19^{bA}$ | $1.40 \pm 0.15^{aA}$ | $0.78 \pm 0.19^{cA}$ | $1.74 \pm 0.64^{aA}$ |
| | 6 | $0.33 \pm 0.06^{aB}$ | $0.48 \pm 0.03^{aB}$ | $0.37 \pm 0.03^{aB}$ | $0.18 \pm 0.05^{bB}$ | nd | nd |
| β-Myrcene | 2 | $0.15 \pm 0.05^{aB}$ | nd | $0.19 \pm 0.01^{aB}$ | nd | nd | Nd |
| (nd) | 4 | $3.21 \pm 0.52^{bA}$ | $0.98 \pm 0.30^{cA}$ | $0.84 \pm 0.12^{cA}$ | $0.26 \pm 0.05^{dA}$ | $5.19 \pm 1.73^{aA}$ | $0.91 \pm 0.14^{cA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |
| Limonene | 2 | $1.01 \pm 0.15^{bB}$ | $3.05 \pm 0.25^{aC}$ | $1.8 \pm 0.24^{bC}$ | $1.3 \pm 0.36^{bC}$ | $1.66 \pm 0.40^{bC}$ | $0.65 \pm 0.15^{cC}$ |
| (nd) | 4 | $0.64 \pm 0.02^{dB}$ | $7.76 \pm 1.56^{aB}$ | $6.29 \pm 0.92^{bB}$ | $5.75 \pm 0.55^{bB}$ | $3.21 \pm 0.73^{cB}$ | $2.96 \pm 0.69^{cB}$ |
| | 6 | $2.29 \pm 0.09^{eA}$ | $16.76 \pm 0.34^{aA}$ | $12.25 \pm 0.6^{bA}$ | $9.98 \pm 1.48^{cA}$ | $9.61 \pm 0.46^{cA}$ | $4.89 \pm 2.62^{dA}$ |
| β-Phellandrene | 2 | $0.53 \pm 0.10^{aB}$ | $0.32 \pm 0.04^{aB}$ | $0.71 \pm 0.03^{aB}$ | nd | nd | $0.76 \pm 0.48^{aB}$ |
| (nd) | 4 | $3.96 \pm 0.85^{aA}$ | $2.87 \pm 0.88^{aA}$ | $2.50 \pm 0.22^{aA}$ | $3.95 \pm 2.73^{aA}$ | $0.63 \pm 0.19^{bA}$ | $3.22 \pm 3.23^{aA}$ |
| | 6 | $0.10 \pm 0.02^{aB}$ | $0.3 \pm 0.04^{aB}$ | nd | nd | nd | nd |
| α-Farnesene | 2 | nd | nd | nd | nd | nd | nd |
| (nd) | 4 | nd | nd | nd | nd | nd | nd |
| | 6 | nd | nd | $0.10 \pm 0.01^{aA}$ | nd | nd | nd |
| (Z)-Geranylacetone | 2 | $0.23 \pm 0.04^{aB}$ | $0.21 \pm 0.11^{aA}$ | nd | nd | nd | nd |
| (nd) | 4 | $0.41 \pm 0.17^{bA}$ | nd | $0.20 \pm 0.06^{cA}$ | nd | $0.22 \pm 0.14^{cA}$ | $0.78 \pm 0.30^{aA}$ |
| | 6 | nd | nd | nd | nd | nd | nd |

* Detected at harvest (0 months); ** means followed by the same lowercase letter between storage conditions, and uppercase letter between months of storage, did not differ by Scott Knott's test ($p < 0.05$). AMB-20 = ambient atmosphere and 20 °C; $pO_2$-20 = 2 kPa $O_2$ + 0.04 kPa $CO_2$, at 20 °C; $pCO_2$-20 = 2 kPa $O_2$ + 15 kPa $CO_2$, at 20 °C; AMB-10 = ambient atmosphere and 10 °C; $pO_2$-10 = 2 kPa $O_2$ + 0.04 kPa $CO_2$, at 10 °C; $pCO_2$-10 = 2 kPa $O_2$ + 15 kPa $CO_2$, at 10 °C; Nd = not detected.

months. 2-Butanone, 2-pentanone, and 2-heptanone had a lower occurrence in the sixth month.

*Esters*. In general, this class of compounds tended to increase during storage except for methyl ethanoate and ethyl ethanoate, which decreased. Ethyl ethanoate showed larger areas in treatments under CA at 10 °C and in $pCO_2$-20 until the fourth month. In the fourth month, this class increased in the peak area and new esters formation, such as methyl butanoate, 2-methyl ethyl butanoate, and 2-methyl-1-butyl ethanoate, were observed. Butyl hexanoate and methyl nonanoate were detected only in the sixth month. Ethyl butanoate and methyl butanoate increased with the storage time; ethyl hexanoate also increased with elapsed time, more notably in treatments at 10 °C and $pCO_2$-20. Ethyl decanoate, ethyl dodecanoate, and diethyl succinate were only detected under CA conditions at 10 °C.

*Terpenes*. The monoterpenes myrcene, geranylacetone, the cyclics sabinene, phellandrene, and limonene, and the sesquiterpene pharnecene were detected. Sabinene, β-phellandrene, and α-myrcene were detected as of the fourth month. α-Myrcene and sabinene showed larger

peak areas in the $pCO_2$-10 treatment and β-myrcene in AMB-20 and $pO_2$-10. In addition, the $pCO_2$-10 treatment showed higher values in the area of geranylacetone in the fourth month. Limonene was detected in all periods analyzed, being the most abundant compound of this class, especially at six months in $pO_2$-20 and $pCO_2$-20.

*Aromatics*, *furans*, and *lactones*. The m-xylene and p-xylene compounds were detected after the fourth month, with smaller peak areas in the $pCO_2$-10 treatment. Furans and lactones were more abundant in nuts at 20 °C, primarily the compounds 5,5-dimethyl 2(5H)-furanone, dihydro 2(3H)-furanone, and γ-lactone, with the highest peak areas in the $pCO_2$-20 treatment.

### 3.7. Exploratory multivariate analysis - PCA

The exploratory multivariate analysis was performed by PCA to visualize the influence of atmospheric conditions, temperature, and storage time on shelled pecan nuts (Figs. 4 and 5). The first three principal components (PC) accounted for 58.26% of the global variation. The main factor of separation among samples was the storage time. The PC1 (26.64%) separated the treatments in the second and fourth months, which were positioned in the left quadrants, from the sixth-month treatments, on the right quadrants of the PCA score graph. We observed in the second month that the samples were correlated to higher MC, the largest peak area of ethanal, 2-methyl butanal, 3-methyl butanal, and more luminosity (L*); the parameter a* is also located in this region and correlated to samples at 20 °C. Correlated to an increase in PVs, consequently, a higher concentration of compounds from oxidation, such as some aldehydes (hexanal, 2-undecenal, and (E)-2-nonenal), acids (C4-C7, C9, and C11), and alcohols (C5-C7 and C9), were found the treatments with six months of storage (mainly those submitted to 10 °C and $pCO_2$-20).

The PC2 (17.14%; Fig. 4A and 4B) separated the second and sixth months of treatments (lower region) from the fourth month of storage (upper region). The CA treatments at 10 °C (on the fourth month) showed a more significant relationship with terpenes (sabinene, α-phelandrene, β-phelandrene, geranylacetone, and myrcene), p-xylene, m-xylene, ethyl decanoate, and ethyl dodecanoate. Interestingly, these two mentioned esters are products of the reaction of ethanol with decanoic acid and dodecanoic acid, which are also correlated with treatments in the four-month period. In addition, the PC2 also showed that there is a relationship between PV and compounds from oxidation in the fourth month of storage, indicating that lipid oxidation occurs over time, among other biochemical reactions, such as enzyme reactions and autoxidation. Nevertheless, PC3 (14.47%) revealed the relationship among the samples AMB-20 (more strongly) and $pO_2$-20 treatments at the fourth and sixth months had higher AV, PV, respiration, and 1-octen-3-ol (Fig. 5A and 5B). Another parameter observed was higher 1-octen-3-ol, which is associated with fungal metabolism, in the AMB-20 treatment in the fourth and sixth months, indicating that this treatment has more significant potential for these microorganisms' development.

## 4. Discussion

This study investigated the quality parameters of 'Barton' shelled pecan nuts before storage (initial point) and after different storage times under different atmospheric conditions and temperatures (results in Supplementary Material 1). Pecan storage under atmospheric conditions with lower $O_2$ concentrations was evaluated as a method of controlling oxidation reactions (Ribeiro, Ribeiro et al., 2020; Maskan, & Karataş, 1999). Ribeiro, Ribeiro et al. (2020) demonstrated that temperatures below 10 °C better preserve the initial kernel color of the unshelled pecans than 20 °C, although these alterations were minimized when the $pO_2$ was reduced (1 and 3 kPa) at 20 °C.

In this study, with the storage of shelled pecan, the data corroborate the aforementioned authors; CA storage, even at 20 °C, contributes significantly ($p < 0.05$) in maintaining L*, lower a* values, and the highest b* values in the color of pecans kernels compared to the AMB (without reducing $pO_2$ or reducing $pO_2$ and increasing $pCO_2$). The more intense red color (higher a* value) is undesirable in pecan nuts as it is correlated to browning due to oxidation processes (Pannico et al., 2015). Nonetheless, higher b* value is desirable because it is associated with the yellow/golden color, indicating the quality of fresh nuts (Ribeiro, Ribeiro et al., 2020; Bagheri et al., 2019). According to Prado et al. (2013), the oxidation of compounds related to oily pigments can cause discoloration or compound formation with different colors found in nuts. Hence, it is pivotal to maintain pecans under adequate storage conditions to inhibit more intense red colors (Ribeiro, Ribeiro et al., 2020). For more extended shelled pecan storage periods (e.g., six months), better color maintenance was acquired at 10 °C with 2 kPa of $O_2$ or 2 kPa of $O_2$ + 15 kPa of $CO_2$. Additionally, the kernel color of pecan stored with low $pO_2$ preserved higher L* and hue angle values at 20 °C, showing low darkness despite the lower b* values. C variable, represents the distance from the luminosity axis (L*), initiating at 0 and, as higher the C value, indicates greater saturation/luminosity of the color. On the other hand, the lower hue angle (hue) is mainly related to the more intense red color in pecans (Thewes, Both, Thewes, Brackmann, Schultz et al., 2021). Our results indicated a greater relationship of C and hue angle with the b* value, indicating that higher values of these attributes are associated with the protection of the desirable golden color.

It is well known that the pecan nut respiration rate is very low (Ribeiro, Ribeiro et al., 2020; Thewes, Both, Thewes, Brackmann, Schultz et al., 2021), albeit that evaluations were performed with pecans in shell. Even the shelled nuts used in this study presented low respiration rates, and differences among storage conditions were observed. High $pCO_2$ at 20 °C had a positive effect on maintaining lower respiration rates, although these rates were low in all treatments at 10 °C. Higher respiration rates indicate more significant metabolic activity (Both et al., 2017), which may be associated with degradation in the long-term storage. The $CO_2$ effect is most pronounced when associated with lower temperatures (i.e., 10 °C); this may be attributed to the high $CO_2$ solubility in the liquid and consequently in aqueous nut portions (Calvo et al., 1994; Zhao, & Wells, 1995), acting as a barrier at the oil/air interface and thus reducing the oxygen consumption rate of the sample.

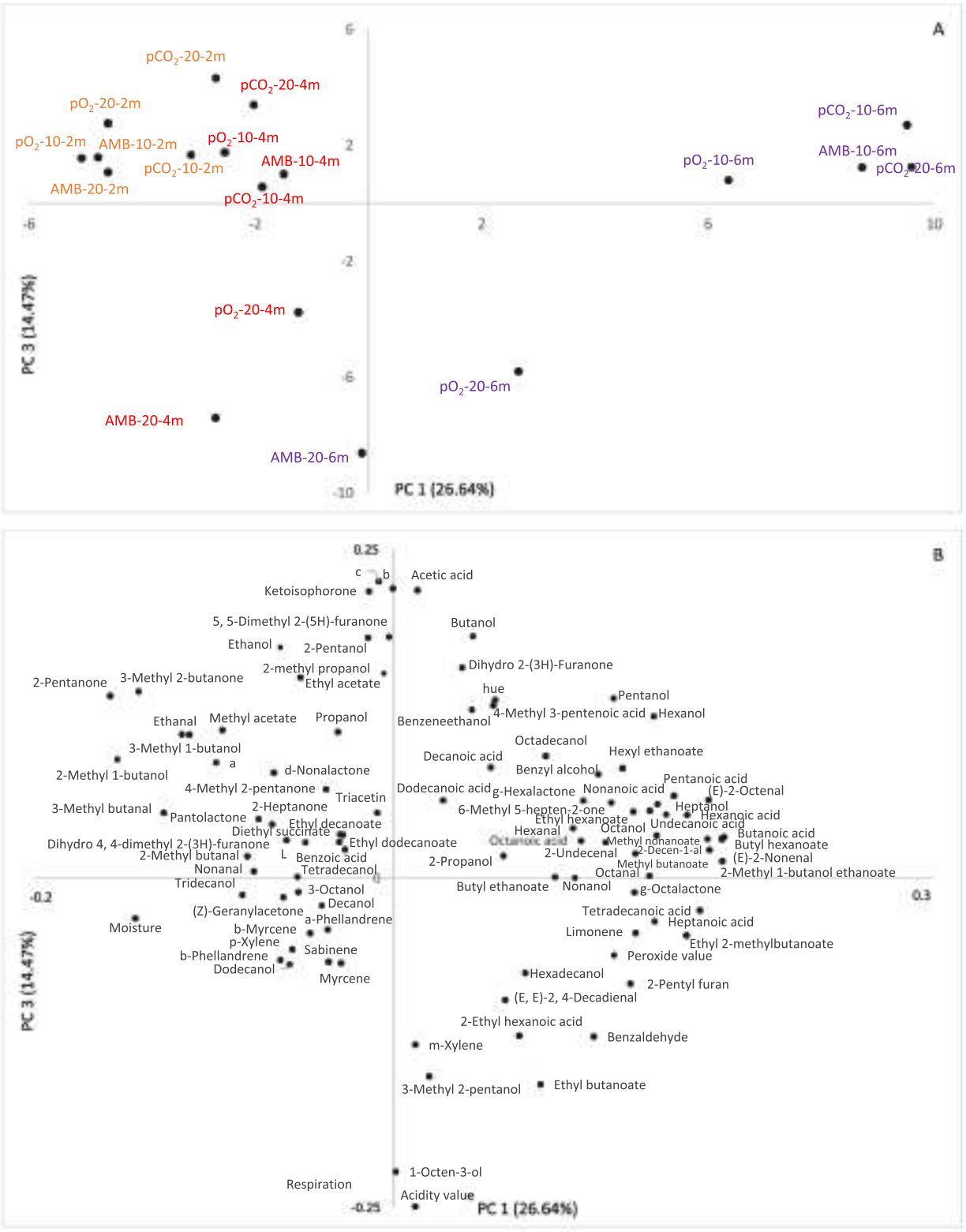
It is paramount to control the MC of nuts as it affects numerous other quality factors, including color, texture, and fungal growth (Lima, & Borges, 2004; Ajith et al., 2015; Costa et al., 2016). Aw and MC below 0.7 and 6%, respectively, are recommended for nut storage (FDA, 2017). Studies conducted with cashew nuts and macadamias, stored with MC above these values, presented fungal growth and internal browning, respectively (Balasubrahmanyam et al., 1985; Walton et al., 2013). In the present study, it was possible to observe that during storage and at the end of the sixth month, CA treatments (regardless of the temperature) and AMB-10 contributed to maintaining lower MC and aw values, which can prevent microorganism development and browning reactions (Costa et al., 2016; Walton et al., 2013). Garcia et al. (2012) reported that fungal growth depends on the interaction between temperature and moisture, which are the most critical variables for their development. After the fourth month of storage, AMB-20 showed higher MC values ($p < 0.05$) and visible mold development. This treatment reflects the real condition in which pecans are commonly stored in warehouses or containers after harvest. Hence, nuts must not remain for four months in AMB-20 after shelling under high MC and aw values conditions.

Before storage, the nuts' AV was 0.29 g of oleic acid 100 $g^{-1}$ of oil. Fresh pistachios have 0.36 g of oleic acid 100 $g^{-1}$ of oil (Maskan, & Karataş, 1999), and fresh hazelnuts can range from 0.5 to 1.30 g oleic acid 100 $g^{-1}$ of oil (Pannico et al., 2015). In this study, the significantly higher ($p < 0.05$) AVs of nuts stored under AMB and low $pO_2$ conditions at 20 °C indicate that these conditions drive oil degradation reactions in nuts. Nevertheless, the use of CA with high $pCO_2$, regardless of the temperature, and AMB-10 maintain lower hydrolytic rancidity values,




AMB – ambient atmosphere, $pO_2$ – 2kPa of $O_2$, $pCO_2$ – 15 kPa of $CO_2$ + 2 kPa of $O_2$, 2m, 4m, 6m

**Fig. 4. A and B.** - Analysis of principal components of 'Barton' pecan nuts during storage in different temperatures, oxygen partial pressure and high $CO_2$. PCA 1 and 2, **(A)** graph of the samples (loadings); (B) graph of the variables (scores) (color, moisture content, aw, respiration, acidity, peroxides indexes and volatile compounds,).




AMB – ambient atmosphere, $pO_2$ – 2kPa of $O_2$, $pCO_2$ – 15 kPa of $CO_2$ + 2 kPa of $O_2$, 2m, 4m, 6m

**Fig. 5. A and B.** - Analysis of principal components of 'Barton' pecan nuts during storage in different temperatures, oxygen partial pressure and high $CO_2$. PCA 1 and 3, (A) - graph of the samples (loadings); (B) graph of the variables (scores) (color, moisture content, aw, respiration, acidity, peroxides indexes and volatile compounds,).

indicating that these conditions better preserve the quality of the nuts throughout the six months. The observed differences were attributed to the hydrolytic process producing free FAs (Gecgel et al., 2011), which is also driven by a reaction between moisture and oil (Phatanayindee et al., 2012), and subsequent loss of FAs occurs by chemical and enzymatic oxidation at higher temperatures. Thus, the AVs are closely related to the quality of raw materials and their conservation conditions (Christopoulos, & Tsantili, 2015; IAL, 2008). According to Resolution RDC No. 270 (ANVISA, 2005), crude olive oil, which has a lipid profile similar to pecan oil (Ribeiro, Klein et al., 2020), must not exceed 1.5 g oleic acid 100 $g^{-1}$ of oil to guarantee product quality.

Pistachios stored under high $CO_2$ (98 kPa) conditions, irrespective of the temperature (10, 20, and 30 °C), had lower AVs (<0.9 g oleic acid 100 $g^{-1}$ oil) than under ambient conditions (20–30 °C, 20 kPa of $O_2$; ~1.2 g oleic acid 100 $g^{-1}$ of oil) (Maskan, & Karataş, 1999). Moreover, these authors reported that storage with high $CO_2$ increases the activation energy of FA hydrolysis. Temperature is another important factor that affects AVs in nuts. In 12 months, unshelled pecans stored at low temperatures (10 and 1.5 °C) had significantly lower AVs ($p < 0.05$) than pecans stored at 20 °C (Ribeiro, Ribeiro et al., 2020), being 0.36, 0.33, and 0.90 g of oleic acid 100 $g^{-1}$ of oil, respectively. Hazelnuts stored for 12 months at 4 °C had lower AVs (0.13 g of oleic acid 100 $g^{-1}$ of oil) than at ambient temperatures (~20 °C; 0.47 g of oleic acid 100 $g^{-1}$ of oil) (Ghirardello et al., 2013).

Free FAs in oils indicate lipid hydrolysis reactions, while PVs indicate lipid oxidation (Maskan & Karataş, 1999; Buransompob et al., 2003; Gecgel et al., 2011). In this study, the PVs were lower in the first two months of storage, although they significantly increased ($p < 0.05$) after the fourth month. However, after six months, treatments at 10 °C had an effect on reducing the higher PVs, regardless of the storage condition. In 12 months of storage, pecans in shell subjected to low temperatures (10 and 1.5 °C) and low $pO_2$ (3 and 1 kPa) showed lower PVs than under ambient conditions (20 °C and 20 kPa of $O_2$) (Ribeiro, Ribeiro et al., 2020). After 15 months, pistachio nuts stored under ambient conditions (20–30 °C and 20 kPa of $O_2$) resulted in increased PVs with significantly higher values ($p < 0.05$) than samples stored at lower temperatures (10 °C) and associated with CA conditions (98 kPa $CO_2$).

The PVs increase rapidly during the propagation stage of free radicals from lipid oxidation (Maskan & Karataş, 1999), mainly at higher temperatures and high availability of $O_2$ (Ribeiro, Ribeiro et al., 2020). As tocopherols, pigments, and other antioxidants substances reduce, FAs begin to be oxidized, and the presence of peroxides and derivates increases (Rudolph et al., 1992). Hydroperoxides accumulate as the main oxidation products, subsequently breaking down to form secondary oxidation products such as alcohols, aldehydes, ketones, and free FA, resulting in rancidity (Pannico et al., 2015). These secondary metabolites are often volatile substances and may cause off-odor and off-flavor in foods (Ivanova-Petropulos et al., 2015). Furthermore, secondary metabolites, peroxides, and lipid-free radicals can react with proteins and vitamins, severely decreasing nutritional value (Gardner, 1979).

Storage had a significant ($p < 0.05$) effect on the VC profile, leading to the disappearance of some initial volatiles and the subsequent formation of new ones, with significant changes in volatile composition in all treatments (Table 2). In general, the formation of VCs in pecans increases with storage at higher temperatures (Ribeiro, Ribeiro et al., 2020). Nonetheless, the results indicated a large formation of volatiles at lower temperatures, and no records were found regarding the use of high $CO_2$ in raw shelled pecan storage.

The VCs may be generated mainly from nut metabolism of amino acids, sugars, lipids, protein degradation, and the Maillard reaction that occurs mainly between amino acids and reducing sugars (Ghidurus et al., 2010; Valdés et al., 2015). Pecan nuts contain about 50–70% oil, with 90% UFAs. Oleic acid (18:1n-9) and linoleic acid (18:2n-6) make up major UFAs (Ribeiro, Klein et al., 2020; Salvador et al., 2016). 'Barton' pecan nuts (used in this study) are rich in UFAs (~91%, with 66.9% of C18:1n9, 22.5% of C18:2n6, and 1.0% of C18:3n3) (Ribeiro, Klein et al., 2020).

Oleic acid is a MUFA less susceptible to oxidation than linoleic acid that has two double bonds (Xu, 2017), although these double bonds and allyl groups, which are highly susceptible to free-radical reactions, undergo natural decomposition (oxidation) in the presence of oxygen, even at low temperatures. The aldehydes hexanal, octanal, nonanal, and 2,4-decadienal are characteristic products of the oxidation process of these aforementioned FAs. Hexanal and 2,4-decadienal are typically regarded as the oxidation products of linoleic acid but from different hydroperoxide sources. Nonanal and octanal originate from the autoxidation of oleic acid (Frankel 1983). Hexanal concentration is directly linked to rancidity and kernel quality (Bai et al., 2019; Bakkalbaşı et al., 2012; Ghirardello et al., 2016). Hexanal production in nuts is mainly influenced by temperature due to the secondary product of lipid oxidation from hydrogen peroxide breakdown (Arena et al., 2013; Jensen et al., 2001, 2003; Gama et al., 2020; Miraliakbari, & Shahidi, 2008). Unshelled pecan storage at 1.5 and 10 °C, when associated with 1 or 3 kPa $O_2$, were efficient in maintaining very low hexanal levels (Ribeiro, Ribeiro et al., 2020). However, in shelled pecan stored in ambient condition (17 ± 5 °C) during six months, the hexanal concentration was significantly higher than those unshelled, despite the 1-methylcyclopropene application reduced this aldehyde concentration (Thewes, Both, Thewes, Brackmann, Wagner et al., 2021). In the present study, hexanal was detected under all conditions and presented lower values at the initial time sample, indicating good nut quality and corroborating Erten and Cadwallader (2017). However, hexanal content increased with time at 10 °C and $pCO_2$-20. Interestingly, hexanal had higher values at the beginning and lower values at the end of storage in samples at AMB-20. This treatment showed evident contamination by fungi, which may affect VC composition.

2-Pentylfuran is an oxidation product generated from linoleic and other n-6 PUFAs (Valdés, 2015; Durmaz, & Gökmen, 2010). Fan (2015) reported that furans derived from FAs are formed even without heat treatment. Other VCs detected derived from PUFA degradation were ketones, 2-propanone being the most abundant, in treatments under ambient atmospheric conditions, followed by 2-pentanone and 2-heptanone in the AMB-10 treatment. Fungal activity can also induce ketone formation (Beier et al., 2014). Thus, a more significant presence of this class in AMB treatments corroborates Ribeiro et al. (2021), who showed lower fungal activity with CA-stored nuts.

Moreover, we observed that organic acids (e.g., C4-C10) tended to appear and/or increase with storage time, more notably at 10 °C. The $pCO_2$-10 treatment showed the highest values in the area from C8 to C10, however, compared with $pO_2$-10, only the use of low $O_2$ at this temperature maintained lower peak area values of this compounds. According to Xu (2017), organic acids may be produced through the oxidation of their corresponding aldehydes.

In one study, long-term storage of sweet almonds also increased lipid oxidation VCs, especially aldehydes, ketones, and alcohols (Mexis, & Kontominas, 2010). Generally, the main compounds that characterize nut odor are aldehydes (Erten, 2016). Benzaldehyde, which is also known as a primary component of almonds, is mainly found in raw samples, increasing with heat processing and contributing to a characteristic bitter almond taste and sweet aromatic flavor (Valdés, 2015). This VC was detected after the fourth month of storage, and according to Contis et al. (2002), it is common the amount increase during nuts storage.

Several alcohols were also identified, ethanol and 1-hexanol being the most abundant. The concentrations of most alcohols intensified during the storage process, mainly in $pCO_2$ treatments. At $pCO_2$-20, the alcohols hexanol, heptanol, octanol, and nonanol presented larger areas after six months and can be associated with floral, herbal, nutty, and oily odors, respectively. The majority of the alcohols detected may be formed by hydroperoxide decomposition of FAs or reducing aldehydes (Valdés, 2015; Scrimgeourn, 2005).

Methyl butanoate, ethyl butanoate, ethyl hexanoate, and 2-methyl-1-

butyl ethanoate tended to increase with storage time except for ethyl hexanoate, which decreased in the sixth month at AMB-20. The higher ester concentrations are seen positively as they enrich the aroma with more fruity notes. However, excessive amounts of ethyl acetate can be associated with fermentation processes (Both et al., 2017), and the CA conditions employed in this study showed that this ester was higher in $pCO_2$ at 20 and 10 °C and $pO_2$ at 10 °C.

Lastly, lipid oxidation is one of the main mechanisms that increase rancidness characteristics in pecans during storage, consequently forming characteristic VCs (Ribeiro, Ribeiro et al., 2020; Wang et al., 2017). Altogether, at the end of the six months, esters, lactones, unsaturated and saturated aldehydes (mainly hexanal), and limonene (terpene class) tended to increase. Nonetheless, higher temperatures generally stimulate the formation of VC, and in this study samples at 10 °C had higher levels of acids, hexanal, and other VCs such as ethanol, ethanal, and esters. On the other hand, nuts submitted to 20 °C showed higher values in the class of furans, terpenes, branched alcohols, and aliphatic alcohols.

## 5. Conclusions

Given our findings, the analyzed conditions for the storage of shelled pecan nuts indicate that lower temperatures (10 °C) remain the ideal and most effective strategy to maintain pecan quality attributes, resulting in lower AV, PV, and high L* and yellow color (b*) values. Therefore, the advantage of storing raw shelled nuts at low temperatures is the leading factor to extend their shelf life. Nonetheless, low $pO_2$ and high $pCO_2$ values better maintain nut quality than storage under normal $O_2$ conditions (20 kPa), even at temperatures above refrigeration. The present study corroborates data already published regarding the storage of unshelled pecans in controlled atmospheric conditions by the authors, thereby being a step forward in pecan nut storage techniques that require lower energy costs with refrigeration.

### *CRediT authorship contribution statement*

**Stephanie Reis Ribeiro:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Bruna Klein:** Formal analysis, Data curation, Writing – review & editing. **Ingrid Duarte dos Santos:** Formal analysis, Data curation, Writing – review & editing. **Flavio Roberto Thewes:** Formal analysis, Data curation. **Auri Brackmann:** Methodology, Writing – original draft, Writing – review & editing. **Vanderlei Both:** Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Roger Wagner:** Conceptualization, Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111498.

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# The influence of production units and seasons on the physicochemical characteristics, mineral and fatty acid content, and texture profile of the artisanal cheeses from Serra da Canastra, Brazil

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ABSTRACT

Canastra cheese is a product with an indication of origin and obtained from raw cow milk using endogenous culture (*pingo*). Since this type of cheese is considered one of the best-known and most consumed artisanal dairy products in Brazil, this study aimed to provide relevant information on its characteristics and evaluate the variability related to the production units and seasons. Thirty-six samples (ripened for 22 days) were produced in winter (n = 18) and summer (n = 18) and analyzed for proximate composition, pH, water activity, mineral content, fatty acid profile, fat health indicators, and texture parameters. Our findings revealed that the season and production units affected the composition and texture properties of the samples due to the fact that pH, Ca, Cu, K, and C4:0, C16:0, C14:1, C16:1, C18:1n-9 t, C18:1n-9c, C18:2n-6c levels were higher in cheese produced in summer, while C6:0, C10:0, C12:0, C14:0, and C18:0 content increased in winter samples. Fat content showed low atherogenic and thrombogenic indexs and high desirable hypocholesterolemic fatty acids, and the resilience was the only texture parameter that did not differ among the samples. The principal component analysis separated the cheese produced in different seasons, making this differentiation clear in all production units. The results provide information for consumers about the characteristics and nutritional composition of these products. In addition, they demonstrated that although it has produced in the same region, these cheeses are heterogeneous and have their peculiarities.

## 1. Introduction

Artisanal cheese is a dairy product produced on a small scale, manufactured on the farm itself, and employs traditional and rudimentary manufacturing techniques (de Jesús Ramírez-Rivera et al., 2018; Guerrero et al., 2009). This cheese class has significant historical, social, and cultural importance and contributes to the income of small producers (Margalho et al., 2020; Rodrigues et al., 2020; Kamimura et al., 2019). Each type of cheese has a distinct production process, and the coagulation time, ingredients, ripening time, among other factors, characterize the final product. According to Margalho et al. (2020), these variations among artisanal cheese varieties maintain their unique characteristics compared to industrialized products.

Minas Gerais is the most recognized Brazilian state in the matter of artisanal cheese production and diversity given its climate, native pastures, microrganisms and artisanal processes that give its products unique flavors (Martin et al., 2023; Pineda et al., 2021; Borelli et al., 2006). Products produced in Serra da Canastra stand out from other regions in terms of added value, indication of source, quality, special sensory characteristics, and are described as buttery with yellowish crusts and sweet flavor (Rodrigues et al., 2020). These features have been pivotal for Serra da Canastra cheese to reach a prominent position in the Brazilian market and be recognized in other countries, as some cheese varieties produced in this region have been awarded in national

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competitions, including Queijo Brasil, and international competitions such as the Mondial du Fromage et des Produits Laitiers in France (Campos et al., 2021).

Due to the growing appreciation and renown of artisanal products, research has been carried out in physicochemical (Andrade et al., 2022; Margalho et al., 2021; Borges et al., 2018; Oliveira et al., 2013; Nóbrega, 2012; Junior et al., 2009), sensory (Rodrigues et al., 2020; Bemfeito et al., 2016) and microbiological (Campos et al., 2021; Kamimura et al., 2020; Margalho et al., 2020) of cheeses produced in Serra da Canastra. Júnior et al. (2009) and Oliveira et al. (2013) focused on the physicochemical investigation of Canastra cheese and determined the proximate composition, while Nóbrega (2012) evaluated the visual characteristics, pH, acidity, moisture, chloride, and fat content of three samples. Borges et al. (2018) investigated the pH, water content, proteolysis, and texture parameters (firmness and fracturability) of cheese produced using three different *Cynara cardunculus* L. concentrations as milk coagulating agent, while Margalho et al. (2021) determined the crude composition and free fatty acid profile, and Andrade et al. (2022) analysed the mineral content.

A characterization is important to understand how seasonal and geographic factors impact the quality of Canastra cheeses (de Souza et al., 2021). A recent study by Martin et al. (2023), concluded that the mycobiota of these products differed according to farm and season and that these microrganisms are responsible for contributing to the peculiarities of the food matrix. Moreover, there is a scarcity of comprehensive research data on how aspects related to the environment and raw material can affect the profile of the final product, and this shows the importance of this work. Therefore, this is the first known study to describe the physical, physicochemical, and chemical aspects of Canastra cheeses involving numerous samples from different production units. Hence, we aimed to characterize cheeses produced in the Serra da Canastra region, identify the influence of the rainy (summer) and dry (winter) seasons and the different production units regarding the physicochemical properties, mineral content, fatty acid and texture profile, and determine the fat health indicators of these cheeses.

## 2. Materials and methods

### 2.1. Reagents and standard solutions

The reagents and solutions employed herein and their respective manufacturers are as follows: chloroform 100% (Synth, Diadema, Brazil); methanol 99.8% (JT Baker, Phillipsburg, USA); sodium sulfate 99%, hydrochloric acid 100%, and sodium hydroxide 100% (Exôdo, Sumaré, Brazil); hexane 98.5% (Fisher Scientific, Minnesota, USA); sodium chloride 100% (Synth, Diadema, Brazil); ethyl alcohol 99.5%, potassium hydroxide 98.5%, and sodium sulfate 100% (Vetec Química Fina, Duque de Caxias, Brazil); sulfuric acid 100% and boric acid solution 99% (Fmaia, Belo Horizonte, Brazil); nitric acid 65% (Merck, Darmstadt, Germany); boron trifluoride 96%, cesium chloride 98%, lanthanum 99%, fatty acid methyl esters standards (C4-C24) 95% and the internal standard (C23:0) 99% (Sigma-Aldrich, Burlington,USA); sodium, potassium, magnesium, manganese, iron, copper, and zinc standard solution 99.5% (Quemis, Joinville, Brazil).

### 2.2. Sample collection and preparation

Thirty-six samples from the Serra da Canastra region (Minas Gerais, Brazil) ripened for 22 days and produced in winter (n = 18) and summer (n = 18) were used. The basic steps of Canastra cheese production consisted of obtaining the milk, adding rennet and "*pingo*" (endogenous yeast), coagulation, curd cutting, stirring, draining, molding, pressing, and dry salting and ripening.

The farms (production units) were coded and characterized according to their location, herd, and animal feed provided in both seasons of the year in which the cheese was produced (Table 1). It was verified whether the climatic conditions of winter (June, July, and August of 2018) and summer (January, February, and March of 2019) in which the samples were produced did not follow an atypical behavior of the region. For this, the average temperature, relative humidity, and rainfall data were collected using the meteorological database of the Instituto Nacional de Meterologia (INMET) for 2015, 2016, 2017, 2018, and 2019 for comparison. No significant difference was found between the years for the variables investigated (data not shown).

The production units were selected according to facility characteristics, herd health, milking hygiene, good manufacturing practices, which represents the traditional cheese-making process, and being registered with the Associaç ão dos Produtores de Queijo Canastra (APROCAN). In addition, the production process needed to comply with the stage the establishes the hygienic-sanitary standards and good practices of artisanal Minas cheese production (IMA, 2006).

The cheeses (~1 kg) of each producer were directly collected from the production units and sent under refrigeration to the laboratories for analysis, and five cheese samples from each production unit were considered repetition. In order to avoid oxidation, the samples were prepared under refrigeration. The cheese was cut into 50 g portions using knives, vacuum packed in polyethylene bags, and stored at − 22 °C until analysis.

### 2.3. Physicochemical characterization

Water content was determined by weight loss in a vacuum oven (AOAC 925.09/17), ash by muffle furnace at 550 °C (AOAC 923.03), protein by the Kjeldahl method (AOAC 960.2), total fat according to Gerber's method (AOAC, 2006), and carbohydrates by difference. The pH was measured by the potentiometric method, and for this, grated cheese samples (10 g) were mixed with distilled water at 40 °C, homogenized, transferred to 100 mL volumetric flasks, diluted, and filtered (Jia et al., 2021). Water activity was determined using a thermo-hygrometer at 25 °C (Aqualab 4TE Series, Decagon Devices Inc., Pullman, WA, USA). Color analyses were performed with a colorimeter (CM-5, Konica Minolta, Tokyo, Japan) to obtain the coordinates L* , a* , b* , chroma (C), and hue angle (h). The color was measured directly on the surface of a standard amount of cheese, and five measurements were made for each sample in all trials (Aktypis et al., 2018).

### 2.4. Mineral content determination

The calcium, magnesium, iron, zinc, potassium, copper, manganese, and sodium contents were determined according to Neves et al. (2019) and using a Perkin-Elmer spectrometer (AAnalyst-200, USA) in flame atomic emission mode for Na and K and atomic absorption mode for the other minerals. A freshly cheese sample (0.5 g) was placed in nitric acid (6 mL) and left at room temperature overnight for mineralization. Subsequently, the tubes were left for 2 h at 110 °C in a digester block (MA-4025, Marconi, Brazil). Then, nitric acid (2 mL) and 30% hydrogen peroxide (2 mL) were added to the tubes and heated once again at 130 °C for 2 h. The digested extract was transferred to a volumetric flask, and the volume was measured up to 25 mL with ultrapure water (Arium Comfort, Sartorius, Germany), followed by filtration on ash-free filter paper (Nalgon, 9 cm in diameter). Samples were diluted as necessary to fit the linear range of each mineral. Lanthanum (0.5%) was added for Ca and Mg analyses, and cesium chloride (0.1%) was used for K and Na. Quantification was performed using external calibration.

### 2.5. Fatty acid composition

The fatty acid profile was obtained by extracting the lipids according to Bligh and Dyer (1959) and esterified in line with Joseph and Ackman (1992). The chromatographic conditions were based on Ballus et al. (2014), with modifications. Methyl ester separation was performed on a gas chromatograph (GC; 7890 A, Agilent Technologies, Germany)

**Table 1**
Characterization of the cheese production units (farms) regarding the cow breed used to obtain the milk, season of the year the cheese was produced, and the feed given to the animals.

| Farm | Cow breed | Season | Diet |
|---|---|---|---|
| F1[a] | Jersey | Summer | Pasture and feed only at milking (soybean meal and corn) |
| | | Winter | Corn silage, mineral salt, soybean bran, and pasture |
| F2[b] | Girolando and Jersolando | Summer | Pasture and mineral salt |
| | | Winter | Corn silage |
| F3[a] | Girolando and Jersolando | Summer | Pasture, mineral salt, and feed |
| | | Winter | Corn silage, feed (corn and soybean), and pasture |
| F4[a] | Jersey and Caracu | Summer | Pasture and feed only at milking time (soybean and wheat bran) |
| | | Winter | Corn silage and feed (soybean, cottonseed, wheat bran, ground corn, mineral supplement, and probiotics) |
| F5[a] | Caracu and Girolando | Summer | Pasture, mineral salt, and feed (cotton bran, corn, wheat, soybean, and mineral supplement) |
| | | Winter | Pasture, corn silage, and feed (soybean meal and corn) |
| F6[a] | Dutch and Jersolando | Summer | Pasture |
| | | Winter | Corn silage and feed (corn and soybean) |
| F7[a] | Girolando, Caracu, Jersey, and Dutch | Summer | Pasture and feed only at milking (corn and soybean) |
| | | Winter | Corn silage |
| F8[a] | Dutch and Jersolando | Summer | Pasture, feed (soybean meal, wheat, and ground corn), and mineral salt |
| | | Winter | Corn silage, pasture, and feed (soybean, corn, cottonseed, and wheat and urea) |
| F9[a] | Jersey, Holstein, and Girolando | Summer | Pasture and feed (wheat bran and soybean meal) |
| | | Winter | Corn silage |
| F10[b] | Caracu and Girolando | Summer | Pasture and feed (corn and soybean meal) |
| | | Winter | Corn silage, feed (corn and soybean), sugarcane, and pasture |
| F11[a] | Girolando | Summer | Pasture and feed at milking (corn and soybean meal, mineral supplement, and bicarbonate) |
| | | Winter | Corn silage and feed (corn and soybean) |
| F12[a] | Girolando | Summer | Pasture and feed (corn bran, cotton bran, soybean meal, and mineral supplement) |
| | | Winter | Feed (corn and soybean), corn silage, and pasture |
| F13[a] | Caracu, Girolando, and Jersolando | Summer | Pasture and feed (corn and soybean) |
| | | Winter | Corn silage and pasture |
| F14[a] | Girolanda | Summer | Pasture and feed (corn, soybean, mineral salt, and wheat bran) |
| | | Winter | Feed (soybean and corn), corn silage, and pasture |
| F15[a] | Girolando and Caracu | Summer | Pasture and feed (corn, soybean, mineral salt, and wheat bran) |
| | | Winter | Corn silage, pasture, and feed (corn, soybean, and mineral salt) |
| F16[a] | Dutch | Summer | Pasture and feed (soybean and cotton bran, whole ground corn, and calcite limestone) |
| | | Winter | Corn silage and feed (wheat and soybean) |
| F17[c] | Girolando and Jersey | Summer | Pasture, mineral salt, and feed (corn and soybean bran, and mineral supplement) |
| | | Winter | Corn silage and feed (wheat and soybean) |
| F18[a] | Dutch, Girolando, and Jersey | Summer | Pasture and feed (wheat, cotton, and corn bran) |
| | | Winter | Corn silage, sugarcane, and feed (soybean meal and corn) |

[a]Farms located in the city of São Roque de Minas.
[b]Farms located in the city of Delfinópolis.
[c]Farm located in the city of Tapiraí .
The pasture is composed of *Brachiaria* (*Brachiaria*), Mombasa (*Panicum maximum* Jaqc. cv. Mombasa), and/or Tanzania grass (*Panicum maximum* cv. Tanzania-1).

equipped with a flame ionization detector (FID). The methyl esters were separated using a DB-23 capillary column (60 m × 0.25 mm id; 0.25-μm thickness film; Agilent Technologies, USA). An aliquot of the extract (1 μL) was introduced in a split/splitless injector operating in split mode (1:50). Injector and detector temperatures were maintained at 250 and 280 °C, respectively. The column oven temperature was 50 °C (5 min isotherm), increasing to 175 at 25 °C $min^{-1}$ until 230 °C at 4 °C $min^{-1}$ and maintained for 25 min. The flow rate of the carrier gas ($N_2$) was 1 mL $min^{-1}$, and the flow rate of the detector gases ($N_2$:$H_2$:synthetic air) were 30, 30, and 300 mL $min^{-1}$, respectively. The methyl esters were identified by comparing their retention times with those obtained with the standards (FAME mix C4-C24) under the same chromatographic conditions. Quantification was performed by internal standardization using C23:0 as internal standard. Correction factors and fatty acid concentration (mg 100 $g^{-1}$ fat) were calculated according to Joseph and Ackman (1992).

The Atherogenic Index (AI) and Thrombogenic Index (TI) proposed by Batista et al. (2017) and Desirable Hypocholesterolemic Fatty acids (DHFA) according to Barlowska et al. (2018) were calculated according to Eqs. 1, 2, and 3.

$$AI = (C12{:}0 + 4 \times C14{:}0 + C16{:}0) / [\sum MUFA + \sum PUFA\ (n\text{-}6) \text{ and } (n\text{-}3)] \quad (1)$$

$$TI = (C14{:}0 + C16{:}0 + C18{:}0) / [0{\cdot}5 \times \sum MUFA + 0{\cdot}5 \times \sum PUFA\ (n\text{-}6) + 3 \times \sum PUFA\ (n\text{-}3) + (n\text{-}3) / (n\text{-}6)] \quad (2)$$

$$DHFA: MUFA + PUFA + C18{:}0 \quad (3)$$

MUFA are monounsaturated fatty acids, and PUFA are polyunsaturated fatty acids.

### 2.6. Texture profile analysis

The cheese was evaluated for the following texture parameters: elasticity, hardness, adhesiveness, cohesiveness, gumminess, chewiness, and resilience using a texture analyzer (CT3, Brookfield, São Paulo, Brazil). The analyzer was equipped with a 10 kg load cell and TA44 probe. Several test conditions were defined, including pre-test speed (2 mm $s^{-1}$), test speed (1 mm $s^{-1}$), and post-test speed (1 mm $s^{-1}$). Three holes were drilled in different regions of each cheese sample analyzed (n = 3). The samples had a triangular form and dimensions approximately 4 cm height x 8 cm width.

### 2.7. Statistical analysis

All analyses were performed in triplicate, and the results were evaluated by analysis of variance (ANOVA) and Tukey's test at 5% significance using the SISVAR software (version 5.6). The effects of two factors on the response variables were investigated: production units and seasons. Principal component analysis (PCA) was performed using MatLab R2019a (MathWorks, USA) with PLS-toolbox (version 8.6, Eigenvector Research Inc, 2010). Chemometric analysis was carried out by processing the data by auto-scaling to give the same importance to all variables with different magnitudes.

## 3. Results and discussion

### 3.1. Physicochemical characterization

The mean values of pH, water activity, proximate composition, and colorimetric parameters of the cheese were significantly influenced ($p < 0.05$) by the production unit and season (winter and summer) in which they were produced (Table 2). This may be because of the following factors: location (Vatavali et al., 2020), cattle breed (Hayaloglu et al., 2013), agroclimatic conditions (Vatavali et al., 2020; Gatzias et al., 2020), season (Junior et al., 2009), feed type (O'Callaghan et al., 2017; Morand-Fehr et al., 2007), milking season (Vatavali et al., 2020; Gatzias et al., 2020), local pasture (Morand-Fehr et al., 2007), milk microbial composition (Campos et al., 2021; Gori et al., 2012), "*pingo*" (Kamimura et al., 2020), cheese ripening conditions, among other variables.

The pH is a critical parameter because it can causes changes in other chemical properties of cheese and influence the microbiological quality and safety of the final product (Pastorino et al., 2003). For the Canastra cheese analyzed, pH values ranged from 5.03 to 5.65, and most of the summer samples showed higher values than the winter ones (Table 2). These findings corroborate Campos et al. (2021) (pH 5.46–5.54) and Borges et al. (2018) (pH 5.40) and differ from Nóbrega (2012) (pH 4.77), who also investigated ripened cheese (~22 days) produced in the Canastra region. Differences in the pH values may have been caused by the distinct composition of lactic acid bacteria present in the "*pingo*," the raw materials, and lactose levels.

The water activity and water content of the cheeses varied from 0.90 to 0.98 and 28.08–45.23%, respectively (Table 2). This oscillation is expected since the ripening temperature (room temperature) is not controlled, in addition to being different throughout the seasons of the year (Nóbrega et al., 2012), consequently interfering in water evaporating from the cheese to the environment. According to Brazilian Legislation, 44.5% of the investigated samples are classified as low moisture cheese (hard cheese) and 55.5% as medium moisture cheese (semi-hard cheese) (Brazil, 1996). Moreover, according to state legislation on Minas artisanal cheese production, all samples conform to moisture content (≤45.9%). Campos et al. (2021) reported mean water activity values between 0.90 and 0.91 in cheese produced in Serra da Canastra, which is also within the range observed herein. Regarding water content, Nóbrega et al. (2012) noted 52% in samples produced in the winter and 38.8% in the summer samples, while Borges et al. (2018), Oliveira et al. (2013), Resende (2010), and Junior (2009) obtained mean values of 20.72%, 44.90%, 42.39%, and 39.22%, respectively, in Canastra cheese.

The ash content (2.53–5.63%), proteins (22.9–33.6%), fat (22.63–41.84%), and carbohydrates (0.30–2.87%) of the analyzed cheese are listed in Table 2. The mean values obtained by Margalho et al. (2021) for Serra da Canastra cheese are similar to the data proposed herein (4.00% ash; 23.70% proteins, and 27.00% fat) and diverge in terms of carbohydrate values (3.57%). Moreover, the data by Oliveira et al. (2013) of Canastra cheese is within the range obtained by our study for lipids (overall mean of 23.62%) and lower for proteins (overall mean of 18.51%); nonetheless, this study had a smaller sample universe (4 production units). When evaluating the levels of these components, the differences between the cheese produced in the different production units and seasons of the year (winter and summer), for most samples, can be explained by the effects of the intrinsic and extrinsic factors mentioned above.

Machado (2002) concluded that water, protein, and fat levels are directly affected by pressing and how the cheese is handled. Severely shaking the cheese may cause structure breakage, releasing lipids, proteins, and other substances that will be consequently lost during drying. The force applied during pressing also influences the compaction of the mass, resulting in the production of cheese with different levels of these macromolecules. Unlike this study, Resende (2010) did not find any significant influence ($p > 0.05$) for the fat content of Canastra cheese of different production units and reported a mean of 28.5%. However, Nóbrega et al. (2009) observed variations between Canastra cheese produced in winter and summer for ash (3.6% and 4.6%), protein (19.9% and 22.3%), and fat (21.8% and 27.5%) contents.

The variable L* represents the luminosity of the samples and presented values between 70.40 and 82.49 (Table 2), thus having a lighter coloration. As for a* , which varies between red/green, and b* , between yellow/blue, the values ranged from 1.32 to 8.11 and 17.28–29.53 (Table 2), respectively, showing that the samples are closer to red and yellow tones. These data are consistent considering that some types of cheese analyzed presented a reddish-orange color on their surface, which is likely due to pigments synthesized by fungi. The measured values corroborate the evidence presented by Rodrigues et al. (2020), as the Canastra cheese was characterized with yellowish crusts. A correlation between fat and b* was observed in all samples (Table 2), meaning that the higher the fat content, the higher the positive b* values. Furthermore, it is likely that the higher b* coordinate values are related to the high content of liposoluble carotenoids present in the pasture, which is transferred to the milk. (Di Grigoli et al., 2022). Color saturation (C) and tone angle (h) were also determined and corresponded to means between 17.34 and 30.66 and 73.11 and 86.44, respectively.

### 3.2. Mineral content

There were differences ($p < 0.05$) in mineral levels between the winter and summer cheese and samples produced in different production units. Among the eight minerals evaluated, sodium, calcium, potassium, and magnesium were the most abundant, while iron and manganese content presented lower concentrations than the quantification limit in all samples, and it was only possible to quantify copper in the summer samples. The Ca, Mg, Zn, K, Na, and Cu levels ranged from 4117.32 to 9647.94 mg $kg^{-1}$, 230.70–481.49 mg $kg^{-1}$, 20.25–41.68 mg $kg^{-1}$, 307.93–1283.85 mg $kg^{-1}$, 2355.80–11832.11 mg $kg^{-1}$, and 0.60–2.21 mg $kg^{-1}$, respectively (Table 3).

Barbara et al.(2022) when evaluating Canastra cheeses from different locations, found these minerals in some samples in concentrations higher than those obtained in this study: calcium (8400–72900 mg $kg^{-1}$), magnesium (3.0–2750 mg $kg^{-1}$), zinc (44.43–178.70 mg $kg^{-1}$), potassium (1700–11680 mg $kg^{-1}$), sodium (9770–96980 mg $kg^{-1}$), manganese (0.001–2.33 mg $kg^{-1}$) and copper (2.05–10.79 mg $kg^{-1}$).

Matera et al. (2018) analyzed the mineral content of typical Brazilian cheese varieties. The authors reported that the mean levels of calcium, magnesium, zinc, and sodium of coalho (6548; 293; 43; 8279 mg $kg^{-1}$), prato (6821; 268; 42; 4339 mg $kg^{-1}$), minas padrão (6779; 271; 40; 4655 mg $kg^{-1}$), and minas frescal cheese (5685; 246; 39; 2749 mg $kg^{-1}$), and potassium of prato (471 mg $kg^{-1}$), minas padrão (642 mg $kg^{-1}$), and minas frescal cheese (764 mg $kg^{-1}$) were similar to Canastra cheese. Nonetheless, higher potassium concentrations were found in Canastra cheese (307.93–1283.85 mg $kg^{-1}$) compared to coalho cheese (0.51 mg $kg^{-1}$).

Based on the results obtained, the estimated contribution of Ca, Mg, Zn, and Cu for the Recommended Daily Intake (RDI) established by ANVISA (2005) was determined. A 30 g portion of cheese and approximate RDI for Ca (1000 mg), Mg (260 mg), Zn (6 mg), and Cu (0.9 mg) were considered (ANVISA, 2003). Given the lowest and highest level of each mineral in the different samples, the intake of one portion of cheese will contribute with 17.65–26.96% of Ca, 2.93–4.52% of Mg, 9.70–15.17% of Zn, and 2.00–4.00% of Cu of the RDI.

The World Health Organization established that ≤ 2000 mg of sodium and at least 3510 mg of potassium should be consumed per day (WHO, 2012). Individuals with high sodium and low potassium levels may be at risk for high blood pressure, which raises the likelihood of heart disease and stroke. In this sense, Canastra cheese provides 113.17–288.49 mg of sodium (daily value of 5.66–14.42%) and 18.92–34.05 mg of potassium (0.54–0.97% of the RDI), and according to

**Table 2**
Physicochemical characterization of cheese produced in the Canastra region in different production units in winter and summer.

| Samples | Season | pH | Water activity | Water content (%) | Ash (%) | Proteins (%) | Fat (%) | Carbohydrates (%) | L* | a* | b* | C | h |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F1 | Winter | 5.06 ± 0.01$^{a}$ | 0.94 ± 0.01$^{a}$ | 30.82 ± 0.43$^{a}$ | 5.38 ± 0.12$^{a}$ | 30.21 ± 0.26$^{a}$ | 31.00 ± 5.07$^{a}$ | 2.59 ± 0.14$^{a}$ | 76.07 ± 0.06$^{a}$ | 2.44 ± 0.02$^{a}$ | 24.23 ± 0.06$^{b}$ | 24.35 ± 0.06$^{b}$ | 84.27 ± 0.03$^{a}$ |
| | Summer | 5.23 ± 0.04$^{a}$ | 0.94 ± 0.01$^{a}$ | 31.57 ± 0.51$^{a}$ | 4.93 ± 0.03$^{b}$ | 28.66 ± 1.77$^{a}$ | 33.76 ± 1.18$^{a}$ | 1.08 ± 0.09$^{b}$ | 75.14 ± 0.18$^{b}$ | 2.37 ± 0.05$^{a}$ | 25.06 ± 0.10$^{a}$ | 25.99 ± 0.12$^{a}$ | 86.13 ± 0.10$^{b}$ |
| | *Mean* | *5.15 ± 0.11$^{C}$* | *0.94 ± 0.01$^{C}$* | *31.20 ± 0.59$^{DC}$* | *5.16 ± 0.26$^{AB}$* | *29.44 ± 1.40$^{AB}$* | *32.34 ± 2.17$^{AB}$* | *1.84 ± 0.10$^{C}$* | *75.60 ± 0.53$^{BC}$* | *2.40 ± 0.05$^{J}$* | *24.65 ± 0.46$^{ABC}$* | *25.17 ± 0.90$^{B}$* | *85.20 ± 1.20$^{A}$* |
| F2 | Winter | 5.14 ± 0.02$^{b}$ | 0.94 ± 0.01$^{a}$ | 28.23 ± 0.28$^{b}$ | 3.78 ± 0.29$^{a}$ | 29.48 ± 0.77$^{a}$ | 36.60 ± 1.11$^{a}$ | 1.91 ± 0.04$^{a}$ | 73.74 ± 0.15$^{b}$ | 7.71 ± 0.03$^{b}$ | 29.53 ± 0.08$^{a}$ | 30.66 ± 0.29$^{a}$ | 75.28 ± 0.13$^{a}$ |
| | Summer | 5.36 ± 0.04$^{a}$ | 0.95 ± 0.01$^{a}$ | 39.84 ± 1.76$^{a}$ | 2.96 ± 0.46$^{b}$ | 26.85 ± 0.37$^{a}$ | 29.52 ± 5.71$^{b}$ | 0.83 ± 0.08$^{b}$ | 76.99 ± 0.38$^{a}$ | 8.11 ± 0.11$^{a}$ | 26.46 ± 0.45$^{b}$ | 28.52 ± 0.43$^{b}$ | 73.11 ± 0.22$^{b}$ |
| | *Mean* | *5.25 ± 0.12$^{BC}$* | *0.95 ± 0.01$^{C}$* | *34.03 ± 6.46$^{BCD}$* | *3.37 ± 0.54$^{FGH}$* | *28.17 ± 2.84$^{ABC}$* | *33.08 ± 4.56$^{AB}$* | *1.42 ± 0.06$^{D}$* | *75.37 ± 1.80$^{BCD}$* | *7.92 ± 0.23$^{A}$* | *28.00 ± 1.70$^{A}$* | *29.88 ± 1.21$^{A}$* | *74.19 ± 1.19$^{H}$* |
| F3 | Winter | 5.07 ± 0.0$^{b}$ | 0.96 ± 0.01$^{a}$ | 41.21 ± 0.98$^{a}$ | 5.41 ± 0.57$^{a}$ | 24.73 ± 0.13$^{a}$ | 26.27 ± 0.39$^{b}$ | 2.38 ± 0.56$^{a}$ | 78.72 ± 0.43$^{a}$ | 6.55 ± 0.38$^{a}$ | 25.67 ± 0.24$^{b}$ | 26.79 ± 0.16$^{b}$ | 78.33 ± 0.36$^{b}$ |
| | Summer | 5.53 ± 0.03$^{a}$ | 0.95 ± 0.01$^{a}$ | 38.51 ± 0.37$^{b}$ | 3.83 ± 0.28$^{b}$ | 25.14 ± 0.73$^{a}$ | 31.58 ± 0.01$^{a}$ | 0.94 + 02$^{b}$ | 75.58 ± 0.35$^{b}$ | 4.71 ± 0.26$^{b}$ | 27.86 ± 0.07$^{a}$ | 28.97 ± 0.08$^{a}$ | 81.01 ± 0.98$^{a}$ |
| | *Mean* | *5.32 ± 0.27$^{ABC}$* | *0.96 ± 0.01$^{B}$* | *39.86 ± 1.62$^{AB}$* | *4.62 ± 0.96$^{ABCD}$* | *24.95 ± 0.51$^{C}$* | *28.93 ± 2.91$^{BC}$* | *1.66 ± 0.37$^{E}$* | *77.15 ± 1.76$^{B}$* | *5.63 ± 0.94$^{B}$* | *26.77 ± 1.20$^{AB}$* | *27.88 ± 1.19$^{AB}$* | *79.67 ± 1.60$^{DE}$* |
| F4 | Winter | 5.06 ± 0.04$^{a}$ | 0.95 ± 0.01$^{a}$ | 37.09 ± 0.10$^{a}$ | 3.91 ± 0.08$^{a}$ | 25.09 ± 0.53$^{b}$ | 31.73 ± 2.13$^{a}$ | 2.18 ± 0.04$^{a}$ | 75.33 ± 0.55$^{a}$ | 5.37 ± 0.05$^{b}$ | 25.63 ± 0.10$^{a}$ | 26.23 ± 0.07$^{a}$ | 78.13 ± 0.05$^{a}$ |
| | Summer | 5.14 ± 0.04$^{a}$ | 0.95 ± 0.01$^{a}$ | 38.59 ± 0.98$^{a}$ | 4.26 ± 0.31$^{a}$ | 25.95 ± 0.14$^{a}$ | 30.90 ± 3.83$^{a}$ | 0.30 ± 0.02$^{b}$ | 74.93 ± 0.06$^{a}$ | 5.88 ± 0.06$^{a}$ | 25.40 ± 0.06$^{b}$ | 26.09 ± 0.05$^{b}$ | 77.57 ± 0.24$^{b}$ |
| | *Mean* | *5.10 ± 0.05$^{C}$* | *0.95 ± 0.01$^{C}$* | *37.84 ± 1.03$^{ABC}$* | *4.08 ± 0.28$^{CDEFG}$* | *25.52 ± 0.48$^{C}$* | *31.31 ± 2.80$^{ABC}$* | *1.24 ± 0.04$^{E}$* | *75.13 ± 0.42$^{BCDE}$* | *5.62 ± 0.27$^{B}$* | *25.51 ± 0.15$^{ABC}$* | *26.16 ± 0.09$^{AB}$* | *77.85 ± 0.34$^{FG}$* |
| F5 | Winter | 5.03 ± 0.02$^{b}$ | 0.96 ± 0.01$^{a}$ | 38.51 ± 0.83$^{a}$ | 2.90 ± 0.47$^{b}$ | 29.01 ± 0.29$^{b}$ | 29.16 ± 4.36$^{a}$ | 0.42 ± 0.01$^{b}$ | 74.30 ± 0.21$^{a}$ | 3.96 ± 0.03$^{a}$ | 27.28 ± 0.05$^{b}$ | 27.56 ± 0.06$^{b}$ | 81.72 ± 0.06$^{b}$ |
| | Summer | 5.41 ± 0.04$^{a}$ | 0.95 ± 0.01$^{a}$ | 31.58 ± 0.61$^{b}$ | 5.08 ± 0.27$^{a}$ | 29.84 ± 0.27$^{a}$ | 31.04 ± 2.47$^{a}$ | 2.46 ± 0.08$^{a}$ | 73.61 ± 0.30$^{b}$ | 3.80 ± 0.11$^{a}$ | 28.20 ± 0.11$^{a}$ | 28.47 ± 0.16$^{a}$ | 82.08 ± 0.03$^{a}$ |
| | *Mean* | *5.22 ± 0.21$^{BC}$* | *0.96 ± 0.01$^{B}$* | *35.05 ± 3.85$^{BCD}$* | *3.49 ± 0.73$^{FGH}$* | *29.43 ± 0.50$^{AB}$* | *30.10 ± 3.00$^{BC}$* | *1.44 ± 0.06$^{E}$* | *73.95 ± 0.44$^{CDEF}$* | *3.88 ± 0.11$^{H}$* | *27.74 ± 0.51$^{A}$* | *28.02 ± 0.50$^{AB}$* | *81.90 ± 0.20$^{BC}$* |
| F6 | Winter | 5.22 ± 0.03$^{b}$ | 0.96 ± 0.01$^{a}$ | 37.88 ± 1.21$^{a}$ | 3.59 ± 0.54$^{a}$ | 28.85 ± 1.24$^{a}$ | 26.81 ± 0.59$^{a}$ | 2.87 ± 0.13$^{a}$ | 74.64 ± 0.21$^{a}$ | 3.04 ± 0.01$^{b}$ | 21.57 ± 0.15$^{b}$ | 21.78 ± 0.15$^{b}$ | 81.98 ± 0.04$^{b}$ |
| | Summer | 5.32 ± 0.02$^{a}$ | 0.96 ± 0.01$^{a}$ | 38.88 ± 1.09$^{a}$ | 3.94 ± 0.55$^{a}$ | 24.58 ± 0.32$^{b}$ | 31.77 ± 4.79$^{a}$ | 0.83 ± 0.02$^{b}$ | 72.44 ± 0.35$^{b}$ | 3.33 ± 0.11$^{a}$ | 22.99 ± 0.14$^{a}$ | 22.17 ± 0.04$^{a}$ | 83.09 ± 0.04$^{a}$ |
| | *Mean* | *5.27 ± 0.05$^{BC}$* | *0.96 ± 0.01$^{B}$* | *38.38 ± 1.17$^{ABC}$* | *3.77 ± 0.52$^{DEFG}$* | *26.72 ± 2.46$^{BC}$* | *29.29 ± 4.08$^{BC}$* | *1.85 ± 0.10$^{C}$* | *73.54 ± 1.23$^{CDEF}$* | *3.19 ± 0.17$^{I}$* | *22.28 ± 0.79$^{B}$* | *21.98 ± 0.24$^{C}$* | *82.53 ± 0.60$^{B}$* |
| F7 | Winter | 5.44 ± 0.02$^{b}$ | 0.98 ± 0.01$^{a}$ | 45.23 ± 1.41$^{a}$ | 4.45 ± 0.44$^{a}$ | 26.17 ± 1.56$^{a}$ | 22.63 ± 0.60$^{b}$ | 1.52 ± 0.01$^{b}$ | 82.49 ± 0.42$^{a}$ | 1.41 ± 0.01$^{a}$ | 17.28 ± 0.03$^{b}$ | 17.34 ± 0.02$^{b}$ | 85.28 ± 0.02$^{b}$ |
| | Summer | 5.65 ± 0.07$^{a}$ | 0.96 ± 0.01$^{b}$ | 39.73 ± 1.25$^{b}$ | 2.61 ± 0.27$^{b}$ | 28.39 ± 0.64$^{a}$ | 27.72 ± 4.22$^{a}$ | 1.55 ± 0.04$^{a}$ | 79.23 ± 0.09$^{b}$ | 1.32 ± 0.07$^{a}$ | 18.43 ± 2.27$^{a}$ | 18.34 ± 0.47$^{a}$ | 86.44 ± 0.21$^{a}$ |
| | *Mean* | *5.54 ± 0.12$^{A}$* | *0.97 ± 0.01$^{A}$* | *44.48 ± 5.33$^{A}$* | *2.53 ± 0.34$^{H}$* | *26.78 ± 2.45$^{BC}$* | *25.18 ± 3.17$^{C}$* | *1.04 ± 0.02$^{F}$* | *80.86 ± 1.80$^{A}$* | *1.37 ± 0.07$^{K}$* | *17.86 ± 0.65$^{D}$* | *17.84 ± 0.62$^{C}$* | *85.86 ± 0.64$^{A}$* |
| F8 | Winter | 5.19 ± 0.03$^{b}$ | 0.96 ± 0.01$^{a}$ | 43.98 ± 0.38$^{a}$ | 4.12 ± 0.25$^{a}$ | 26.15 ± 1.74$^{a}$ | 23.56 ± 1.07$^{a}$ | 2.19 ± 0.09$^{a}$ | 74.75 ± 0.30$^{a}$ | 4.02 ± 0.01$^{a}$ | 25.05 ± 0.04$^{b}$ | 25.29 ± 0.16$^{a}$ | 80.92 ± 0.08$^{b}$ |
| | Summer | 5.42 ± 0.05$^{a}$ | 0.94 ± 0.01$^{b}$ | 35.63 ± 1.04$^{b}$ | 4.92 ± 0.47$^{a}$ | 26.77 ± 0.32$^{a}$ | 31.79 ± 3.93$^{a}$ | 0.89 ± 0.01$^{b}$ | 71.52 ± 0.32$^{b}$ | 4.07 ± 0.05$^{a}$ | 27.05 ± 0.14$^{a}$ | 25.16 ± 0.93$^{b}$ | 82.60 ± 0.35$^{a}$ |
| | *Mean* | *5.30 ± 0.13$^{ABC}$* | *0.95 ± 0.01$^{C}$* | *39.81 ± 4.63$^{AB}$* | *4.52 ± 0.55$^{ABCDE}$* | *26.46 ± 1.12$^{BC}$* | *27.67 ± 7.55$^{BC}$* | *1.54 ± 0.05$^{D}$* | *73.14 ± 1.80$^{DEF}$* | *4.04 ± 0.04$^{GH}$* | *26.05 ± 1.10$^{BC}$* | *26.22 ± 1.05$^{AB}$* | *81.76 ± 0.94$^{BC}$* |
| F9 | Winter | 5.04 ± 0.05$^{b}$ | 0.90 ± 0.01$^{b}$ | 29.48 ± 0.39$^{a}$ | 5.00 ± 0.25$^{b}$ | 33.60 ± 0.62$^{a}$ | 29.93 ± 2.70$^{a}$ | 1.99 ± 0.03$^{a}$ | 74.33 ± 0.05$^{a}$ | 4.63 ± 0.02$^{a}$ | 24.84 ± 0.04$^{a}$ | 25.26 ± 0.04$^{b}$ | 79.36 ± 0.01$^{b}$ |
| | Summer | 5.33 ± 0.06$^{a}$ | 0.92 ± 0.01$^{a}$ | 31.09 ± 1.01$^{a}$ | 5.63 ± 0.32$^{a}$ | 30.54 ± 0.20$^{b}$ | 30.53 ± 1.16$^{a}$ | 2.21 ± 0.29$^{a}$ | 74.00 ± 0.12$^{b}$ | 4.30 ± 0.06$^{b}$ | 24.99 ± 0.90$^{a}$ | 25.88 ± 0.09$^{a}$ | 80.26 ± 0.36$^{a}$ |
| | *Mean* | *5.18 ± 0.16$^{C}$* | *0.91 ± 0.01$^{E}$* | *30.28 ± 1.18$^{D}$* | *5.32 ± 0.42$^{A}$* | *32.07 ± 1.70$^{A}$* | *30.23 ± 1.34$^{BC}$* | *2.10 ± 0.16$^{B}$* | *74.16 ± 0.20$^{CDEF}$* | *4.47 ± 0.18$^{EFGH}$* | *24.91 ± 0.11$^{ABC}$* | *25.57 ± 0.34$^{AB}$* | *79.81 ± 0.54$^{D}$* |
| F10 | Winter | 5.21 ± 0.04$^{b}$ | 0.95 ± 0.01$^{a}$ | 37.23 ± 1.20$^{b}$ | 4.11 ± 0.29$^{a}$ | 26.74 ± 0.61$^{a}$ | 29.44 ± 2.54$^{a}$ | 2.48 ± 0.23$^{a}$ | 74.12 ± 0.11$^{b}$ | 5.01 ± 0.04$^{a}$ | 25.00 ± 0.04$^{a}$ | 26.07 ± 0.11$^{a}$ | 79.85 ± 0.05$^{a}$ |
| | Summer | 5.41 ± 0.03$^{a}$ | 0.96 ± 0.01$^{a}$ | 40.66 ± 0.96$^{a}$ | 4.39 ± 0.08$^{a}$ | 25.48 ± 0.13$^{b}$ | 29.13 ± 0.65$^{a}$ | 0.34 ± 0.01$^{b}$ | 74.97 ± 0.08$^{a}$ | 4.33 ± 0.10$^{b}$ | 24.01 ± 0.04$^{b}$ | 25.51 ± 0.07$^{b}$ | 78.95 ± 0.24$^{b}$ |
| | *Mean* | *5.31 ± 0.11$^{ABC}$* | *0.96 ± 0.01$^{B}$* | *38.95 ± 2.07$^{AB}$* | *4.25 ± 0.25$^{BCDEF}$* | *26.11 ± 0.77$^{BC}$* | *29.28 ± 0.68$^{BC}$* | *1.41 ± 0.17$^{E}$* | *74.55 ± 0.48$^{CDE}$* | *4.67 ± 0.38$^{DEFG}$* | *24.51 ± 0.54$^{ABC}$* | *25.79 ± 0.32$^{AB}$* | *79.40 ± 0.52$^{DEF}$* |
| F11 | Winter | 5.19 ± 0.04$^{b}$ | 0.93 ± 0.01$^{a}$ | 31.85 ± 0.25$^{a}$ | 3.29 ± 0.32$^{a}$ | 29.06 ± 0.57$^{a}$ | 33.40 ± 0.79$^{b}$ | 2.40 ± 0.26$^{a}$ | 73.42 ± 0.31$^{a}$ | 4.17 ± 0.05$^{b}$ | 26.29 ± 0.07$^{b}$ | 26.33 ± 0.10$^{b}$ | 80.17 ± 0.05$^{a}$ |
| | Summer | 5.34 ± 0.04$^{a}$ | 0.93 ± 0.01$^{a}$ | 30.90 ± 0.77$^{a}$ | 3.09 ± 0.24$^{a}$ | 22.90 ± 0.12$^{b}$ | 41.84 ± 1.29$^{a}$ | 1.27 + 0.26$^{b}$ | 72.08 ± 0.06$^{b}$ | 4.44 ± 0.09$^{a}$ | 26.86 ± 0.10$^{a}$ | 27.11 ± 0.12$^{a}$ | 80.73 ± 0.39$^{a}$ |
| | *Mean* | *5.31 ± 0.11$^{BC}$* | *0.93 ± 0.01$^{D}$* | *31.38 ± 0.73$^{DC}$* | *3.24 ± 0.27$^{GH}$* | *25.98 ± 2.45$^{BC}$* | *37.62 ± 4.71$^{A}$* | *1.84 ± 0.26$^{C}$* | *72.75 ± 0.76$^{EF}$* | *4.30 ± 0.16$^{EFGH}$* | *26.58 ± 0.32$^{AB}$* | *26.72 ± 0.44$^{AB}$* | *80.45 ± 0.39$^{CD}$* |
| F12 | Winter | 5.38 ± 0.04$^{b}$ | 0.96 ± 0.01$^{a}$ | 38.56 ± 0.37$^{a}$ | 4.10 ± 0.27$^{a}$ | 27.04 ± 0.10$^{a}$ | 27.99 ± 2.00$^{a}$ | 2.31 ± 0.08$^{a}$ | 75.75 ± 0.12$^{a}$ | 4.18 ± 0.06$^{a}$ | 24.16 ± 0.05$^{b}$ | 26.51 ± 0.16$^{b}$ | 78.95 ± 0.03$^{b}$ |
| | Summer | 5.53 ± 0.05$^{a}$ | 0.95 ± 0.01$^{a}$ | 34.88 ± 0.12$^{b}$ | 4.10 ± 0.28$^{a}$ | 30.63 ± 0.14$^{a}$ | 29.14 ± 2.16$^{a}$ | 1.25 ± 0.02$^{b}$ | 75.07 ± 0.06$^{b}$ | 4.21 ± 0.10$^{a}$ | 24.61 ± 0.06$^{a}$ | 26.88 ± 0.10$^{a}$ | 79.29 ± 0.07$^{a}$ |
| | *Mean* | *5.45 ± 0.09$^{AB}$* | *0.96 ± 0.01$^{B}$* | *36.12 ± 2.03$^{BCD}$* | *4.10 ± 0.39$^{CDEFG}$* | *28.36 ± 2.68$^{ABC}$* | *28.56 ± 1.89$^{BC}$* | *1.78 ± 0.06$^{D}$* | *75.41 ± 0.38$^{BCD}$* | *4.20 ± 0.07$^{FGH}$* | *24.39 ± 0.25$^{ABC}$* | *26.69 ± 0.23$^{AB}$* | *79.12 ± 0.19$^{DEFG}$* |
| F13 | Winter | 5,24 ± 0,03a | 0.93 ± 0.01$^{b}$ | 29.85 ± 1.48$^{b}$ | 5.28 ± 0.25$^{a}$ | 28.15 ± 0.41$^{a}$ | 34.26 ± 2.56$^{a}$ | 2.46 ± 0.04$^{a}$ | 73.18 ± 0.06$^{b}$ | 5.27 ± 0.04$^{a}$ | 26.23 ± 0.05$^{a}$ | 27.40 ± 0.33$^{a}$ | 75.21 ± 0.15$^{a}$ |
| | Summer | 5,27 ± 0,01a | 0.95 ± 0.01$^{a}$ | 37.58 ± 1.21$^{a}$ | 4.29 ± 0.34$^{b}$ | 26.38 ± 0.46$^{a}$ | 29.34 ± 1.58$^{b}$ | 2.41 ± 0.09$^{a}$ | 76.24 ± 0.10$^{a}$ | 5.25 ± 0.07$^{a}$ | 25.43 ± 0.12$^{b}$ | 26.58 ± 0.11$^{b}$ | 78.33 ± 0.51$^{b}$ |
| | *Mean* | *5.25 ± 0.03BC* | *0.94 ± 0.01$^{C}$* | *33.71 ± 4.40$^{BCD}$* | *4.79 ± 0.69$^{ABC}$* | *27.26 ± 1.54$^{BC}$* | *31.80 ± 3.17$^{ABC}$* | *2.44 ± 0.07$^{A}$* | *74.71 ± 1.68$^{BCDE}$* | *5.26 ± 0.05$^{BCD}$* | *25.83 ± 0.45$^{ABC}$* | *26.99 ± 0.50$^{AB}$* | *76.77 ± 0.30$^{AB}$* |
| F14 | Winter | 5.13 ± 0.04$^{b}$ | 0.95 ± 0.01$^{a}$ | 37.95 ± 0.41$^{a}$ | 3.79 ± 0.56$^{a}$ | 27.91 ± 0.23$^{b}$ | 28.18 ± 2.92$^{b}$ | 2.17 ± 0.06$^{a}$ | 76.50 ± 0.44$^{a}$ | 4.54 ± 0.32$^{a}$ | 25.38 ± 0.16$^{b}$ | 26.43 ± 0.17$^{b}$ | 77.82 ± 0.95$^{a}$ |
| | Summer | 5.41 ± 0.04$^{a}$ | 0.91 ± 0.01$^{b}$ | 28.08 ± 0.23$^{b}$ | 3.93 ± 0.46$^{a}$ | 31.39 ± 0.79$^{a}$ | 34.52 ± 2.38$^{a}$ | 2.08 ± 0.19$^{a}$ | 73.65 ± 1.37$^{b}$ | 4.48 ± 0.27$^{a}$ | 26.60 ± 0.21$^{a}$ | 27.33 ± 0.12$^{a}$ | 78.44 ± 0.27$^{a}$ |
| | *Mean* | *5.28 ± 0.16$^{BC}$* | *0.93 ± 0.01$^{D}$* | *33.02 ± 5.42$^{BCD}$* | *3.86 ± 0.47$^{CDEFG}$* | *29.65 ± 2.04$^{AB}$* | *31.35 ± 3.76$^{ABC}$* | *2.13 ± 0.12$^{B}$* | *75.07 ± 1.60$^{BCDE}$* | *4.51 ± 0.27$^{EFGH}$* | *25.99 ± 0.68$^{ABC}$* | *26.88 ± 0.50$^{AB}$* | *78.13 ± 0.71$^{EFG}$* |
| F15 | Winter | 5.14 ± 0.04$^{b}$ | 0.95 ± 0.01$^{b}$ | 34.47 ± 0.55$^{b}$ | 3.29 ± 0.26$^{a}$ | 28.48 ± 0.58$^{a}$ | 32.65 ± 1.08$^{a}$ | 1.11 ± 0.22$^{a}$ | 73.55 ± 0.50$^{b}$ | 5.33 ± 0.10$^{a}$ | 26.54 ± 0.20$^{a}$ | 28.32 ± 0.10$^{a}$ | 78.99 ± 0.67$^{a}$ |
| | Summer | 5.26 ± 0.04$^{a}$ | 0.97 ± 0.01$^{a}$ | 43.83 ± 0.73$^{a}$ | 3.78 ± 0.29$^{a}$ | 25.27 ± 0.40$^{b}$ | 26.73 ± 0.82$^{b}$ | 0.39 ± 0.01$^{b}$ | 74.65 ± 0.30$^{a}$ | 5.40 ± 0.04$^{a}$ | 24.40 ± 0.22$^{b}$ | 26.30 ± 0.16$^{b}$ | 77.34 ± 0.18$^{b}$ |
| | *Mean* | *5.20 ± 0.08$^{BC}$* | *0.96 ± 0.01$^{B}$* | *39.15 ± 5.16$^{AB}$* | *3.54 ± 0.35$^{EFH}$* | *26.88 ± 1.90$^{BC}$* | *29.69 ± 3.49$^{BC}$* | *0.75 ± 0.18$^{G}$* | *74.10 ± 0.70$^{CDEF}$* | *5.36 ± 0.07$^{BC}$* | *25.47 ± 1.18$^{ABC}$* | *27.31 ± 1.11$^{AB}$* | *78.16 ± 1.00$^{EFG}$* |
| F16 | Winter | 5.22 ± 0.03$^{b}$ | 0.96 ± 0.01$^{a}$ | 38.09 ± 3.75$^{a}$ | 3.55 ± 0.26$^{b}$ | 27.56 ± 0.18$^{a}$ | 28.13 ± 0.60$^{a}$ | 2.67 ± 0.23$^{a}$ | 74.25 ± 0.06$^{b}$ | 4.50 ± 0.06$^{a}$ | 25.35 ± 0.16$^{a}$ | 28.73 ± 0.34$^{a}$ | 78.13 ± 0.09$^{a}$ |
| | Summer | 5.39 ± 0.03$^{a}$ | 0.97 ± 0.01$^{a}$ | 41.74 ± 0.63$^{a}$ | 4.45 ± 0.05$^{a}$ | 25.39 ± 0.64$^{a}$ | 27.55 ± 1.35$^{a}$ | 0.87 ± 0.03$^{b}$ | 75.40 ± 0.60$^{a}$ | 4.48 ± 0.06$^{a}$ | 24.33 ± 0.11$^{b}$ | 26.11 ± 0.11$^{b}$ | 77.23 ± 0.09$^{b}$ |
| | *Mean* | *5.31 ± 0.10$^{BC}$* | *0.97 ± 0.01$^{A}$* | *39.91 ± 3.12$^{AB}$* | *4.00 ± 0.51$^{CDEFG}$* | *26.47 ± 1.54$^{BC}$* | *27.84 ± 1.49$^{BC}$* | *1.77 ± 0.19$^{D}$* | *74.83 ± 0.63$^{BCDE}$* | *4.49 ± 0.06$^{EFGH}$* | *24.84 ± 0.57$^{ABC}$* | *26.42 ± 0.40$^{AB}$* | *77.68 ± 0.50$^{G}$* |
| F17 | Winter | 5.30 ± 0.02$^{b}$ | 0.95 ± 0.01$^{a}$ | 35.21 ± 0.77$^{a}$ | 4.46 ± 0.05$^{a}$ | 30.87 ± 0.86$^{a}$ | 27.65 ± 4.05$^{b}$ | 1.81 ± 0.21$^{a}$ | 74.37 ± 0.23$^{a}$ | 4.50 ± 0.06$^{b}$ | 24.22 ± 0.11$^{b}$ | 26.73 ± 0.34$^{a}$ | 78.33 ± 0.19$^{a}$ |
| | Summer | 5.38 ± 0.05$^{a}$ | 0.95 ± 0.01$^{a}$ | 33.23 ± 0.83$^{b}$ | 4.10 ± 0.09$^{b}$ | 28.61 ± 0.58$^{b}$ | 32.54 ± 1.42$^{a}$ | 1.52 ± 0.06$^{a}$ | 73.25 ± 0.17$^{b}$ | 5.40 ± 0.21$^{a}$ | 25.35 ± 0.16$^{a}$ | 26.51 ± 0.05$^{a}$ | 78.25 ± 0.15$^{a}$ |
| | *Mean* | *5.35 ± 0.05$^{ABC}$* | *0.95 ± 0.01$^{C}$* | *34.22 ± 1.30$^{BCD}$* | *4.28 ± 0.20$^{BCDEF}$* | *29.75 ± 1.40$^{AB}$* | *30.09 ± 2.90$^{BC}$* | *1.67 ± 0.14$^{D}$* | *73.81 ± 0.64$^{CDEF}$* | *4.96 ± 0.51$^{CDE}$* | *24.79 ± 0.63$^{ABC}$* | *26.62 ± 0.25$^{AB}$* | *78.29 ± 0.16$^{EFG}$* |
| F18 | Winter | 5.09 ± 0.02$^{b}$ | 0.96 ± 0.01$^{a}$ | 40.47 ± 1.06$^{a}$ | 4.74 ± 0.24$^{a}$ | 29.84 ± 0.18$^{a}$ | 24.43 ± 0.86$^{b}$ | 0.52 ± 0.01$^{b}$ | 70.40 ± 0.32$^{b}$ | 4.73 ± 0.27$^{a}$ | 25.40 ± 0.15$^{b}$ | 28.29 ± 0.16$^{a}$ | 79.33 ± 0.18$^{b}$ |
| | Summer | 5.21 ± 0.03$^{a}$ | 0.95 ± 0.01$^{a}$ | 34.87 ± 1.19$^{b}$ | 4.81 ± 0.08$^{a}$ | 29.46 ± 0.72$^{a}$ | 28.05 ± 1.22$^{a}$ | 2.81 ± 0.03$^{a}$ | 73.41 ± 0.32$^{a}$ | 4.88 ± 0.02$^{a}$ | 26.26 ± 0.49$^{a}$ | 26.51 ± 1.09$^{a}$ | 80.73 ± 0.53$^{a}$ |
| | *Mean* | *5.16 ± 0.07$^{C}$* | *0.96 ± 0.01$^{B}$* | *37.67 ± 3.23$^{ABC}$* | *4.77 ± 0.17$^{ABC}$* | *29.65 ± 1.07$^{AB}$* | *26.24 ± 2.14$^{BC}$* | *1.67 ± 0.03$^{D}$* | *71.91 ± 1.67$^{F}$* | *4.81 ± 0.19$^{CDEF}$* | *25.83 ± 0.57$^{ABC}$* | *27.40 ± 1.19$^{AB}$* | *80.03 ± 0.84$^{D}$* |

Data presented on wet basis. Mean ± standard deviation (n=3). Means followed by the same lower case letter in the column for each sample and capital letters between samples do not differ statistically (p>0.05) between them by Tukey's test.

**Table 3**
Mean values of mineral concentration (mg $kg^{-1}$ cheese) analyzed in Canastra cheese.

| Samples | Season | Ca | Mg | Zn | K | Na | Cu | Fe | Mn |
|---|---|---|---|---|---|---|---|---|---|
| F1 | Winter | 6057.66 ± 234.35$^{b}$ | 254.48 ± 15.18$^{b}$ | 23.18 ± 1.02$^{a}$ | 475.28 ± 27.61$^{b}$ | 8616.20 ± 171.64$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 7399.68 ± 255.11$^{a}$ | 302.09 ± 22.13$^{a}$ | 22.08 ± 0.04$^{b}$ | 1149.49 ± 5.85$^{a}$ | 8701.80 ± 626.68$^{a}$ | 2.18 ± 0.04$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *6728.67 ± 240.73$^{H}$* | *278.28 ± 18.14$^{H}$* | *22.63 ± 0.02$^{H}$* | *812.38 ± 10.24$^{I}$* | *8659.00 ± 398.56$^{E}$* | *1.19 ± 0.04$^{A}$* | *<LOQ* | *<LOQ* |
| F2 | Winter | 4117.32 ± 209.04$^{b}$ | 232.08 ± 6.46$^{b}$ | 21.03 ± 0.53$^{b}$ | 1100.79 ± 93.33$^{b}$ | 2418.29 ± 83.98$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 9253.13 ± 650.67$^{a}$ | 276.10 ± 3.07$^{a}$ | 34.99 ± 3.30$^{a}$ | 1169.19 ± 110.87$^{a}$ | 5126.35 ± 438.01$^{a}$ | 2.17 ± 0.05$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *6685.22 ± 429.85$^{I}$* | *254.09 ± 4.08$^{I}$* | *28.01 ± 1.31$^{E}$* | *1134.99 ± 101.88$^{A}$* | *3772.32 ± 260.54$^{K}$* | *1.18 ± 0.05$^{A}$* | *<LOQ* | *<LOQ* |
| F3 | Winter | 5288.71 ± 73.49$^{b}$ | 419.94 ± 8.38$^{a}$ | 26.94 ± 0.66$^{a}$ | 714.63 ± 38.88$^{b}$ | 9776.29 ± 469.58$^{a}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 6342.98 ± 256.46$^{a}$ | 287.58 ± 10.12$^{b}$ | 21.87 ± 2.02$^{b}$ | 1029.48 ± 35.05$^{a}$ | 8999.90 ± 577.34$^{b}$ | 1.59 ± 0.02$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *5815.84 ± 164.47$^{K}$* | *353.76 ± 9.13$^{C}$* | *24.40 ± 1.03$^{G}$* | *872.05 ± 35.06$^{F}$* | *9388.09 ± 523.35$^{C}$* | *0.90 ± 0.02$^{B}$* | *<LOQ* | *<LOQ* |
| F4 | Winter | 6007.95 ± 123.12$^{b}$ | 326.67 ± 23.57$^{a}$ | 40.62 ± 1.25$^{a}$ | 545.64 ± 39.10$^{b}$ | 7199.20 ± 654.25$^{a}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 7499.05 ± 489.91$^{a}$ | 286.46 ± 25.07$^{b}$ | 24.74 ± 0.46$^{b}$ | 1067.19 ± 41.75$^{a}$ | 6667.02 ± 166.44$^{b}$ | 2.21 ± 0.03$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *6753.50 ± 305.52$^{H}$* | *306.56 ± 24.08$^{E}$* | *32.68 ± 0.85$^{B}$* | *806.41 ± 40.40$^{I}$* | *6933.11 ± 410.30$^{G}$* | *1.20 ± 0.03$^{A}$* | *<LOQ* | *<LOQ* |
| F5 | Winter | 6184.74 ± 278.34$^{b}$ | 270.54 ± 15.19$^{b}$ | 27.79 ± 1.07$^{b}$ | 560.00 ± 28.30$^{b}$ | 11832.11 ± 1219.91$^{a}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 8606.25 ± 378.32$^{a}$ | 382.82 ± 22.18$^{a}$ | 28.11 ± 0.31$^{a}$ | 1267.89 ± 52.86$^{a}$ | 6979.75 ± 219.34$^{b}$ | 1.57 ± 0.04$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *7395.49 ± 328.33$^{F}$* | *326.68 ± 18.40$^{D}$* | *27.95 ± 0.63$^{E}$* | *913.94 ± 39.57$^{C}$* | *9405.93 ± 719.25$^{B}$* | *0.88 ± 0.04$^{C}$* | *<LOQ* | *<LOQ* |
| F6 | Winter | 6746.65 ± 160.60$^{b}$ | 276.52 ± 13.36$^{b}$ | 27.04 ± 1.73$^{a}$ | 536.61 ± 44.64$^{b}$ | 10276.25 ± 550.85$^{a}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 6829.10 ± 175.18$^{a}$ | 284.24 ± 18.41$^{a}$ | 20.52 ± 0.84$^{b}$ | 1112.68 ± 107.57$^{a}$ | 8940.77 ± 844.43$^{b}$ | 2.16 ± 0.07$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *6787.87 ± 167.19$^{H}$* | *280.38 ± 15.32$^{G}$* | *23.78 ± 1.25$^{G}$* | *824.64 ± 76.18$^{H}$* | *9608.51 ± 697.64$^{A}$* | *1.18 ± 0.07$^{A}$* | *<LOQ* | *<LOQ* |
| F7 | Winter | 5758.76 ± 293.37$^{b}$ | 230.70 ± 16.69$^{b}$ | 24.48 ± 1.37$^{b}$ | 581.27 ± 30.62$^{b}$ | 2683.01 ± 57.43$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 8997.48 ± 260.72$^{a}$ | 324.83 ± 4.99$^{a}$ | 31.58 ± 1.14$^{a}$ | 1190.46 ± 31.25$^{a}$ | 6223.75 ± 542.95$^{a}$ | 2.18 ± 0.04$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *7378.12 ± 276.04$^{F}$* | *277.76 ± 10.81$^{H}$* | *28.03 ± 1.20$^{E}$* | *885.86 ± 30.93$^{E}$* | *4453.38 ± 292.05$^{J}$* | *1.19 ± 0.04$^{A}$* | *<LOQ* | *<LOQ* |
| F8 | Winter | 8877.97 ± 279.00$^{b}$ | 270.33 ± 5.87$^{b}$ | 36.47 ± 2.50$^{a}$ | 371.48 ± 35.64$^{b}$ | 9356.94 ± 973.72$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 9093.35 ± 459.67$^{a}$ | 312.69 ± 6.11$^{a}$ | 24.09 ± 0.51$^{b}$ | 889.9 ± 17.90$^{a}$ | 9600.78 ± 253.70$^{a}$ | 1.57 ± 0.03$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *8985.66 ± 369.21$^{A}$* | *291.50 ± 5.78$^{F}$* | *30.28 ± 1.50$^{C}$* | *630.69 ± 26.11$^{L}$* | *9478.86 ± 613.21$^{B}$* | *0.88 ± 0.03$^{C}$* | *<LOQ* | *<LOQ* |
| F9 | Winter | 6765.87 ± 55.45$^{b}$ | 308.93 ± 13.74$^{b}$ | 33.55 ± 1.47$^{a}$ | 635.37 ± 47.26$^{b}$ | 7462.73 ± 720.62$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 9647.94 ± 827.85$^{a}$ | 341.28 ± 24.45$^{a}$ | 32.81 ± 2.00$^{b}$ | 1283.85 ± 88.02$^{a}$ | 10472.48 ± 853.39$^{a}$ | 1.58 ± 0.02$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *8206.90 ± 468.86$^{B}$* | *325.10 ± 18.67$^{D}$* | *33.18 ± 1.71$^{B}$* | *959.61 ± 67.03$^{B}$* | *8967.50 ± 785.40$^{D}$* | *0.89 ± 0.02$^{B}$* | *<LOQ* | *<LOQ* |
| F10 | Winter | 5765.62 ± 232.26$^{b}$ | 259.32 ± 15.74$^{b}$ | 29.92 ± 2.33$^{a}$ | 471.93 ± 32.06$^{b}$ | 6281.65 ± 402.40$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 6001.52 ± 175.99$^{a}$ | 292.28 ± 18.16$^{a}$ | 20.61 ± 0.93$^{b}$ | 1224.91 ± 45.41$^{a}$ | 9064.55 ± 370.41$^{a}$ | 2.17 ± 0.05$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *5883.57 ± 204.10$^{K}$* | *275.80 ± 16.90$^{H}$* | *25.26 ± 1.60$^{F}$* | *848.41 ± 38.73$^{G}$* | *7673.10 ± 386.42$^{F}$* | *1.18 ± 0.04$^{A}$* | *<LOQ* | *<LOQ* |
| F11 | Winter | 6501.68 ± 211.48$^{b}$ | 307.21 ± 13.73$^{b}$ | 33.63 ± 1.73$^{a}$ | 411.86 ± 21.69$^{b}$ | 5384.44 ± 381.56$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 6642.01 ± 366.00$^{a}$ | 350.39 ± 18.06$^{a}$ | 24.79 ± 2.28$^{b}$ | 1038.40 ± 95.51$^{a}$ | 7821.60 ± 682.40$^{a}$ | 1.55 ± 0.03$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *6571.89 ± 288.74$^{J}$* | *328.80 ± 15.80$^{D}$* | *29.21 ± 2.00$^{D}$* | *725.13 ± 58.50$^{J}$* | *6603.02 ± 530.98$^{G}$* | *0.88 ± 0.03$^{C}$* | *<LOQ* | *<LOQ* |
| F12 | Winter | 7104.77 ± 362.98$^{b}$ | 335.06 ± 15.68$^{a}$ | 35.96 ± 1.77$^{a}$ | 344.34 ± 4.17$^{b}$ | 6172.00 ± 385.84$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 7617.73 ± 164.53$^{a}$ | 283.89 ± 15.75$^{b}$ | 25.19 ± 2.49$^{b}$ | 1118.10 ± 147.67$^{a}$ | 6235.82 ± 385.85$^{a}$ | 2.19 ± 0.02$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *7361.25 ± 263.75$^{F}$* | *309.47 ± 15.67$^{E}$* | *30.57 ± 2.10$^{C}$* | *731.22 ± 74.91$^{J}$* | *6203.91 ± 385.84$^{H}$* | *1.20 ± 0.02$^{A}$* | *<LOQ* | *<LOQ* |
| F13 | Winter | 6686.13 ± 454.45$^{b}$ | 349.55 ± 4.41$^{a}$ | 27.29 ± 1.87$^{b}$ | 654.38 ± 30.80$^{b}$ | 8641.71 ± 545.94$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 8922.53 ± 508.68$^{a}$ | 381.51 ± 6.61$^{b}$ | 32.86 ± 2.58$^{a}$ | 1064.89 ± 47.32$^{a}$ | 9966.84 ± 756.74$^{a}$ | 2.16 ± 0.03$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *7804.33 ± 480.56$^{D}$* | *365.53 ± 5.42$^{B}$* | *30.07 ± 2.22$^{C}$* | *859.63 ± 39.06$^{F}$* | *9304.27 ± 651.30$^{C}$* | *1.18 ± 0.03$^{A}$* | *<LOQ* | *<LOQ* |
| F14 | Winter | 6225.07 ± 365.76$^{b}$ | 276.83 ± 7.31$^{b}$ | 28.10 ± 2.17$^{a}$ | 423.50 ± 37.21$^{b}$ | 5739.61 ± 603.35$^{a}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 8487.52 ± 658.34$^{a}$ | 313.03 ± 12.22$^{a}$ | 24.94 ± 0.41$^{b}$ | 1370.13 ± 73.63$^{a}$ | 5448.57 ± 372.56$^{b}$ | 1.60 ± 0.01$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *7356.29 ± 512.05$^{F}$* | *294.93 ± 9.76$^{F}$* | *26.52 ± 1.24$^{F}$* | *896.80 ± 55.42$^{D}$* | *5594.09 ± 487.95$^{I}$* | *0.90 ± 0.01$^{B}$* | *<LOQ* | *<LOQ* |
| F15 | Winter | 7235.43 ± 487.12$^{a}$ | 481.49 ± 17.54$^{a}$ | 30.44 ± 0.44$^{a}$ | 307.93 ± 10.04$^{b}$ | 2355.80 ± 171.02$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 7279.30 ± 252.37$^{b}$ | 302.09 ± 28.99$^{b}$ | 20.25 ± 1.93$^{b}$ | 1047.62 ± 47.00$^{a}$ | 8278.42 ± 629.61$^{a}$ | 2.19 ± 0.03$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *7257.36 ± 369.34$^{G}$* | *391.79 ± 23.26$^{A}$* | *23.34 ± 1.16$^{G}$* | *677.77 ± 28.01$^{K}$* | *5317.11 ± 400.12$^{I}$* | *1.20 ± 0.03$^{A}$* | *<LOQ* | *<LOQ* |
| F16 | Winter | 5913.75 ± 318.43$^{b}$ | 283.16 ± 16.62$^{b}$ | 34.03 ± 1.56$^{a}$ | 413.34 ± 32.01$^{b}$ | 6251.71 ± 634.47$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 8515.92 ± 642.64$^{a}$ | 308.15 ± 22.10$^{a}$ | 31.68 ± 0.63$^{b}$ | 1080.85 ± 65.91$^{a}$ | 9693.37 ± 789.12$^{a}$ | 0.97 ± 0.03$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *7214.83 ± 480.53$^{G}$* | *295.65 ± 19.31$^{F}$* | *32.85 ± 1.09$^{B}$* | *747.09 ± 48.92$^{J}$* | *7972.54 ± 711.79$^{F}$* | *0.60 ± 0.03$^{E}$* | *<LOQ* | *<LOQ* |
| F17 | Winter | 7463.49 ± 719.28$^{b}$ | 346.21 ± 20.58$^{b}$ | 34.12 ± 2.64$^{a}$ | 518.27 ± 22.33$^{b}$ | 5340.35 ± 174.10$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 7622.81 ± 66.03$^{a}$ | 363.86 ± 19.92$^{a}$ | 29.68 ± 1.85$^{b}$ | 1301.48 ± 3.20$^{a}$ | 7797.82 ± 140.77$^{a}$ | 1.52 ± 0.01$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *7543.15 ± 392.65$^{E}$* | *355.03 ± 20.20$^{C}$* | *31.90 ± 2.21$^{C}$* | *909.88 ± 12.76$^{C}$* | *6569.08 ± 157.40$^{G}$* | *0.86 ± 0.01$^{D}$* | *<LOQ* | *<LOQ* |
| F18 | Winter | 7166.18 ± 391.44$^{b}$ | 336.41 ± 8.81$^{b}$ | 41.68 ± 2.34$^{a}$ | 387.53 ± 41.98$^{b}$ | 6663.32 ± 691.99$^{b}$ | <LOQ$^{b}$ | <LOQ | <LOQ |
| | Summer | 8872.23 ± 711.99$^{a}$ | 372.24 ± 9.63$^{a}$ | 29.11 ± 1.68$^{b}$ | 1117.29 ± 44.76$^{a}$ | 9236.54 ± 571.51$^{a}$ | 1.59 ± 0.01$^{a}$ | <LOQ | <LOQ |
| | *Mean* | *8019.20 ± 551.71$^{C}$* | *354.32 ± 9.22$^{C}$* | *35.39 ± 2.01$^{A}$* | *752.41 ± 43.37$^{J}$* | *7949.93 ± 633.50$^{F}$* | *0.90 ± 0.01$^{B}$* | *<LOQ* | *<LOQ* |

Data presented on wet basis. Mean ± standard deviation (n=3). Means followed by the same lower case letter in the column for each sample and capital letters between samples do not differ statistically (p>0.05) between them by Tukey's test. LOQ: Limit of quantification (Cu: 0.20 mg kg-1; Fe: 1.25 mg kg-1; Mn: 0.25 mg kg-1).

Brazilian legislation, a food is considered a source of minerals when it has at least 15% of the RDI of reference per 100 g of solid sample (ANVISA, 1998). Thus, all Canastra cheese samples analyzed herein were considered sources of calcium and zinc, and some samples of magnesium.

### 3.3. Fatty acids and health indexs of fat

The fatty acid (FA) composition of Canastra cheese is listed in Tables 4 and 5, and 19 FAs were identified in all samples, including 12 saturated, 5 monounsaturated, and 2 polyunsaturated fatty acids. Short (n = 2), medium (n = 4), and long (n = 13) chain fatty acids were also detected. Variations ($p < 0.05$) were observed regarding the average concentration of all identified FAs in samples from different production units and winter and summer seasons. Palmitic acid (C16:0) was the most abundant FA in all samples, and the lowest level was found in the F18 winter sample (206.50 mg 100 $g^{-1}$ fat) and the highest level in the F11 summer sample (305.70 mg 100 $g^{-1}$ fat). The second most abundant FA was oleic acid (C18:1n-9c), ranging from 154.90 (F7 winter) to 277.90 mg 100 $g^{-1}$ fat (F11 summer). Stearic acid (C18:0) was the third most abundant and varied from 78.70 (F12 summer) to 152.30 mg 100 $g^{-1}$ fat (F5 winter). Notably, all cheese samples mentioned above were from the city of São Roque de Minas, and these results corroborate Correddu et al. (2021), Gatzias et al. (2020), and Vargas-Bello-Pérez et al. (2018). In the characterization study of the fatty acid profile of Canastra cheese conducted by Margalho et al. (2021), the average concentration of palmitic acid was 258.00 mg 100 $g^{-1}$ fat, oleic acid was 130.00 mg 100 $g^{-1}$ fat, and stearic acid was 152.00 mg 100 $g^{-1}$ fat. The

**Table 4**
Saturated fatty acid concentration (mg 100 $g^{-1}$ fat) identified in Canastra cheese.

| Samples | Season | C4:0 | C6:0 | C8:0 | C10:0 | C11:0 | C12:0 | C13:0 | C14:0 | C15:0 | C16:0 | C17:0 | C18:0 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| F1 | Winter | 74.00 ±0.30$^{b}$ | 28.00±0.10$^{a}$ | 43.00 ±0.50$^{a}$ | 30.00±0.20$^{a}$ | 0.09 ±0.01$^{b}$ | 42.30±0.30$^{a}$ | 0.06 ±0.01$^{b}$ | 97.60±0.13$^{a}$ | 16.00 ±0.20$^{a}$ | 243.50±1.91$^{b}$ | 0.40 ±0.02$^{a}$ | 143.40±0.22$^{a}$ |
| | Summer | 99.00±0.10$^{a}$ | 22.40±0.10$^{a}$ | 34.00±0.40$^{b}$ | 27.80±0.20$^{a}$ | 0.15±0.01$^{a}$ | 31.30±0.80$^{b}$ | 0.10±0.01$^{a}$ | 83.20±0.21$^{b}$ | 16.50±0.40$^{a}$ | 261.30±2.01$^{a}$ | 0.23±0.02$^{b}$ | 101.00±0.56$^{b}$ |
| | *Mean* | *87.00±0.10$^{G}$* | *25.20±0.10$^{F}$* | *39.00±0.30$^{B}$* | *28.90±0.20$^{F}$* | *0.12±0.01$^{C}$* | *36.80±0.60$^{I}$* | *0.08±0.02$^{C}$* | *90.40±0.17$^{C}$* | *16.30±0.03$^{C}$* | *252.40±1.96$^{B}$* | *0.32±0.02$^{C}$* | *122.20±0.39$^{B}$* |
| F2 | Winter | 74.00±0.30$^{b}$ | 26.30±0.20$^{a}$ | 55.00±0.15$^{a}$ | 32.01±0.11$^{a}$ | 0.12±0.01$^{a}$ | 40.10±0.11$^{a}$ | 0.13±0.01$^{a}$ | 101.10±0.12$^{a}$ | 11.47±0.10$^{a}$ | 213.40±1.01$^{b}$ | 0.17±0.03$^{b}$ | 126.70±0.32$^{a}$ |
| | Summer | 80.00±0.10$^{a}$ | 20.20±0.30$^{b}$ | 61.00±0.30$^{a}$ | 22.50±0.10$^{b}$ | 0.08±0.01$^{b}$ | 30.10±0.20$^{a}$ | 0.09±0.01$^{b}$ | 82.10±0.32$^{b}$ | 14.40±0.10$^{a}$ | 229.90±3.11$^{a}$ | 0.22±0.01$^{a}$ | 95.80±0.91$^{b}$ |
| | *Mean* | *77.00±0.20$^{G}$* | *23.20±0.30$^{H}$* | *58.00±0.20$^{A}$* | *27.30±0.60$^{H}$* | *0.10±0.01$^{D}$* | *35.10±0.70$^{J}$* | *0.11±0.01$^{B}$* | *91.60±0.22$^{B}$* | *11.46±0.10$^{G}$* | *221.70±2.06$^{DE}$* | *0.20±0.02$^{GH}$* | *111.30±0.62$^{C}$* |
| F3 | Winter | 52.00±0.20$^{b}$ | 24.80±0.20$^{a}$ | 62.00±0.30$^{a}$ | 29.90±0.20$^{a}$ | 0.15±0.02$^{a}$ | 39.40±0.40$^{a}$ | 0.10±0.02$^{a}$ | 91.20±0.90$^{a}$ | 15.50±0.20$^{a}$ | 233.30±0.65$^{b}$ | 0.32±0.01$^{b}$ | 138.20±0.22$^{a}$ |
| | Summer | 65.00±0.30$^{a}$ | 20.90±0.40$^{b}$ | 22.00±0.40$^{b}$ | 22.40±0.30$^{b}$ | 0.11±0.02$^{a}$ | 31.20±0.10$^{b}$ | 0.12±0.02$^{a}$ | 73.50±0.40$^{b}$ | 14.30±0.20$^{a}$ | 241.70±1.67$^{a}$ | 0.45±0.02$^{a}$ | 114.50±0.73$^{b}$ |
| | *Mean* | *59.00±0.30$^{K}$* | *22.90±0.30$^{H}$* | *42.00±0.40$^{B}$* | *26.20±0.30$^{J}$* | *0.13±0.02$^{C}$* | *35.30±0.30$^{J}$* | *0.11±0.02$^{B}$* | *82.40±0.70$^{E}$* | *14.90±0.20$^{F}$* | *237.50±1.16$^{C}$* | *0.39±0.02$^{B}$* | *126.40±0.48$^{B}$* |
| F4 | Winter | 84.00±0.20$^{b}$ | 30.10±0.20$^{a}$ | 23.00±0.30$^{a}$ | 32.90±0.40$^{a}$ | 0.17±0.02$^{a}$ | 41.10±0.30$^{a}$ | 0.07±0.02$^{a}$ | 100.20±0.80$^{a}$ | 16.30±0.10$^{a}$ | 233.30±0.65$^{b}$ | 0.31±0.01$^{a}$ | 122.10±0.13$^{a}$ |
| | Summer | 26.00±0.20$^{a}$ | 21.10±0.30$^{b}$ | 26.00±0.20$^{a}$ | 23.20±0.50$^{b}$ | 0.15±0.02$^{a}$ | 31.20±0.40$^{b}$ | 0.08±0.02$^{a}$ | 75.60±0.10$^{b}$ | 15.60±0.10$^{b}$ | 241.70±1.67$^{a}$ | 0.25±0.01$^{b}$ | 96.50±0.19$^{b}$ |
| | *Mean* | *55.00±0.20$^{C}$* | *25.60±0.30$^{E}$* | *25.00±0.30$^{EF}$* | *28.00±0.50$^{G}$* | *0.16±0.02$^{B}$* | *36.20±0.40$^{I}$* | *0.08±0.02$^{C}$* | *87.90±0.90$^{D}$* | *16.00±0.10$^{C}$* | *237.50±1.16$^{C}$* | *0.28±0.01$^{E}$* | *109.30±0.16$^{D}$* |
| F5 | Winter | 50.00 ±0.10$^{b}$ | 31.20 ±0.10$^{a}$ | 32.00 ±0.40$^{a}$ | 39.90 ±0.20$^{a}$ | 0.12 ±0.01$^{a}$ | 55.40 ±0.30$^{a}$ | 0.11 ±0.01$^{a}$ | 82.30 ±0.15$^{a}$ | 14.90 ±0.10$^{a}$ | 272.10 ±2.55$^{b}$ | 0.40 ±0.01$^{a}$ | 152.30 ±0.32$^{a}$ |
| | Summer | 65.00 ±0.10$^{a}$ | 22.30 ±0.20$^{b}$ | 39.00 ±0.20$^{a}$ | 27.80 ±0.30$^{b}$ | 0.03 ±0.01$^{b}$ | 30.70 ±0.50$^{b}$ | 0.10 ±0.01$^{a}$ | 68.70 ±0.76$^{b}$ | 15.30 ±0.10$^{a}$ | 284.50 ±3.16$^{a}$ | 0.26 ±0.01$^{b}$ | 119.20 ±0.87$^{b}$ |
| | *Mean* | *58.00 ±0.10$^{K}$* | *26.80 ±0.20$^{C}$* | *36.00 ±0.30$^{C}$* | *33.90 ±0.30$^{B}$* | *0.08 ±0.01$^{D}$* | *43.00 ±0.40$^{F}$* | *0.10±0.01$^{C}$* | *75.50 ±0.46$^{G}$* | *15.10±0.20$^{F}$* | *278.30 ±2.86$^{A}$* | *0.33 ±0.01$^{C}$* | *135.80 ±0.60$^{A}$* |
| F6 | Winter | 38.00 ±0.20$^{b}$ | 31.00 ±0.50$^{a}$ | 54.00 ±0.40$^{a}$ | 40.10 ±0.40$^{a}$ | 0.04 ±0.01$^{b}$ | 54.30 ±0.23$^{a}$ | 0.12 ±0.01$^{a}$ | 105.60 ±0.78$^{a}$ | 15.50 ±0.20$^{b}$ | 233.30 ±3.10$^{b}$ | 0.31 ±0.01$^{a}$ | 141.90 ±0.15$^{a}$ |
| | Summer | 65.00 ±0.30$^{a}$ | 22.10 ±0.40$^{b}$ | 32.00 ±0.40$^{b}$ | 28.80 ±0.30$^{b}$ | 0.15 ±0.01$^{a}$ | 31.20 ±0.51$^{b}$ | 0.09 ±0.01$^{b}$ | 77.70 ±0.75$^{b}$ | 16.20 ±0.20$^{a}$ | 251.20 ±2.06$^{a}$ | 0.17 ±0.02$^{b}$ | 118.40 ±0.47$^{b}$ |
| | *Mean* | *51.50 ±0.30$^{F}$* | *26.50 ±0.50$^{C}$* | *43.00 ±0.40$^{B}$* | *34.40 ±0.30$^{B}$* | *0.09 ±0.01$^{D}$* | *42.80 ±0.37$^{F}$* | *0.10±0.01$^{C}$* | *91.70 ±0.77$^{B}$* | *15.80 ±0.20$^{D}$* | *242.20 ±2.58$^{C}$* | *0.24 ±0.02$^{F}$* | *130.20 ±0.31$^{A}$* |
| F7 | Winter | 59.00 ±0.30$^{b}$ | 29.80 ±0.30$^{a}$ | 12.00 ±0.50$^{b}$ | 36.60 ±0.40$^{a}$ | 0.20 ±0.03$^{a}$ | 41.80 ±0.30$^{a}$ | 0.08 ±0.01$^{a}$ | 78.80 ±0.13$^{a}$ | 14.90 ±0.20$^{b}$ | 207.40 ±1.27$^{b}$ | 0.36 ±0.03$^{a}$ | 102.10 ±0.32$^{a}$ |
| | Summer | 94.00 ±0.20$^{a}$ | 22.10 ±0.20$^{b}$ | 33.00 ±0.30$^{a}$ | 30.50 ±0.10$^{a}$ | 0.18 ±0.02$^{a}$ | 31.40 ±0.30$^{b}$ | 0.07 ±0.02$^{a}$ | 61.50 ±0.23$^{b}$ | 16.30 ±0.20$^{a}$ | 227.80 ±1.62$^{a}$ | 0.34 ±0.02$^{a}$ | 86.70 ±0.17$^{b}$ |
| | *Mean* | *77.00 ±0.30$^{I}$* | *26.00 ±0.30$^{D}$* | *23.00 ±0.40$^{F}$* | *33.40 ±0.30$^{C}$* | *0.19 ±0.03$^{A}$* | *36.60 ±0.30$^{I}$* | *0.08±0.02$^{C}$* | *70.20 ±0.18$^{H}$* | *15.60 ±0.20$^{D}$* | *216.60 ±1.45$^{DE}$* | *0.35 ±0.03$^{C}$* | *94.40 ±0.24$^{E}$* |
| F8 | Winter | 58.00±0.30$^{b}$ | 27.30 ±0.10$^{a}$ | 53.00 ±0.40$^{a}$ | 30.40 ±0.10$^{a}$ | 0.05 ±0.02$^{b}$ | 39.90 ±0.30$^{a}$ | 0.09 ±0.01$^{b}$ | 96.80 ±0.11$^{a}$ | 16.10 ±0.30$^{a}$ | 243.60±1.99$^{b}$ | 0.18 ±0.02$^{b}$ | 133.20 ±0.19$^{a}$ |
| | Summer | 91.00 ±0.20$^{a}$ | 20.60 ±0.10$^{b}$ | 31.00 ±0.20$^{b}$ | 25.60 ±0.20$^{b}$ | 0.17 ±0.02$^{a}$ | 30.30 ±0.40$^{a}$ | 0.13 ±0.02$^{a}$ | 81.30 ±0.34$^{b}$ | 13.90 ±0.30$^{b}$ | 273.20 ±4.29$^{a}$ | 0.20 ±0.02$^{a}$ | 104.40 ±0.12$^{b}$ |
| | *Mean* | *75.00 ±0.30$^{I}$* | *24.00 ±0.10$^{G}$* | *42.00 ±0.30$^{B}$* | *28.00 ±0.20$^{G}$* | *0.11 ±0.02$^{D}$* | *35.10 ±0.40$^{J}$* | *0.11 ±0.02$^{B}$* | *89.00 ±0.23$^{D}$* | *15.00 ±0.30$^{F}$* | *258.40 ±6.28$^{B}$* | *0.19±0.02$^{H}$* | *118.80 ±0.16$^{C}$* |
| F9 | Winter | 25.00 ±0.30$^{b}$ | 32.10 ±0.23$^{a}$ | 22.00 ±0.20$^{b}$ | 40.00 ±0.10$^{a}$ | 0.15 ±0.01$^{a}$ | 50.20 ±0.11$^{a}$ | 0.14 ±0.01$^{a}$ | 103.20 ±0.19$^{a}$ | 14.50 ±0.20$^{b}$ | 272.90 ±0.73$^{b}$ | 0.32 ±0.01$^{a}$ | 150.20 ±0.15$^{a}$ |
| | Summer | 33.00 ±0.30$^{a}$ | 24.40 ±0.18$^{b}$ | 41.00 ±0.30$^{a}$ | 31.60 ±0.10$^{b}$ | 0.17 ±0.01$^{a}$ | 42.10 ±0.21$^{b}$ | 0.15 ±0.02$^{a}$ | 81.10 ±0.41$^{b}$ | 20.00 ±0.10$^{a}$ | 294.10 ±1.19$^{a}$ | 0.21 ±0.01$^{b}$ | 103.60 ±0.21$^{b}$ |
| | *Mean* | *29.00 ±0.30$^{B}$* | *28.30 ±0.20$^{B}$* | *32.00 ±0.30$^{C}$* | *35.80 ±0.10$^{A}$* | *0.16 ±0.01$^{B}$* | *46.20 ±0.16$^{D}$* | *0.15 ±0.02$^{A}$* | *92.20 ±0.30$^{A}$* | *17.30 ±0.20$^{B}$* | *283.50 ±0.96$^{A}$* | *0.27 ±0.01$^{E}$* | *126.90 ±0.18$^{B}$* |
| F10 | Winter | 67.00 ±0.20$^{b}$ | 30.10 ±0.20$^{a}$ | 32.00 ±0.19$^{a}$ | 41.00 ±0.30$^{a}$ | 0.16 ±0.02$^{a}$ | 52.10 ±0.07$^{a}$ | 0.12 ±0.02$^{a}$ | 88.40 ±0.87$^{a}$ | 15.50 ±0.20$^{b}$ | 245.70 ±0.98$^{b}$ | 0.17 ±0.02$^{b}$ | 111.30 ±0.15$^{a}$ |
| | Summer | 78.00 ±0.20$^{a}$ | 21.10 ±0.10$^{b}$ | 35.00±0.40$^{a}$ | 25.10 ±0.20$^{b}$ | 0.15 ±0.02$^{a}$ | 31.10 ±0.10$^{b}$ | 0.11 ±0.02$^{a}$ | 77.60 ±0.21$^{b}$ | 17.60 ±0.10$^{a}$ | 263.70 ±2.05$^{a}$ | 0.21 ±0.02$^{a}$ | 99.90 ±0.17$^{b}$ |
| | *Mean* | *73.00 ±0.20$^{I}$* | *25.60 ±0.20$^{E}$* | *34.00 ±0.30$^{C}$* | *33.00 ±0.30$^{C}$* | *0.16 ±0.02$^{B}$* | *41.60 ±0.40$^{G}$* | *0.12±0.02$^{B}$* | *83.00 ±0.54$^{E}$* | *16.60 ±0.20$^{C}$* | *254.80 ±1.52$^{B}$* | *0.19 ±0.02$^{H}$* | *105.60 ±0.16$^{D}$* |
| F11 | Winter | 25.00 ±0.30$^{b}$ | 30.00 ±0.20$^{a}$ | 25.00 ±0.50$^{a}$ | 32.30 ±0.30$^{a}$ | 0.18 ±0.03$^{a}$ | 53.20 ±0.20$^{a}$ | 0.08 ±0.01$^{a}$ | 93.10 ±0.87$^{a}$ | 12.90 ±0.10$^{b}$ | 281.70 ±3.19$^{b}$ | 0.33 ±0.03$^{b}$ | 135.40 ±0.98$^{a}$ |
| | Summer | 32.00 ±0.20$^{a}$ | 20.10 ±0.01$^{b}$ | 27.00 ±0.20$^{a}$ | 21.50 ±0.20$^{b}$ | 0.19 ±0.02$^{a}$ | 42.10 ±0.60$^{b}$ | 0.10 ±0.02$^{a}$ | 67.10 ±0.29$^{b}$ | 14.10 ±0.10$^{a}$ | 305.70 ±3.35$^{a}$ | 1.40 ±0.02$^{a}$ | 120.10 ±0.60$^{b}$ |
| | *Mean* | *28.50 ±0.30$^{H}$* | *25.00 ±0.20$^{F}$* | *26.00 ±0.40$^{DE}$* | *26.90 ±0.30$^{I}$* | *0.19 ±0.03$^{A}$* | *47.70 ±0.40$^{C}$* | *0.09 ±0.02$^{C}$* | *80.01 ±0.58$^{F}$* | *13.50 ±0.10$^{H}$* | *293.70 ±3.27$^{A}$* | *0.87 ±0.03$^{A}$* | *127.80 ±0.79$^{B}$* |
| F12 | Winter | 22.00 ±0.30$^{a}$ | 30.50 ±0.40$^{a}$ | 29.00 ±0.10$^{b}$ | 40.30 ±0.80$^{a}$ | 0.09 ±0.01$^{a}$ | 62.10 ±0.11$^{a}$ | 0.03 ±0.01$^{a}$ | 74.40 ±0.21$^{a}$ | 16.60 ±0.10$^{a}$ | 213.70 ±1.53$^{b}$ | 0.13 ±0.02$^{a}$ | 101.30 ±0.43$^{a}$ |
| | Summer | 25.00 ±0.10$^{a}$ | 20.40 ±0.30$^{b}$ | 34.00 ±0.20$^{a}$ | 21.10 ±0.50$^{b}$ | 0.08 ±0.01a | 38.50 ±0.30$^{b}$ | 0.03 ±0.01$^{a}$ | 65.70 ±0.20$^{b}$ | 15.00 ±0.20$^{b}$ | 241.30 ±1.24$^{a}$ | 0.15 ±0.01$^{a}$ | 78.70 ±0.32$^{b}$ |
| | *Mean* | *23.50 ±0.20$^{E}$* | *25.50 ±0.40$^{E}$* | *32.00 ±0.20$^{C}$* | *30.70 ±0.70$^{E}$* | *0.09 ±0.01$^{D}$* | *50.30 ±0.70$^{A}$* | *0.03 ±0.01$^{D}$* | *70.00 ±0.11$^{H}$* | *15.80 ±0.20$^{DE}$* | *227.50 ±1.39$^{DE}$* | *0.14 ±0.02$^{I}$* | *90.00 ±0.38$^{E}$* |
| F13 | Winter | 56.00 ±0.20$^{b}$ | 31.70 ±0.20$^{a}$ | 42.00 ±0.20$^{a}$ | 37.80±0.40$^{a}$ | 0.03 ±0.01$^{b}$ | 55.50 ±0.60$^{a}$ | 0.08 ±0.01$^{b}$ | 93.20 ±0.30$^{a}$ | 16.90 ±0.10$^{b}$ | 242.90 ±2.76$^{b}$ | 0.18 ±0.03$^{b}$ | 143.20 ±0.43$^{a}$ |
| | Summer | 78.00 ±0.20$^{a}$ | 25.50 ±0.10$^{b}$ | 23.00 ±0.10$^{b}$ | 31.00 ±0.20$^{a}$ | 0.13 ±0.02$^{a}$ | 43.20 ±0.50$^{b}$ | 0.13 ±0.02$^{a}$ | 61.11 ±0.20$^{b}$ | 18.20 ±0.20$^{a}$ | 274.50 ±3.11$^{a}$ | 0.28 ±0.02$^{a}$ | 123.20 ±0.32$^{b}$ |
| | *Mean* | *67.00 ±0.20$^{J}$* | *28.60 ±0.20$^{B}$* | *33.00 ±0.20$^{C}$* | *34.04 ±0.30$^{B}$* | *0.08 ±0.02$^{D}$* | *49.40 ±0.60$^{B}$* | *0.10 ±0.02$^{C}$* | *77.20 ±0.30$^{G}$* | *17.60 ±0.20$^{B}$* | *258.70 ±2.94$^{B}$* | *0.23 ±0.03$^{FG}$* | *133.20 ±0.38$^{A}$* |
| F14 | Winter | 21.00 ±0.20$^{a}$ | 29.50 ±0.50$^{a}$ | 33.00 ±0.10$^{a}$ | 36.60 ±0.80$^{a}$ | 0.11 ±0.01$^{a}$ | 42.10 ±0.11$^{a}$ | 0.09 ±0.01$^{a}$ | 103.20 ±0.12$^{a}$ | 13.30 ±0.20$^{b}$ | 246.70 ±3.19$^{b}$ | 0.41 ±0.01$^{a}$ | 136.70 ±0.32$^{a}$ |
| | Summer | 32.00 ±0.10$^{a}$ | 21.60 ±0.20$^{b}$ | 21.00 ±0.10$^{b}$ | 27.80 ±0.20$^{a}$ | 0.15 ±0.01$^{a}$ | 34.50 ±0.80$^{b}$ | 0.09 ±0.01$^{a}$ | 81.00 ±0.20$^{b}$ | 17.70 ±0.20$^{a}$ | 280.40 ±3.65$^{a}$ | 0.21 ±0.01$^{b}$ | 110.00 ±0.08$^{b}$ |
| | *Mean* | *26.00 ±0.20$^{D}$* | *25.50 ±0.40$^{E}$* | *27.00 ±0.19$^{D}$* | *32.20 ±0.50$^{D}$* | *0.13 ±0.01$^{C}$* | *38.30 ±0.90$^{H}$* | *0.09±0.01$^{C}$* | *92.10 ±0.70$^{AB}$* | *15.50 ±0.20$^{E}$* | *263.60 ± 3.42$^{B}$* | *0.31 ±0.01$^{D}$* | *123.40 ±0.20$^{B}$* |
| F15 | Winter | 55.00 ±0.10$^{b}$ | 33.60 ±0.10$^{a}$ | 29.00 ±0.20$^{a}$ | 41.10 ±0.20$^{a}$ | 0.13 ±0.01$^{a}$ | 51.00±0.30$^{a}$ | 0.12 ±0.01$^{a}$ | 88.80 ±0.15$^{a}$ | 17.00 ±0.10$^{b}$ | 263.40 ±2.00$^{b}$ | 0.15 ±0.01$^{b}$ | 126.50 ±0.22$^{a}$ |
| | Summer | 87.00 ±0.10$^{a}$ | 25.30 ±0.10$^{b}$ | 28.00 ±0.18$^{a}$ | 23.30 ±0.30$^{b}$ | 0.17 ±0.01$^{a}$ | 47.80 ±0.70$^{b}$ | 0.13 ±0.01$^{a}$ | 54.50 ±0.11$^{b}$ | 20.30 ±0.20$^{a}$ | 281.70 ±1.09$^{a}$ | 0.22 ±0.01$^{a}$ | 104.20 ±0.32$^{b}$ |
| | *Mean* | *71.00 ±0.10$^{I}$* | *29.50 ±0.10$^{A}$* | *29.00 ±0.20$^{D}$* | *32.20 ±0.30$^{D}$* | *0.15 ±0.01$^{B}$* | *49.40 ±0.50$^{B}$* | *0.13 ±0.01$^{B}$* | *71.70 ±0.13$^{H}$* | *18.70 ±0.20$^{A}$* | *272.50 ±1.50$^{A}$* | *0.19 ±0.01$^{H}$* | *115.30 ±0.27$^{C}$* |
| F16 | Winter | 20.00 ±0.30$^{a}$ | 31.70 ±0.20$^{a}$ | 33.00 ±0.30$^{b}$ | 39.90 ±0.40$^{a}$ | 0.07 ±0.01$^{b}$ | 44.90 ±0.60$^{a}$ | 0.11 ±0.01$^{a}$ | 102.10 ±0.10$^{a}$ | 17.70 ±0.20$^{b}$ | 228.60±1.65$^{b}$ | 0.32 ±0.01$^{a}$ | 131.00 ±0.15$^{a}$ |
| | Summer | 32.00 ±0.10$^{a}$ | 25.50 ±0.30$^{b}$ | 40.00 ±0.10$^{a}$ | 20.90 ±0.20$^{b}$ | 0.18 ±0.01$^{a}$ | 40.20 ±0.20$^{a}$ | 0.08 ±0.01$^{b}$ | 81.10 ±0.34$^{b}$ | 18.90 ±0.20$^{a}$ | 250.40 ±3.21$^{a}$ | 0.30 ±0.01$^{b}$ | 119.80 ±0.25$^{b}$ |
| | *Mean* | *26.00 ±0.20$^{D}$* | *28.60 ±0.30$^{B}$* | *37.00 ±0.20$^{C}$* | *30.40 ±0.30$^{E}$* | *0.13 ±0.01$^{C}$* | *42.60 ±0.80* | *0.09 ±0.01$^{C}$* | *91.60 ±0.22$^{B}$* | *18.30 ±0.20$^{A}$* | *239.50 ±2.43$^{CD}$* | *0.31 ±0.01$^{D}$* | *125.40 ±0.20$^{B}$* |
| F17 | Winter | 36.00 ±0.20$^{b}$ | 31.00 ±0.21$^{a}$ | 20.00 ±0.10$^{a}$ | 40.00 ±0.10$^{a}$ | 0.20 ±0.02$^{a}$ | 52.20 ±0.11$^{a}$ | 0.08 ±0.01$^{a}$ | 111.70 ±0.40$^{a}$ | 13.00 ±0.20$^{b}$ | 248.80 ±0.87$^{b}$ | 0.42 ±0.02$^{a}$ | 148.70 ±0.77$^{a}$ |
| | Summer | 22.00 ±0.20$^{a}$ | 19.80 ±0.10$^{b}$ | 19.00 ±0.20$^{a}$ | 21.10 ±0.10$^{b}$ | 0.14 ±0.01$^{b}$ | 38.20 ±0.10$^{a}$ | 0.09 ±0.01$^{a}$ | 70.20 ±0.02$^{b}$ | 14.50 ±0.20$^{a}$ | 278.60 ±3.17$^{a}$ | 0.31 ±0.02$^{b}$ | 113.20 ±0.54$^{b}$ |
| | *Mean* | *29.00 ±0.20$^{A}$* | *25.40 ±0.11$^{E}$* | *19.00 ±0.20$^{G}$* | *30.60 ±0.60$^{E}$* | *0.17 ±0.02$^{AB}$* | *45.02 ±0.60$^{E}$* | *0.09 ±0.01$^{C}$* | *90.90 ±0.21$^{C}$* | *13.80 ±0.20$^{E}$* | *263.70 ±2.02$^{B}$* | *0.37 ±0.02$^{B}$* | *130.90 ±0.66$^{A}$* |
| F18 | Winter | 24.00 ±0.30$^{b}$ | 27.60 ±0.10$^{a}$ | 22.00±0.20$^{b}$ | 32.70 ±0.10$^{a}$ | 0.12 ±0.01$^{a}$ | 53.20 ±0.20$^{a}$ | 0.10 ±0.01$^{a}$ | 90.00 ±0.11$^{a}$ | 12.20 ±0.10$^{b}$ | 206.50 ±1.34$^{b}$ | 0.21 ±0.01$^{a}$ | 110.40 ±0.19$^{a}$ |
| | *Summer* | *56.00±0.30$^{a}$* | *20.10 ±0.20$^{b}$* | *26.00±0.21$^{a}$* | *22.50 ±0.10$^{b}$* | *0.11±0.02$^{a}$* | *39.90 ±0.10$^{a}$* | *0.10±0.01$^{a}$* | *72.30 ±0.13$^{b}$* | *17.00 ±0.10$^{a}$* | *265.20 ±2.00$^{a}$* | *0.16±0.02$^{b}$* | *82.30 ±0.20$^{b}$* |
| | *Mean* | *40.00±0.30$^{D}$* | *23.90±0.20$^{G}$* | *24.00±0.20$^{EF}$* | *27.60±0.10$^{H}$* | *0.11±0.02$^{D}$* | *46.00±0.20$^{D}$* | *0.10±0.01$^{C}$* | *81.20±0.12$^{F}$* | *14.60±0.30$^{G}$* | *235.90±1.67$^{CD}$* | *0.19±0.02$^{H}$* | *96.40±0.20$^{E}$* |

Mean ± standard deviation (n=3). Means followed by the same lower case letter in the column for each sample and capital letters between samples do not differ statistically (p>0.05) between them by Tukey's test.

**Table 5**
Concentration of unsaturated fatty acids (mg 100 $g^{-1}$ fat) identified in Canastra cheese.

| Season | Samples | C14:1 | C16:1 | C17:1 | C18:1n-9t | C18:1n-9c | C18:2n-6c | C18:3n-3 | Samples | C14:1 | C16:1 | C17:1 | C18:1n-9t | C18:1n-9c | C18:2n-6c | C18:3n-3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Winter | F1 | 0.95 ±0.02b | 14.00 ±0.10a | 0.15 ±0.01a | 1.22 ±0.01b | 201.00±1.56b | 69.00 ±0.10a | 0.10±0.01a | F10 | 0.87 ±0.08b | 13.30 ±0.10b | 0.19 ±0.02b | 1.23 ±0.05b | 212.30 ±0.35b | 77.00 ±0.30b | 0.23 ±0.01[a] |
| Summer | | 1.10±0.01a | 14.90±0.10a | 0.13±0.01a | 1.30±0.01a | 221.00±1.10a | 82.00±0.20a | 0.12±0.01a | | 1.05 ±0.02a | 15.20 ±0.20a | 0.23 ±0.02a | 1.30 ±0.02[a] | 228.40 ±0.91[a] | 86.00 ±0.20[a] | 0.15±0.02b |
| *Mean* | | *1.03±0.02B* | *14.50±0.10[C]* | *0.14±0.01[G]* | *1.26±0.01 [B]* | *211.00±1.33 [D]* | *75.50±0.20[C]* | *0.08±0.01 [DE]* | | *0.96 ±0.05[C]* | *14.30 ±0.20[C]* | *0.21±0.02 [BC]* | *1.27 ±0.04 [AB]* | *220.40 ±0.63[C]* | *82.00 ±0.30[F]* | *0.19 ±0.02[A]* |
| Winter | F2 | 0.86±0.02b | 12.20±0.10b | 0.10±0.01b | 1.15±0.01b | 182.40±0.79b | 73.00±0.10b | 0.03±0.01b | F11 | 1.00 ±0.01b | 12.00 ±0.10b | 0.30 ±0.03a | 1.16 ±0.01b | 268.90 ±1.10b | 59.00 ±0.10b | 0.03 ±0.01b |
| Summer | | 0.99±0.02a | 12.40±0.10a | 0.20±0.01a | 1.20±0.01a | 198.70±2.02a | 88.00±0.20a | 0.13±0.01a | | 1.13 ±0.02a | 13.10 ±0.10a | 0.32 ±0.02a | 1.25 ±0.01[a] | 277.90 ±2.62[a] | 86.00 ±0.10[a] | 0.13 ±0.02[a] |
| *Mean* | | *0.93±0.02[C]* | *12.30±0.10 [H]* | *0.15±0.01[G]* | *1.18±0.01[F]* | *190.60±1.40 [E]* | *82.50±0.20[F]* | *0.11±0.01[C]* | | *1.07 ±0.02 [B]* | *12.60 ±0.10 [H]* | *0.31 ±0.03[A]* | *1.21 ±0.01 [E]* | *273.40 ± 1.86[A]* | *73.00 ±0.10[G]* | *0.18 ±0.02[A]* |
| Winter | F3 | 0.88±0.01b | 12.80±0.10b | 0.17±0.02b | 1.21±0.01b | 174.80±2.12b | 69.00±0.30b | 0.04±0.01a | F12 | 0.98 ±0.02a | 12.20 ±0.10b | 0.10 ±0.01a | 1.13 ±0.02b | 161.40 ±0.23b | 88.00 ±0.20b | 0.02 ±0.01b |
| Summer | | 1.02±0.01a | 13.10±0.10a | 0.21±0.02a | 1.28±0.01a | 225.40±2.01a | 92.00±0.50a | 0.06±0.01a | | 1.09 ±0.01a | 13.10 ±0.10a | 0.12 ±0.01a | 1.22 ±0.03[a] | 216.70 ±2.12[a] | 92.00 ±0.40[a] | 0.06 ±0.01[a] |
| *Mean* | | *0.95±0.01[C]* | *13.00±0.10[G]* | *0.19±0.02 [D]* | *1.25±0.01C* | *200.10±2.07 [D]* | *80.50±0.40[F]* | *0.14±0.01 [B]* | | *1.04 ±0.02 [B]* | *12.70 ±0.10 [H]* | *0.11 ±0.01 [H]* | *1.18 ±0.03[F]* | *189.00 ±1.18[F]* | *90.00 ±0.30[C]* | *0.04 ±0.01[F]* |
| Winter | F4 | 0.76±0.01b | 13.60±0.20b | 0.12±0.02b | 1.00±0.01b | 190.10±1.03b | 79.00±0.10a | 0.23±0.02a | F13 | 0.86 ±0.01b | 12.10 ±0.10b | 0.12 ±0.01a | 1.15 ±0.02b | 212.10 ±1.12b | 79.00 ±0.10b | 0.03 ±0.01[a] |
| Summer | | 1.07±0.01a | 14.40±0.20a | 0.19±0.02a | 1.33±0.01a | 221.00±1.01a | 92.00±0.30a | 0.16±0.01b | | 1.10 ±0.01a | 14.30 ±0.10a | 0.15 ±0.02a | 1.23 ±0.01[a] | 249.90 ±2.10[a] | 82.00 ±0.20[a] | 0.03 ±0.01[a] |
| *Mean* | | *0.91±0.01[C]* | *14.00±0.20 [D]* | *0.16±0.02 [FE]* | *1.17±0.01[F]* | *205.50±1.02 [D]* | *85.50±0.20[C]* | *0.05±0.01[F]* | | *0.98 ±0.01[C]* | *13.20 ±0.10[F]* | *0.14±0.02[G]* | *1.19 ±0.02[F]* | *231.00 ±1.61[C]* | *45.50 ±0.10 [B]* | *0.07 ±0.01 [E]* |
| Winter | F5 | 0.89 ±0.02b | 13.30 ±0.10b | 0.21 ±0.01a | 1.10 ±0.01b | 229.90 ±1.23b | 76.00 ±0.50b | 0.20±0.02a | F14 | 1.02 ±0.01a | 12.50 ±0.10b | 0.12 ±0.01b | 1.22 ±0.02b | 201.00 ±1.10b | 76.00 ±0.20b | 0.12 ±0.01[a] |
| Summer | | 0.95 ±0.01a | 14.10 ±0.10a | 0.18 ±0.01b | 1.21 ±0.01a | 265.60 ±1.67a | 88.04 ±0.30a | 0.13±0.01b | | 1.09 ±0.01a | 14.50 ±0.10a | 0.18 ±0.01a | 1.31 ±0.01[a] | 250.90 ±2.08[a] | 81.10 ±0.10[a] | 0.15 ±0.01[a] |
| *Mean* | | *0.92 ±0.02[C]* | *13.70 ±0.10 [E]* | *0.20 ±0.01 [CD]* | *1.16 ±0.01[F]* | *247.80 ±1.45 [B]* | *82.02 ±0.20 [D]* | *0.17±0.02[A]* | | *1.06 ±0.01 [B]* | *13.50 ±0.10 [E]* | *0.15 ±0.01[G]* | *1.27 ±0.02 [AB]* | *226.00 ±1.59[C]* | *78.60 ±0.20[C]* | *0.17 ±0.02[A]* |
| Winter | F6 | 0.78 ±0.01b | 13.50 ±0.20b | 0.22 ±0.01b | 1.17 ±0.01b | 177.80 ±0.65b | 63.00 ±0.20a | 0.08±0.01a | F15 | 1.12 ±0.01a | 15.10 ±0.10b | 0.10 ±0.01a | 1.27 ±0.01b | 211.20 ±4.00b | 81.00 ±0.20b | 0.13 ±0.03[a] |
| Summer | | 1.12 ±0.01a | 14.60 ±0.20a | 0.25 ±0.01a | 1.25 ±0.02a | 203.40 ±0.46a | 69.00 ±0.31a | 0.07±0.01a | | 1.14 ±0.01a | 17.10 ±0.10a | 0.14 ±0.01a | 1.32 ±0.01[a] | 233.40 ±3.12[a] | 84.34 ±0.30[a] | 0.12 ±0.01[a] |
| *Mean* | | *0.95 ±0.01[C]* | *14.10 ±0.20 [D]* | *0.24 ±0.01 [B]* | *1.21 ±0.02 [E]* | *190.60 ±0.55 [E]* | *66.00 ±0.30 [D]* | *0.05±0.02[F]* | | *1.12 ±0.01A* | *16.10 ±0.10[A]* | *0.12 ±0.01[G]* | *1.30 ±0.01[A]* | *222.30 ±3.56[C]* | *82.67 ±0.30[A]* | *0.18 ±0.02[A]* |
| Winter | F7 | 0.74 ±0.01b | 13.80 ±0.10b | 0.15 ±0.03b | 1.12 ±0.01b | 154.90 ±1.77b | 59.00 ±0.20b | 0.13±0.02a | F16 | 1.04 ±0.01a | 12.20 ±0.20b | 0.19 ±0.01a | 1.24 ±0.02b | 212.10 ±2.10b | 88.00 ±0.30b | 0.14 ±0.02[a] |
| Summer | | 1.01 ±0.01a | 15.00 ±0.10a | 0.22 ±0.02a | 1.33 ±0.01a | 191.80 ±0.32a | 75.00 ±0.40a | 0.16±0.02a | | 1.10 ±0.01a | 14.30 ±0.20a | 0.15 ±0.01b | 1.32 ±0.01[a] | 230.80 ±2.32[a] | 92.09 ±0.20[a] | 0.02 ±0.01b |
| *Mean* | | *0.88 ±0.01 [D]* | *14.40 ±0.10[C]* | *0.19 ±0.03 [D]* | *1.23 ±0.01 [D]* | *173.40 ±1.05[F]* | *67.00 ±0.30 [H]* | *0.03±0.01[F]* | | *1.07 ±0.01 [B]* | *13.30 ±0.20[F]* | *0.17 ±0.01 [DE]* | *1.28 ±0.02[A]* | *221.50 ±2.21[C]* | *90.00 ±0.30 [B]* | *0.03 ±0.01[F]* |
| Winter | F8 | 0.98 ±0.01b | 12.40 ±0.20b | 0.18 ±0.02a | 1.26 ±0.01b | 201.30 ±2.72b | 73.00 ±0.40a | 0.10±0.02a | F17 | 1.03 ±0.01b | 11.90 ±0.10b | 0.22 ±0.02b | 1.16 ±0.02b | 199.00 ±0.79b | 61.00 ±0.10a | 0.02 ±0.01[a] |
| Summer | | 1.11 ±0.01a | 13.70 ±0.10a | 0.10 ±0.02b | 1.31 ±0.01a | 262.90 ±3.99a | 82.00 ±0.20a | 0.11±0.01a | | 1.17 ±0.02a | 13.20 ±0.10a | 0.11 ±0.01a | 1.25 ±0.01[a] | 251.20 ±1.11[a] | 65.00 ±0.10[a] | 0.03 ±0.01a |
| *Mean* | | *1.05 ±0.01 [B]* | *13.00 ±0.20[G]* | *0.14 ±0.02[G]* | *1.29 ±0.01[A]* | *232.10 ±3.36[C]* | *77.50 ±0.30 [E]* | *0.17±0.02[A]* | | *1.10 ±0.02[A]* | *12.60 ±0.10 [H]* | *0.17 ±0.02 [DE]* | *1.20 ±0.02 [E]* | *225.10 ±0.95[C]* | *63.00 ±0.10 [H]* | *0.03 ±0.01[F]* |
| Winter | F9 | 0.54 ±0.01b | 13.20 ±0.10b | 0.22 ±0.01b | 1.21 ±0.01b | 238.60 ±1.12b | 62.02 ±0.40a | 0.15±0.01a | F18 | 0.89 ±0.02b | 11.50 ±0.10b | 0.15 ±0.01b | 1.09 ±0.01b | 155.70 ±0.39b | 49.00 ±0.20b | 0.14±0.01[a] |
| Summer | | 0.76 ±0.03a | 16.20 ±0.10a | 0.25 ±0.01a | 1.33 ±0.03a | 269.30 ±2.12a | 72.16 ±0.20a | 0.04±0.02b | | 1.15 ±0.02a | 16.10 ±0.10a | 0.19±0.02a | 1.33 ±0.01[a] | 247.70 ±3.22[a] | 81.00 ±0.20[a] | 0.16±0.02a |
| *Mean* | | *0.65 ±0.02 [E]* | *14.70 ±0.10 [B]* | *0.24 ±0.01 [B]* | *1.27 ±0.02 [AB]* | *254.00 ±1.62 [B]* | *67.09 ±0.30[A]* | *0.09±0.02 [D]* | | *1.02 ±0.02 [B]* | *13.80 ±0.10 [E]* | *0.17 ±0.02 [DE]* | *1.21 ±0.01 [E]* | *201.70 ±1.80 [D]* | *65.00 ±0.20 [H]* | *0.15±0.01 [B]* |

Mean ± standard deviation (n=3). Means followed by the same lower case letter in the column for each sample and capital letters between samples do not differ statistically (p>0.05) between them by Tukey's test.

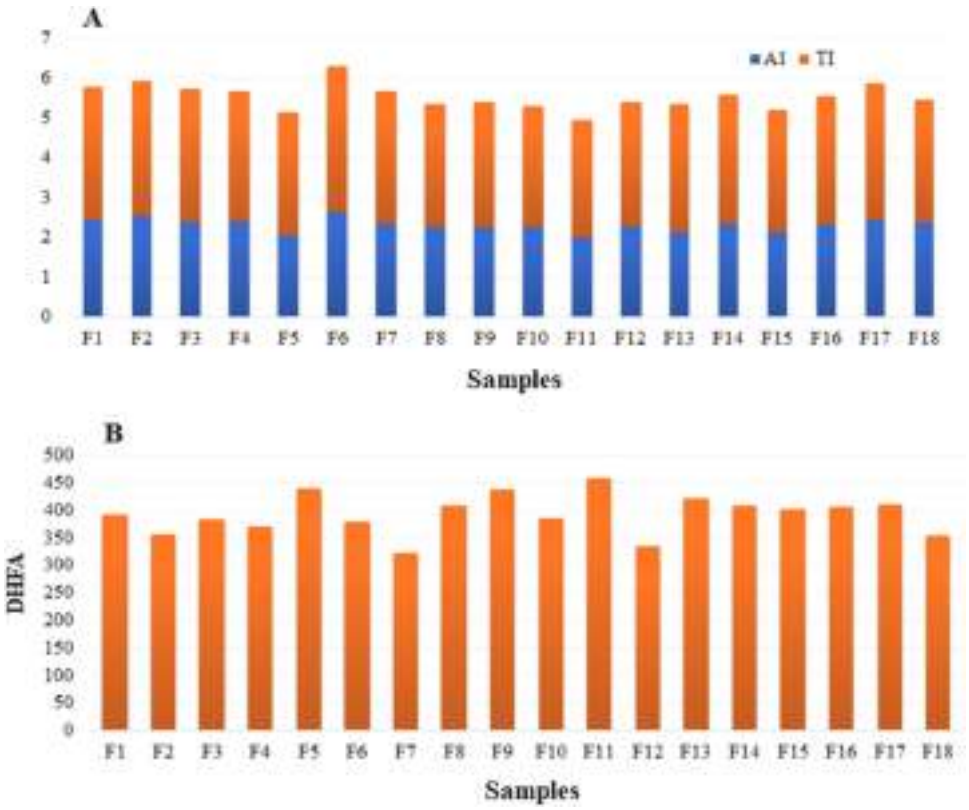



**Fig. 1.** Health indicators of fat from Canastra cheese. AI: Atherogenic index and IT: thrombogenic index (A); DHFA: desirable hypocholesterolemic fatty acids (B).

authors also observed that C16:0, C18:1n-9c, and C18:0 were the predominant fatty acids, although C18:0 content was higher than C18:1n-9c, which was not observed in our experiment.

Regarding saturated fatty acids, the concentrations of C6:0, C10:0, C12:0, C14:0, and C18:0 were higher in winter samples and C4:0 and C16:0 in summer samples. As for unsaturated fatty acids, C14:1, C16:1, C18:1n-9 t, C18:1n-9c, and C18:2n-6c had higher levels in cheese produced in the summer. Some data obtained in this study corroborate Correddu et al. (2021) in Sardinian pecorino cheese as the authors also reported higher C16:0 (21.57–31.62%) and C18:1n-9c (21.21–28.20%) levels in summer samples and higher C6:0 (0.24–2.07%) and C12:0 (2.03–3.35%) levels in winter samples. However, it is worth mentioning that the climatic conditions in summer and winter in Sardinia, Italy, are the opposite of those found in Serra da Canastra, Brazil. Possibly, other factors, such as the diet of the animals, influenced the concentration of some fatty acids to be similar between the samples. According to Meľuchová et al. (2008), pasture plants are rich in linoleic acid and palmitic acid, and their concentrations vary according to the season. This may justify why the Canastra cheese produced in the summer had a higher content of these two compounds, since the food supplied to the cattle in this season is mainly composed of pasture, while in winter, because it is a drier period, the animals consume more silage and feed (Table 1).

The cheese produced in the Serra da Canastra region had low AIs and TIs and similar values in the 18 samples, although high DHFA values were also noted (Fig. 1). The AI ranged from 2.0 (F11) to 2.63 (F6), the TI from 2.93 (F11) to 3.69 (F6), and the DHFA from 322.20 (F7) to 458.80 (F11). O'Callaghan et al. (2017) observed AI values varying from 2.84 to 3.29 and TI from 2.78 to 3.92 for cheddar cheese fat. Compared to our study, Vargas-Bello-Pérez et al. (2018) obtained lower AI values (0.60–1.38) and higher TI values (2.00–5.01) in Chanco cheese. A recent study with Canastra cheese by Margalho et al. (2021) corroborates the data presented herein, given that the authors obtained mean AI values of 2.4, TI of 3.5, and DHFA of 458.00.

Furthermore, the AI exhibited a correlation between the sum of the main saturated fatty acids and the main classes of unsaturated fatty acids, the former being considered atherogenic (favoring lipid adhesion to the cells of the immune and circulatory systems) and the latter being anti-atherogenic (inhibiting plaque aggregation and reducing esterified fatty acid, cholesterol, and phospholipid levels and preventing the appearance of micro- and macro- coronary diseases). The TI quantifies the tendency of blood clot formation in blood vessels and is defined as the ratio between pro-thrombogenic (saturated) and anti-thrombogenic (MUFAs, PUFAs - n6 and PUFAs - n3) (Batista et al., 2017; Apas et al., 2015). Therefore, it is plausible that cheese from F11 contains the fat that provides the most significant health benefit.

### 3.4. Texture profile

The results of the seven texture parameters evaluated in the Canastra cheese are listed in Table 6. Proteolysis has a notable influence on cheese texture (Barracosa et al., 2021), and according to Xia et al. (2020), protein breakdown releases amino and carboxyl groups, increasing hydration and consequently significantly modifying the texture properties of these dairy products. The resilience measured in the samples was the only variable that was not affected ($p > 0.05$) by the seasons or production units, obtaining an overall mean of 0.03.

Hardness and chewability were negatively correlated to water content, thus corroborating Jia et al. (2021), who analyzed semi-hard goat cheese. Sample F16 had the lowest values for these variables in winter and summer, ranging from 6.23 (summer) to 10.01 N (winter) and 85.11 (summer) to 90.11 mJ (winter), respectively. In comparison, the highest values were obtained in sample F9, with means of 51.21 (summer) to 62.80 N (winter) and 476.65 (summer) to 555.65 mJ (winter), respectively, for hardness and chewiness. Differences in hardness can be attributed to variations in the fat content of the raw material and the pH of the cheese (Deshwal et al., 2020). Diezhandino et al. (2016) also mentioned that the salt/moisture ratio could be an influencing factor

**Table 6**
Texture parameters of Canastra cheese made in winter and summer by different producers.

| Samples | Season | Elasticity (mm) | Hardness (N) | Adhesiveness (mJ) | Cohesiveness | Gumminess (N) | Chewability (mJ) | Resilience |
|---|---|---|---|---|---|---|---|---|
| F1 | Winter | 19.77 ± 0.03$^{a}$ | 35.35 ± 0.92$^{a}$ | 136.00 ± 16.97$^{a}$ | 0.46 ± 0.07$^{a}$ | 16.10 ± 2.98$^{a}$ | 318.20 ± 58.55$^{a}$ | 0.01 ± 0.00$^{a}$ |
| | Summer | 19.63 ± 0.06$^{a}$ | 29.34 ± 1.14$^{b}$ | 134.98 ± 10.11$^{b}$ | 0.47 ± 0.03$^{a}$ | 15.23 ± 2.21$^{b}$ | 305.12 ± 18.55$^{b}$ | 0.01 ± 0.00$^{a}$ |
| | *Mean* | *19.70 ± 0.05$^{B}$* | *32.35 ± 1.03$^{B}$* | *135.49 ± 13.54$^{B}$* | *0.47 ± 0.05$^{B}$* | *15.66 ± 2.60$^{B}$* | *311.66 ± 38.55$^{B}$* | *0.01 ± 0.01$^{A}$* |
| F2 | Winter | 17.24 ± 0.58$^{a}$ | 23.34 ± 2.92$^{a}$ | 66.00 ± 7.90$^{a}$ | 0.38 ± 0.07$^{a}$ | 9.19 ± 2.91$^{a}$ | 159.75 ± 20.15$^{a}$ | 0.01 ± 0.00$^{a}$ |
| | Summer | 16.89 ± 0.37$^{b}$ | 16.76 ± 1.23$^{b}$ | 59.00 ± 3.01$^{b}$ | 0.36 ± 0.03$^{b}$ | 8.88 ± 1.42$^{b}$ | 123.65 ± 12.43$^{b}$ | 0.01 ± 0.00$^{a}$ |
| | *Mean* | *17.07 ± 0.48$^{D}$* | *20.05 ± 2.08$^{D}$* | *62.50 ± 5.46$^{G}$* | *0.37 ± 0.05$^{C}$* | *9.04 ± 2.17$^{D}$* | *141.70 ± 16.29$^{F}$* | *0.01 ± 0.01$^{A}$* |
| F3 | Winter | 19.21 ± 0.21$^{a}$ | 16.14 ± 1.50$^{b}$ | 44.00 ± 4.32$^{b}$ | 0.33 ± 0.02$^{b}$ | 10.10 ± 1.51$^{b}$ | 98.75 ± 9.21$^{b}$ | 0.02 ± 0.00$^{a}$ |
| | Summer | 14.21 ± 1.11$^{b}$ | 19.21 ± 1.11$^{a}$ | 45.00 ± 2.91$^{a}$ | 0.39 ± 0.01$^{a}$ | 12.23 ± 0.81$^{a}$ | 100.22 ± 10.12$^{a}$ | 0.01 ± 0.00$^{a}$ |
| | *Mean* | *16.71 ± 0.66$^{E}$* | *17.68 ± 1.31$^{E}$* | *44.50 ± 3.62$^{I}$* | *0.36 ± 0.02$^{C}$* | *11.17 ± 1.16$^{C}$* | *99.49 ± 9.67$^{J}$* | *0.02 ± 0.01$^{A}$* |
| F4 | Winter | 19.75 ± 0.09$^{a}$ | 27.99 ± 5.00$^{a}$ | 87.35 ± 10.10$^{a}$ | 0.42 ± 0.05$^{a}$ | 11.87 ± 2.90$^{a}$ | 234.55 ± 50.21$^{a}$ | 0.01 ± 0.00$^{a}$ |
| | Summer | 19.05 ± 0.10$^{a}$ | 22.87 ± 1.20$^{b}$ | 84.11 ± 5.02$^{b}$ | 0.42 ± 0.03$^{a}$ | 9.99 ± 0.61$^{b}$ | 213.32 ± 20.72$^{b}$ | 0.01 ± 0.00$^{a}$ |
| | *Mean* | *19.40 ± 0.09$^{B}$* | *25.43 ± 3.10$^{C}$* | *85.73 ± 7.56$^{E}$* | *0.42 ± 0.04$^{B}$* | *10.93 ± 1.76$^{C}$* | *223.94 ± 35.47$^{D}$* | *0.01 ± 0.00$^{A}$* |
| F5 | Winter | 19.73 ± 0.03$^{a}$ | 18.82 ± 0.10$^{b}$ | 94.80 ± 8.22$^{b}$ | 0.55 ± 0.01$^{b}$ | 9.35± 0.98$^{b}$ | 204.70 ± 24.04$^{b}$ | 0.02 ± 0.00$^{a}$ |
| | Summer | 19.54 ± 0.07$^{a}$ | 24.11 ± 0.81$^{a}$ | 96.25 ± 5.12$^{a}$ | 0.59 ± 0.03$^{a}$ | 10.38 ± 1.21$^{a}$ | 222.12 ± 14.43$^{a}$ | 0.02 ± 0.00$^{a}$ |
| | *Mean* | *19.64 ± 0.05$^{B}$* | *21.47 ± 0.46$^{D}$* | *95.53 ± 6.67$^{D}$* | *0.57 ± 0.02$^{A}$* | *9.87 ± 1.10$^{D}$* | *213.41 ± 19.24$^{D}$* | *0.02 ± 0.00$^{A}$* |
| F6 | Winter | 19.53 ± 0.19$^{a}$ | 11.50 ± 1.28$^{a}$ | 67.70 ± 4.24$^{a}$ | 0.55 ± 0.01$^{a}$ | 6.60 ± 0.91$^{a}$ | 128.90 ± 19.09$^{a}$ | 0.02 ± 0.00$^{a}$ |
| | Summer | 19.24 ± 0.21$^{a}$ | 8.30 ± 0.79$^{b}$ | 63.21 ± 3.12$^{b}$ | 0.51 ± 0.05$^{b}$ | 6.10 ± 0.21$^{a}$ | 104.65 ± 16.09$^{b}$ | 0.02 ± 0.00$^{a}$ |
| | *Mean* | *19.39 ± 0.20$^{B}$* | *9.90 ± 1.04$^{H}$* | *65.46 ± 3.68$^{G}$* | *0.53 ± 0.03$^{A}$* | *6.35 ± 0.56$^{E}$* | *116.78 ± 17.59$^{H}$* | *0.02 ± 0.00$^{A}$* |
| F7 | Winter | 19.48 ± 0.23$^{b}$ | 13.23 ± 0.06$^{b}$ | 75.60 ± 14.99$^{a}$ | 0.54 ± 0.09$^{a}$ | 7.12 ± 1.19$^{a}$ | 138.95 ± 26.51$^{b}$ | 0.03 ± 0.01$^{a}$ |
| | Summer | 20.12 ± 0.21$^{a}$ | 19.99 ± 0.09$^{a}$ | 72.21 ± 12.11$^{b}$ | 0.53 ± 0.04$^{a}$ | 6.32 ± 0.98$^{b}$ | 175.23 ± 19.45$^{a}$ | 0.02 ± 0.00$^{a}$ |
| | *Mean* | *19.80 ± 0.22$^{B}$* | *16.61 ± 0.08$^{F}$* | *73.91 ± 13.55$^{F}$* | *0.54 ± 0.07$^{A}$* | *6.72 ± 1.09$^{E}$* | *157.09 ± 22.98$^{F}$* | *0.03 ± 0.00$^{A}$* |
| F8 | Winter | 19.66 ± 0.22$^{b}$ | 14.83 ± 3.34$^{b}$ | 76.35 ± 19.91$^{b}$ | 0.50 ± 0.01$^{b}$ | 7.40 ± 1.79$^{a}$ | 145.70 ± 13.23$^{b}$ | 0.02 ± 0.00$^{a}$ |
| | Summer | 20.02 ± 0.12$^{a}$ | 20.22 ± 2.12$^{a}$ | 77.12 ± 12.11$^{a}$ | 0.52 ± 0.05$^{a}$ | 7.65 ± 1.11$^{a}$ | 195.28 ± 10.11$^{a}$ | 0.02 ± 0.00$^{a}$ |
| | *Mean* | *19.84 ± 0.17$^{B}$* | *17.53 ± 2.73$^{E}$* | *76.74 ± 16.01$^{F}$* | *0.51 ± 0.03$^{A}$* | *7.53 ± 1.45$^{E}$* | *170.49 ± 11.67$^{E}$* | *0.02 ± 0.00$^{A}$* |
| F9 | Winter | 19.97 ± 0.38$^{a}$ | 62.80 ± 7.12$^{a}$ | 199.65 ± 34.02$^{a}$ | 0.44 ± 0.09$^{a}$ | 27.84 ± 3.33$^{a}$ | 555.65 ± 32.68$^{a}$ | 0.01 ± 0.00$^{a}$ |
| | Summer | 19.20 ± 0.21$^{a}$ | 51.21 ± 3.09$^{b}$ | 159.21 ± 11.21$^{b}$ | 0.32 ± 0.03$^{b}$ | 23.22 ± 2.21$^{b}$ | 476.65 ± 21.11$^{b}$ | 0.01 ± 0.00$^{a}$ |
| | *Mean* | *19.59 ± 0.30$^{B}$* | *57.00 ± 5.10$^{A}$* | *179.43 ± 22.62$^{A}$* | *0.38 ± 0.06$^{C}$* | *25.53 ± 2.77$^{A}$* | *516.15 ± 26.90$^{A}$* | *0.01 ± 0.00* |
| F10 | Winter | 18.00 ± 0.21$^{a}$ | 17.17 ± 1.07$^{a}$ | 82.22 ± 3.33$^{a}$ | 0.42 ± 0.01$^{a}$ | 8.22 ± 0.11$^{a}$ | 88.23 ± 2.13$^{a}$ | 0.02 ± 0.00$^{a}$ |
| | Summer | 16.23 ± 0.11$^{b}$ | 14.23 ± 0.99$^{b}$ | 78.11 ± 4.23$^{b}$ | 0.40 ± 0.03$^{b}$ | 4.29 ± 0.12$^{b}$ | 65.52 ± 4.10$^{b}$ | 0.03 ± 0.00$^{a}$ |
| | *Mean* | *17.16 ± 0.16$^{D}$* | *15.70 ± 1.03$^{F}$* | *82.22 ± 3.78$^{E}$* | *0.41 ± 0.02$^{B}$* | *6.22 ± 0.12$^{E}$* | *76.88 ± 3.12$^{K}$* | *0.03 ± 0.00$^{A}$* |
| F11 | Winter | 20.03 ± 0.21$^{a}$ | 16.21 ± 1.07$^{a}$ | 81.21 ± 2.43$^{a}$ | 0.50 ± 0.03$^{a}$ | 4.25 ± 0.10$^{a}$ | 123.13 ± 4.21$^{a}$ | 0.01 ± 0.00$^{a}$ |
| | Summer | 17.23 ± 0.21$^{b}$ | 12.23 ± 0.99$^{b}$ | 74.11 ± 6.11$^{b}$ | 0.43 ± 0.01$^{b}$ | 3.89 ± 0.16$^{b}$ | 105.00 ± 5.22$^{b}$ | 0.01 ± 0.00$^{a}$ |
| | *Mean* | *18.63 ± 0.21$^{C}$* | *14.22 ± 1.03$^{F}$* | *81.21 ± 4.27$^{E}$* | *0.47 ± 0.02$^{B}$* | *4.07 ± 0.13$^{F}$* | *114.07 ± 4.72$^{HI}$* | *0.01 ± 0.00$^{A}$* |
| F12 | Winter | 21.34 ± 0.77$^{a}$ | 11.65 ± 1.02$^{b}$ | 97.21 ± 2.31$^{b}$ | 0.30 ± 0.02$^{b}$ | 5.51 ± 0.21$^{b}$ | 109.23 ± 6.87$^{b}$ | 0.01 ± 0.00$^{a}$ |
| | Summer | 20.83 ± 1.24$^{b}$ | 14.31 ± 0.65$^{a}$ | 100.21 ± 3.11$^{a}$ | 0.39 ± 0.01$^{a}$ | 8.29 ± 0.32$^{a}$ | 140.54 ±7.34$^{a}$ | 0.02 ± 0.00$^{a}$ |
| | *Mean* | *20.74 ± 1.00$^{A}$* | *12.98 ± 0.84$^{G}$* | *98.71 ± 2.71$^{D}$* | *0.35 ± 0.02$^{C}$* | *6.90 ± 0.27$^{E}$* | *124.89 ±7.11$^{G}$* | *0.02 ± 0.00$^{A}$* |
| F13 | Winter | 19.21 ± 0.21$^{a}$ | 16.14 ± 1.50$^{b}$ | 44.00 ± 4.32$^{a}$ | 0.33 ± 0.02$^{b}$ | 10.10 ± 1.51$^{b}$ | 98.75 ± 9.21$^{b}$ | 0.01 ± 0.00$^{a}$ |
| | Summer | 14.21 ± 1.11$^{b}$ | 19.21 ± 1.11$^{a}$ | 45.00 ± 2.91$^{a}$ | 0.39 ± 0.01$^{a}$ | 12.23 ± 0.81$^{a}$ | 100.22 ± 10.12$^{a}$ | 0.01 ± 0.00$^{a}$ |
| | *Mean* | *16.71 ± 0.66$^{E}$* | *17.68 ± 1.31$^{E}$* | *44.50 ± 3.62$^{I}$* | *0.36 ± 0.02$^{C}$* | *11.17 ± 1.16$^{C}$* | *99.49 ± 9.67$^{J}$* | *0.01 ± 0.00$^{A}$* |
| F14 | Winter | 16.00 ± 0.35$^{b}$ | 18.24 ± 1.67$^{b}$ | 56.20 ± 1.64$^{a}$ | 0.31 ± 0.01$^{b}$ | 3.11 ± 0.32$^{b}$ | 49.90 ± 4.21$^{b}$ | 0.04 ± 0.00$^{a}$ |
| | Summer | 17.32 ± 0.53$^{a}$ | 25.21 ± 2.87$^{a}$ | 49.21 ± 2.21$^{b}$ | 0.34 ± 0.07$^{a}$ | 5.21 ± 0.45$^{a}$ | 65.22 ± 3.21$^{a}$ | 0.04 ± 0.00$^{a}$ |
| | *Mean* | *16.66 ± 0.44$^{E}$* | *21.73 ± 2.27$^{D}$* | *52.71 ± 1.93$^{H}$* | *0.33 ± 0.04$^{C}$* | *4.26 ± 0.39$^{F}$* | *57.56 ± 3.71$^{L}$* | *0.04 ± 0.00$^{A}$* |
| F15 | Winter | 15.11 ± 0.11$^{a}$ | 10.23 ± 0.72$^{a}$ | 52.98 ± 2.32$^{a}$ | 0.43 ± 0.02$^{a}$ | 6.22 ± 0.43$^{a}$ | 113.21 ± 10.11$^{a}$ | 0.03 ± 0.01$^{a}$ |
| | Summer | 13.47 ± 0.10$^{b}$ | 7.28 ± 0.23$^{b}$ | 48.34 ± 1.23$^{b}$ | 0.36 ± 0.01$^{b}$ | 6.01 ± 0.12$^{a}$ | 98.11 ± 7.11$^{b}$ | 0.02 ± 0.00$^{a}$ |
| | *Mean* | *14.29 ± 0.10$^{F}$* | *8.76 ± 0.48$^{H}$* | *50.66 ± 1.78$^{H}$* | *0.40 ± 0.02$^{B}$* | *6.12 ± 0.28$^{E}$* | *105.66 ± 8.61$^{I}$* | *0.03 ± 0.00$^{A}$* |
| F16 | Winter | 14.21 ± 0.22$^{a}$ | 10.01 ± 0.23$^{a}$ | 43.22 ± 3.34$^{a}$ | 0.35 ± 0.03$^{a}$ | 8.21 ± 0.13$^{a}$ | 90.11 ± 8.09$^{a}$ | 0.03 ± 0.00$^{a}$ |
| | Summer | 10.23 ± 0.34$^{b}$ | 6.23 ± 0.12$^{b}$ | 40.43 ± 2.92$^{b}$ | 0.31 ± 0.02$^{b}$ | 6.81 ± 0.23$^{b}$ | 85.11 ± 6.00$^{b}$ | 0.02 ± 0.00$^{a}$ |
| | *Mean* | *12.22 ± 0.28$^{G}$* | *8.12 ± 0.18$^{H}$* | *43.33 ± 3.13$^{I}$* | *0.33 ± 0.03$^{C}$* | *7.51 ± 0.18$^{E}$* | *94.11 ± 7.05$^{J}$* | *0.03 ± 0.00$^{A}$* |
| F17 | Winter | 18.34 ± 0.34$^{a}$ | 13.23 ± 2.34$^{b}$ | 76.34 ± 1.11$^{a}$ | 0.32 ± 0.03$^{b}$ | 4.23 ± 0.21$^{b}$ | 90.21 ± 3.43$^{a}$ | 0.02 ± 0.00$^{a}$ |
| | Summer | 15.21 ± 1.11$^{b}$ | 15.34 ± 1.11$^{a}$ | 56.45 ± 2.44$^{b}$ | 0.35 ± 0.01$^{a}$ | 7.91 ± 0.13$^{a}$ | 95.14 ± 4.54$^{b}$ | 0.02 ± 0.00$^{a}$ |
| | *Mean* | *16.77 ± 0.73$^{E}$* | *14.29 ± 1.73$^{F}$* | *66.40 ± 1.78$^{G}$* | *0.34 ± 0.02$^{C}$* | *6.07 ± 0.17$^{E}$* | *92.68 ± 3.99$^{J}$* | *0.02 ± 0.00$^{A}$* |
| F18 | Winter | 19.16 ± 0.10$^{b}$ | 32.01 ± 2.90$^{b}$ | 101.30 ± 2.55$^{b}$ | 0.41 ± 0.01$^{b}$ | 13.03 ± 1.40$^{b}$ | 249.55 ± 28.07$^{b}$ | 0.02 ± 0.00$^{a}$ |
| | Summer | 19.34 ± 0.21$^{a}$ | 39.23 ± 3.21$^{a}$ | 104.23 ± 2.22$^{a}$ | 0.44 ± 0.03$^{a}$ | 15.21 ± 1.21$^{a}$ | 278.21 ± 19.32$^{a}$ | 0.02 ± 0.00$^{a}$ |
| | *Mean* | *19.25 ± 0.16$^{B}$* | *35.62 ± 3.06$^{B}$* | *102.77 ± 2.39$^{C}$* | *0.43 ± 0.02$^{B}$* | *14.12 ± 1.31$^{B}$* | *263.88 ± 23.70$^{C}$* | *0.02 ± 0.00$^{A}$* |

Mean ± standard deviation (n=3). Means followed by the same lower case letter in the column for each sample and capital letters between samples do not differ statistically
($p<0.05$) between them by Tukey's test.

and showed that when there was an increase in NaCl per 100 $g^{-1}$ of water, it significantly increased the ionic strength of the cheese, reducing protein solubility due to a salting-out phenomenon. Under these conditions, a higher of protein-protein interactions is established, enabling the lowest level of electrostatic repulsion between the proteins due to pH values being close to the isoelectric point of the caseins. Thus, the hardness of the cheese increases, as there is greater loss of water. This occurs due to the decrease in protein solubility in aqueous medium.

Sample F16 showed the lowest elasticity (10.23 mm in summer and 14.21 mm in winter), and F12 showed the highest elasticity (20.13 mm in summer and 21.34 mm in winter). The lowest mean values of adhesiveness were found for sample F16 (40.43–43.22 mJ) and the highest for sample F9 (159.21–199.65 mJ). The lowest values for gumminess were obtained in sample F11 (3.89 N in summer and 4.25 N in winter), while the highest means were found in sample F9 (23.22 N in summer and 27.84 N in winter). There were also differences among the samples regarding cohesiveness (0.30–0.59), and this may be related to extracellular polysaccharide production by lactic acid bacteria in starter cultures (Adhikari et al., 2003).

All cheese samples that presented the lowest and/or highest values for the texture parameters are from the same city (São Roque de Minas). Given this scenario, it is plausible that the peculiar artisanal way of producing the cheese and environmental conditions are crucial factors that significantly interfered with the differences in the texture profile of the samples.

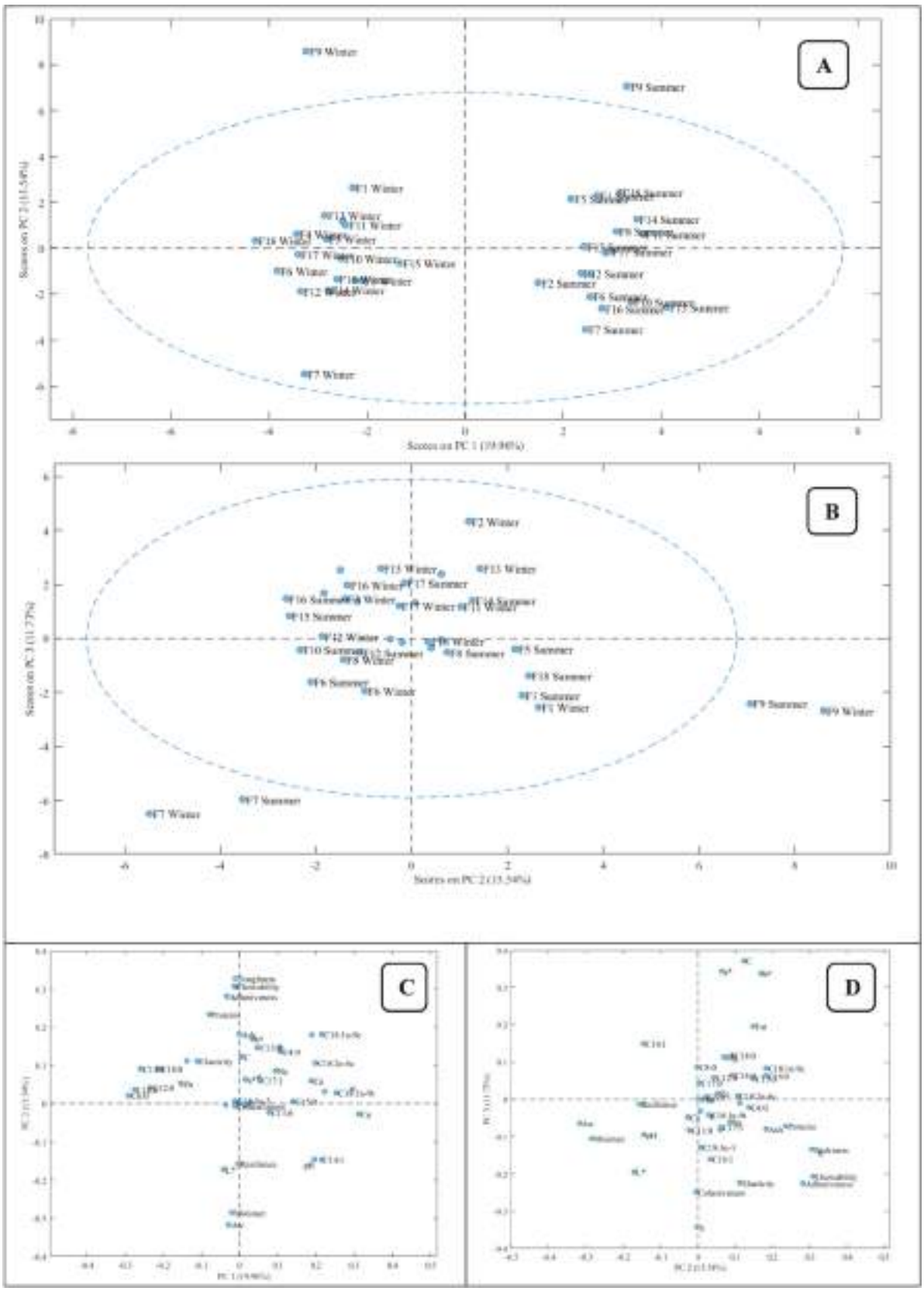



**Fig. 2.** Principal component analysis of Canastra cheese samples produced in winter and summer regarding their profile obtained from 46 variables: A and B (scores); C and D (loadings).

### 3.5. Exploratory data analysis

The principal component analysis (PCA) was performed to explore all the samples investigated and the formation of possible groups of Canastra cheese with similar characteristics according to the variables studied. The data matrix was composed of 36 samples (18 produced in summer and 18 produced in winter) by 46 variables using the mean found among the repetitions to perform this multivariate analysis. The projections of the scores and loadings of the three principal components (PC) are shown in Fig. 2.

The PC1 separated the winter and summer samples, being mainly attributed to differences in fatty acid, mineral, and pH content (Fig. 2A and C). Thus, the cheese produced in summer were correlated to high concentrations of unsaturated fatty acids (C14:1, C16:1, C18:1n-9c, C18:1n-9 t, and C18:2n-6c) and the main fatty acid (C16:0) present in Canastra cheese, in addition to higher Cu, K, Ca and pH values. Nevertheless, winter samples were highly correlated with short-chain (C6:0), medium-chain (C10:0 and C12:0), and long-chain (C14:0 and C18:0) fatty acids and Zn. The PC2 separated three groups, and one contained the F7 winter and summer samples (lower-left quadrant) due to higher water content, water activity, and luminosity (L) (Fig. 2B and D). One group of samples (F9 from summer and winter) was found in the lower right quadrant because it correlated with physicochemical characteristics (protein and ash) and texture parameters (gumminess, chewiness, and stickiness). The third group was formed by most samples in the center, consisting of those with lower values for these measurements. The PC3 is related primarily to the color parameters (Fig. 2B and D). The F7 samples produced in winter and summer showed higher hue angles (h) without significant discrepancies than the other samples. The separation of these two samples is due to the lower values obtained for a* , b* , and C.

In this study, the existing distinctions between samples produced in winter and summer were investigated, and this parameter was well explained in PC1. However, other factors significantly influenced the differentiation between the samples and are embedded in the experiment, albeit not being evaluated in this experiment, including a) processing conditions and technologies; b) microbial composition of the "*pingo*"; c) characteristics of the raw material, among others. As a result, grouping by location of the production units studied was not observed.

## 4. Conclusion

New information on the physicochemical characteristics and texture properties of Canastra cheeses from different producers and seasons were provided. Since these are artisanal products, the differences between the samples from the 18 farms were already expected. Sodium, calcium, potassium, and magnesium are the main minerals in this type of product. The Canastra cheeses are rich in fatty acids, especially C16:0, and have low atherogenic and thrombogenic indexs and high desirable hypocholesterolemic fatty acids. Resilience was the only texture parameter that did not differ between the cheese samples. Furthermore, the principal component analysis could synthesize the interactions between the samples and variables studied, such as the difference between the cheese depending on the season (summer or winter), regardless of the production unit of origin. Given the above, most of the fatty acids detected herein and the minerals copper, potassium, and calcium are good markers for the differentiation of Canastra cheese produced in winter and summer. Therefore, this work provides relevant information to determine the identity, authenticity and quality of these products. Future studies are needed, including production units from all cities that are part of the region, in order to obtain a larger mapping of the profile of Canastra cheeses.

## CRediT authorship contribution statement

**Milton de Jesus Filho:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft. **Bruna Klein:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Luciano José Quintão Teixeira:** Methodology, Formal analysis. **Joyce Grazielle Siqueira:** Methodology, Formal analysis. **Juliana Azevedo Lima Pallone:** Methodology. **Roger Wagner:** Supervision, Writing – review & editing. **Helena Teixeira Godoy:** Project administration, Supervision, Resources, Funding acquisition, Writing – review & editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data Availability

Data will be made available on request.

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# Superficial scald development in 'Granny Smith' and 'Nicoter' apples: The role of key volatile compounds when fruit are stored under dynamic controlled atmosphere

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ABSTRACT

A positive correlation of α-farnesene and its oxidation metabolites with superficial scald is commonly reported in apples stored in air or controlled atmosphere (CA) systems, where $O_2$ levels are above the lower oxygen limit (LOL) tolerated by the fruit. Nevertheless, the LOL can be monitored by the dynamic controlled atmosphere (DCA) techniques and to provide different physiological responses. Therefore, this study aimed to evaluate key volatile metabolites from 'Granny Smith' and 'Nicoter' ('Kanzi®') apples stored under dynamic controlled atmosphere (DCA) monitored by respiratory quotient (RQ), i. e. at extremely low oxygen partial pressures (ELO) and correlate their emissions with the incidence of superficial scald (SS). The volatile compounds (VCs) were isolated by solid phase microextraction (HS-SPME) and analyzed by gas chromatography. For the first time, higher concentrations of α-farnesene and its oxidation metabolites (6-methyl-5-hepten-2-one, and 6-methyl-5-hepten-2-ol) were negatively correlated with SS in DCA-RQ. This is likely due to the higher levels of ethanol in fruit stored under this condition having an inhibitory effect on SS incidence even when α-farnesene, 6-methyl-5-hepten-2-one, and 6-methyl-5-hepten-2-ol accumulate. Additionally, SS is more correlated to internal ethylene concentration (IEC) than α-farnesene accumulation and their metabolites, even when fruit were stored under ELO, where ethylene action is reduced.

## 1. Introduction

Many studies on apples postharvest have reported the influence of the storage technology on overall quality of the apple, physiological disorders, and volatile compounds' profile in a controlled atmosphere (CA) (Eren et al., 2015; Sabban-Amin et al., 2011; Poirier et al., 2020; Tokala et al., 2021). More recent reports have expanded the studies in the relation to dynamic controlled atmosphere (DCA) after long-term storage (Anese et al., 2020a, 2020b; Both et al. 2017; Büchele et al., 2023; Donadel et al., 2019; Klein et al., 2021; Thewes et al., 2022; Weber et al., 2019; Wendt et al., 2023).

'Granny Smith' and 'Nicoter' ('Kanzi®') are popular apple varieties. After harvest, apples are generally stored under controlled atmosphere (CA). During and after CA storage, the main problem of 'Granny Smith' is superficial scald (SS) incidence (Bessemans et al., 2016; Grimm et al., 2012; Zanella, 2003). 'Nicoter' apple is a cross between 'Gala' and 'Braeburn' and is grown in Central Europe (Nicolai, 2006). 'Nicoter' has good acceptance by European consumers due to the high flesh firmness and higher total titratable acidity (TTA) than other varieties, which are characteristics relevant to the German and Dutch markets (Bonany et al., 2013). Adequate storage condition is essential to guarantee apple supply during the off-season. According to Kittemann et al. (2015) 'Nicoter'

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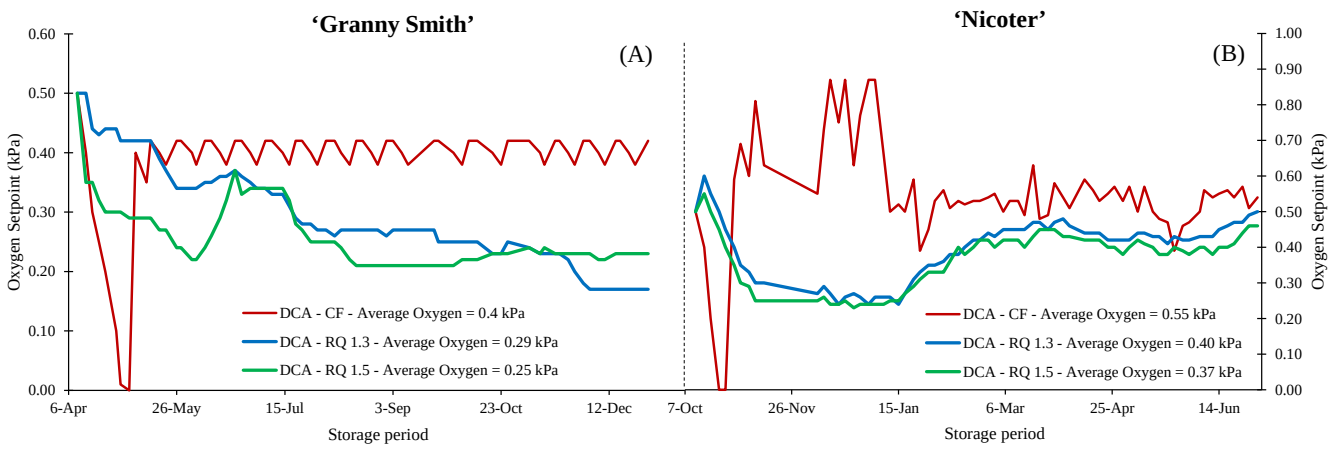



**Fig. 1.** Oxygen setpoint variation according to fruit metabolism based on chlorophyll fluorescence (DCA-CF) and respiratory quotient (DCA-RQ) of 'Granny Smith' (A) and 'Nicoter' (B) apples over 9 months of storage.

apples may be stored under refrigeration, between 1 and 3 °C, or with CA. These authors reported that fruit sensitivity to carbon dioxide increases with advancing fruit maturity and lower storage temperatures. Increased $CO_2$ sensitivity will increase the development of internal and external physiological disorders such as flesh breakdown and SS, respectively (Kittemann et al., 2015).

A high incidence of SS is generally associated with high ethylene production rates and α-farnesene accumulation (Bessemans et al., 2016; Lurie et al., 2005; Mditshwa et al., 2016; Zanella, 2003) as well as α-farnesene oxidation products, such as conjugated trienols (Ding et al., 2019). The use of postharvest antioxidant diphenylamine (DPA) was extremely efficient in controlling SS without significantly affecting ethylene metabolism (Lurie & Watkins, 2012). However, it was recently banned in EU countries (Larrigaudière et al., 2019), highlighting the need for evaluations of other strategies to prevent the development of SS. In this sense, lovastatin with combined treatments such as heat + oil and ethanol vapour + heat were used to control SS and gave the best results in terms of reduced α-farnesene accumulation in peel and control of superficial scald in 'Granny Smith' in comparison with DPA (Savran & Koyuncu, 2016). Exogenous resveratrol application had a similar effect in comparison with 1-methylcyclopropene (1-MCP) and DPA in maintaining quality and inhibiting superficial scald in 'Starkrimson' apples during storage and shelf life (Niu et al., 2018). Other authors have shown the application of 1-MCP also prevents scald development (Farneti et al., 2015; Larrigaudière et al., 2019), but has the disadvantage of reducing the apple aroma (Yang et al., 2016), as well as being a chemical treatment, and its application in organic production system is not allowed. Thus, apples storage with initial low stress oxygen (ILOS) or low oxygen partial pressures conditions has been investigated as an alternative to control SS (Sabban-Amin et al., 2011; Wang & Dilley, 2000; Zanella, 2003, Buccheri et al., 2021). However, the efficacy of extremely low oxygen partial pressures (ELO) in controlling SS in fruit subjected to DCA warrants further investigation.

Recently, studies have reported that the accumulation and application of 6-methyl-5-hepeten-2-one (MHO) induced SS in fruit stored at low temperatures (Farneti et al., 2015; Hui et al., 2016). The accumulation of ethanol and α-farnesene in fruit stored under CA in ultra-low oxygen (ULO; 0.7 kPa $O_2$) are also linked with SS incidence in pears (Larrigaudière et al., 2019). However, Bessemans et al. (2016) reported a reduction in SS incidence under ELO partial pressures based on respiratory quotient (DCA-RQ), despite Thewes et al. (2017) demonstrating that MHO tends to accumulate under the same conditions. A recent study has evaluated flesh breakdown, core breakdown, and cavity formation in 'Nicoter' apples stored in DCA-RQ1.5 with 0.7 kPa $CO_2$ (Weber et al., 2019), but key marker volatile compounds were not investigated in this study. Moreover, there are studies relating a comparison only DCA-RQ with CA or CA + 1-MCP or only dynamic controlled atmosphere monitored by chlorophyll fluorescence (DCA-CF) comparing with 'Granny Smith' apples stored in atmospheric air (Bessemans et al., 2016; Mditshwa et al., 2017b). Nevertheless, there are no studies on the tolerance of 'Nicoter' and 'Granny Smith' fruit stored with DCA-CF and DCA-RQ under extremely low oxygen (<0.4 kPa $O_2$) and the relationship with SS incidence and key volatile metabolites in these cultivars.

Given these conflicting results, it is paramount to evaluate the interactions among α-farnesene, MHO, 6-methyl-5-hepten-2-ol, ethanol accumulation, and SS incidence when 'Granny Smith' and 'Nicoter' apples are stored under ELO conditions (<0.4 kPa). Contrarily to CA storage, this relationship is not well understood in fruit stored in DCA. Future researches should focus on obtaining more insight into alternatives to chemical substances to reduced SS incidence and improve postharvest quality of apples under DCA. Therefore, the present study aimed to evaluate the accumulation of α-farnesene, MHO, 6-methyl-5-hepten-2-ol, ethanol, internal ethylene concentration (IEC), and SS incidence in 'Granny Smith' and 'Nicoter' apples stored under DCA monitored by DCA-CF and DCA-RQ, with two RQ levels: 1.3 and 1.5. Additionally, the interactions between these parameters and average oxygen levels with the incidence of SS were evaluated to verify if the accumulation of these compounds increases SS incidence when apples are stored under DCA, specifically under ELO partial pressures.

## 2. Material and methods

### 2.1. Sampling, setup, and atmosphere monitoring

'Granny Smith' apples were harvested from an orchard located in Vacaria, Rio Grande do Sul State, Brazil. Initial quality analyses were performed with 25 fruit per replicate (n = 4). Other samples of about 100 kg of apples were stored in small experimental chambers (0.18 $m^3$) at 1.5 °C (±0.1 °C) and relative humidity of 94 ± 2 % over the 9 months storage period. 'Nicoter' apples were harvested at the optimal maturity for long-term storage from commercial orchards at the Constance Lake region of Southwest Germany. At-harvest the starch pattern index (SPI, scale: 1 maximal starch to 10 fully hydrolyzed starch) was 3.8 for this cultivar. Immediately after harvest, fruit were transported to the Competence Centre for Fruit Growing at Lake Constance (KOB), Germany, and randomly allocated into replicates of 20 fruit (4 replicates/treatment), for each evaluation after storage. Samples were placed in 0.25 $m^3$ experimental gas-tight chambers. Three DCA conditions were tested for both cultivars: DCA-CF, DCA-RQ1.3, and DCA-RQ1.5. The $CO_2$ partial pressures ($pCO_2$) were constantly maintained at 1.2 kPa for 'Granny Smith' and 1.0 kPa for 'Nicoter' apples. The $pO_2$ varied

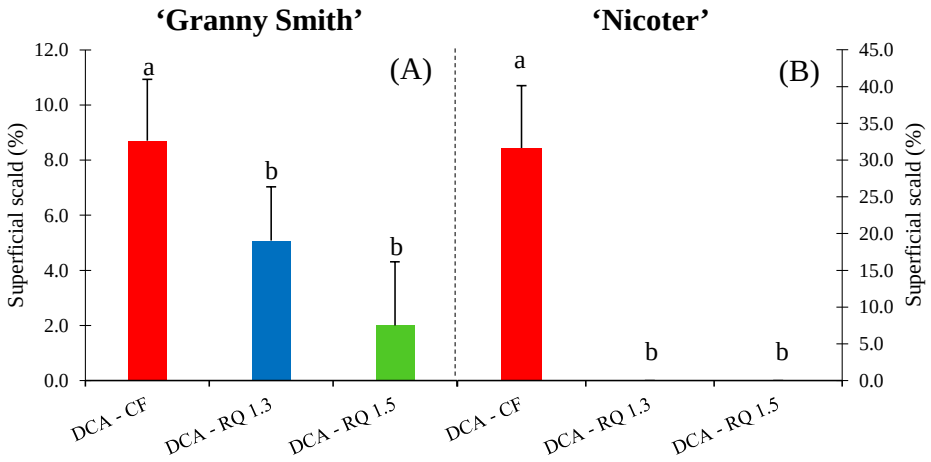



**Fig. 2.** Superficial scald incidence on 'Granny Smith' and 'Nicoter' apples after 9 months of storage under DCA-CF and DCA-RQ plus 7 d of shelf life at 20 °C. The columns accompanied by the same lowercase letters do not differ statistically between storage conditions by LSD test ($P < 0.05$).

according to CF and RQ protocols. Storage conditions were in accordance with Thewes et al. (2017). When the temperature reached the preset level (1.5 °C ± 0.1 for 'Granny Smith' and 3.0 °C ± 0.1 for 'Nicoter'), the chambers were flushed with nitrogen until reaching $pO_2$ of 5 kPa and then gradually reduced to 0.5 kPa over 5 d (reduction around 1 kPa per day with the respiration of the fruit). Thereafter, the oxygen set point was changed according to fruit metabolism for both cultivars (Fig. 1). The $pCO_2$ was obtained by its accumulation in experimental chambers due to fruit respiration.

In 'Granny Smith' experiment, the $pO_2$ and $pCO_2$ within the experimental chambers were monitored by circulating the air through a gas analyzer (Siemens, Ultramat 23, Germany) and corrected by an automatic DCA control system (Valis®, Lajeado, RS, Brazil). For 'Nicoter' apples, each chamber was connected to an automatic CA system (Isollcel®, Bolzano Italy), to continuously control the gas partial pressure setpoints. If the $pO_2$ was too low, cold atmospheric air was injected into the chamber and excess $CO_2$ absorbed with a hydrated lime scrubber.

#### 2.1.1. Monitoring the lowest oxygen limit (LOL) by DCA-CF and DCA-RQ

Throughout the storage period, $pO_2$ was altered according to fruit metabolism. In DCA-CF, 6 apples per cultivar were placed into plastic containers with the fluorescence sensor inside. Containers were placed inside DCA-CF storage chamber and the LOL monitored according to Prange et al. (2007). For DCA-RQ chambers, RQ was determined twice a week according to the method proposed by Weber et al. (2015). Thus, RQ was set at 1.3 and 1.5, and $pO_2$ changed to maintain this RQ. Chambers were closed for 13 to 14 h between the first and second determination of $pO_2$ and $pCO_2$ to calculate the RQ by the ratio between $CO_2$ production and $O_2$ uptake by the fruit.

### 2.2. Evaluated variables

Superficial scald and metabolites were evaluated after 9 months of storage under DCA plus 7 d of shelf life at 20 °C.

#### 2.2.1. Superficial scald incidence

Superficial scald was determined according to the method described by Lu et al. (2011) by the means of quantifying the fruit with symptoms of the disorder. The results are expressed as a percentage of the fruit with scald.

#### 2.2.2. Internal ethylene concentration (IEC)

Internal ethylene concentration was evaluated by withdrawing the internal air of the fruit. Measurements were carried out according to the method proposed by Mannapperuma et al. (1991) with slight modifications. Two samples of the internal air (1 mL) of each replicate were injected into a Varian Star 3400CX gas chromatograph (CA, USA) using chromatographic conditions described by Both et al. (2018). Results were expressed in μg $L^{-1}$.

#### 2.2.3. Volatile compound (VCs) analysis of apple juice

This evaluation was performed to obtain ethanol, α-farnesene, 6-methyl-5-hepten-2-one and 6-methyl-5-hepten-2-ol concentrations. The fruit were cooled to 0.5 °C and the juice prepared and extracted according to Both et al. (2014). The juice was immediately stored at −30 °C until GC analyses. The VC extractions were carried out by HS-SPME, using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/Car/PDMS; Supelco, 50/30 μm × 20 mm) fiber phase. Samples were thawed in tap water for 1 min with the temperature not exceeding 5 °C. A 10 mL aliquot of this juice was placed inside a 20 mL vial following the addition of 3 g of NaCl and 10.0 μL of a 3-octanol standard solution (82.2 μg $mL^{-1}$). Then, the vial was sealed with a screw cap and septum coated with PTFE/silicone. The vial was submerged in a water bath at 35 °C for 15 min without fiber exposition. Afterwards, the fiber was exposed to the headspace of the sample for 60 min under constant stirring at the same temperature for VCs sorption and submitted to GC analysis.

The VCs were identified and quantified on a gas chromatograph coupled to a mass spectrometer (Shimadzu QP2010 Plus - GC/MS, Shimadzu Corporation, Kyoto, Japan). The chromatographic conditions employed were according to Thewes et al. (2017) with slight modifications. The fiber was desorbed at 250 °C for 10 min and the injection split ratio was 1:5. The compounds were separated on ZB-WAX polar phase capillary column (Zebron, Phenomenex, USA; 30 m × 0.25 mm × 0.25 μm). Helium was used as carrier gas at a constant flow 1.2 mL $min^{-1}$. The column temperature began at 35 °C, holding for 3 min, and increased from 2 °C $min^{-1}$ to 80 °C and then increased from 5 °C $min^{-1}$ to 230 °C. The column temperature remained isothermal for 5 min. The GC/MS interface and MS ion source temperature were maintained at 230 °C. The detector operated in the electron impact ionization mode with ionization energy of + 70 eV and scan mass range from 35 to 350 *m/z*. A series of homologous n-alkanes was analyzed under the same conditions to calculate the linear retention index (LRI). The VCs were identified by comparing their mass spectra with those of the GC/MS library spectra bank (NIST 5.0) and comparing the experimental LRI with those found in the literature (Acree and Heinrinch, 2019; El-Sayed, 2019). Quantification was performed with the use of 3-octanol as the internal standard (Both et al., 2014).

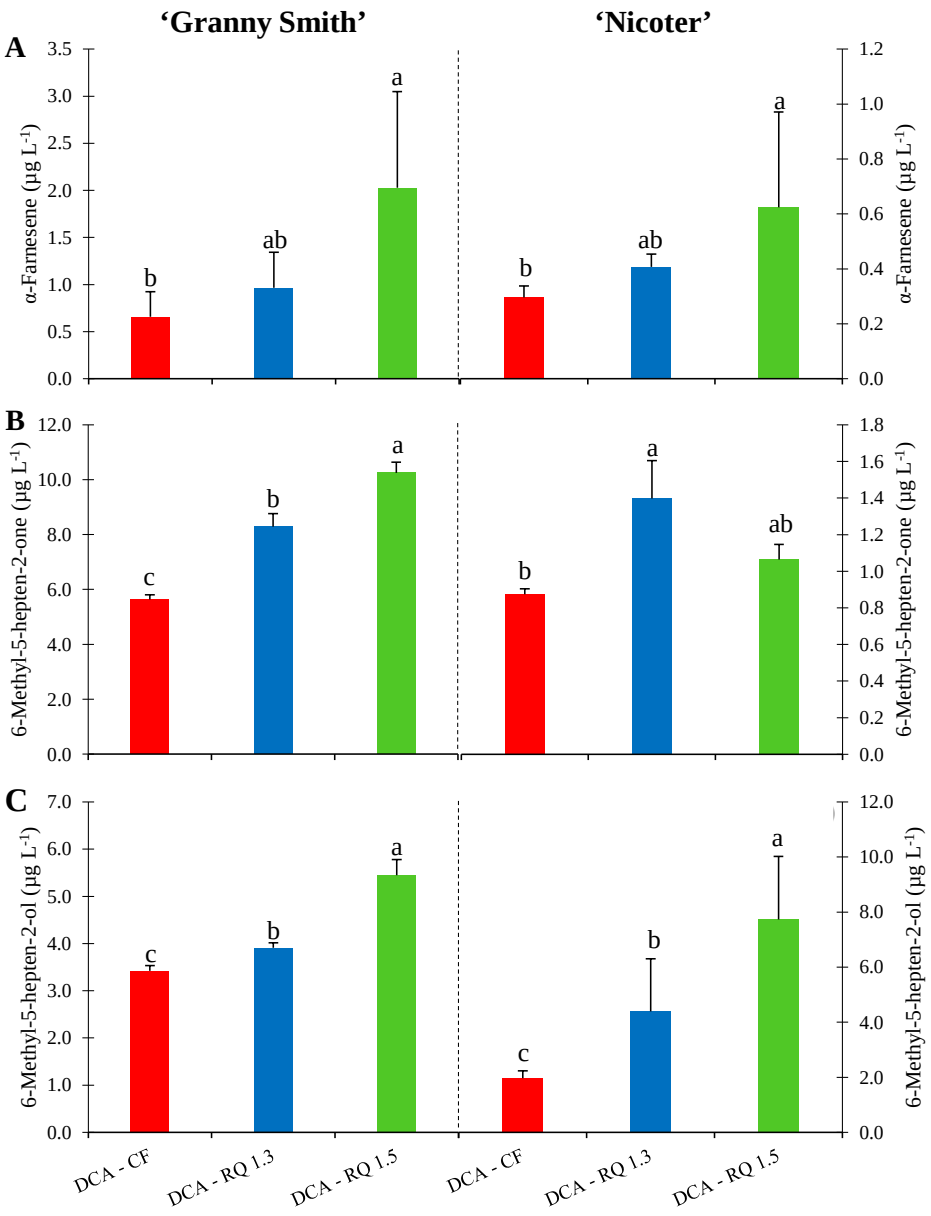



**Fig. 3.** α-Farnesene (A), 6-Methyl-5-hepten-2-one (B), and 6-Methyl-5-hepten-2-ol (C) concentrations in 'Granny Smith' and 'Nicoter' apples after 9 months of storage under DCA-CF and DCA-RQ plus 7 d of shelf life at 20 °C. The columns accompanied by the same lowercase letters do not differ statistically between storage conditions by LSD test ($P < 0.05$) for each compound of both cultivars.

### *2.3. Statistical analysis*

The experimental data were submitted to analysis of variance (ANOVA) followed by the LSD-Fisher's test at 5% error probability ($P < 0.05$). A Pearson's correlation analysis between average oxygen, IEC, and the concentration of VCs emitted by apple juice with superficial scald incidence was performed ($P < 0.05$).

## 3. Results and discussion

A lower incidence of superficial scald was found for storage under DCA-RQ1.5 in comparison to DCA-CF for 'Granny Smith' and this disorder was absent in 'Nicoter' apples stored in DCA-RQ1.3 and DCA-RQ1.5 (Fig. 2A and B). In contrast, higher α-farnesene, and 6-methyl-5-hepten-2-ol concentration (Fig. 3A and C), and also a higher ethanol production was observed in DCA-RQ1.5 in both cultivars, differing from DCA-CF storage (Fig. 4A). MHO, an oxidation product of α-farnesene, was also in higher concentration in 'Granny Smith' apples stored with lower $pO_2$ (DCA-RQ1.5), differing from DCA-RQ1.3 and DCA-CF. However, in 'Nicoter' apples MHO concentration differed only between DCA-RQ1.3 (higher) and DCA-CF (lower concentration), and in DCA-RQ1.5 there was an intermediate concentration (Fig. 3B). The possible hypothesis for this phenomenon may be the influence of the cultivar, and more studies are needed to better understand this statement. Studies have reported that apple storage in DCA-RQ reduced SS incidence compared to CA storage, although the concentration of the VCs such as α-farnesene and MHO were not evaluated, with the exception of ethanol (Bessemans et al., 2016; Tran et al., 2015; Weber et al., 2015). Furthermore, recent studies have evaluated the influence of DCA-CF on α-farnesene and MHO concentration in 'Granny Smith' apples (Mditshwa et al., 2017b, c), compared to regular air (RA) storage and its relationship with SS development. However, as far as we know, this is the first study that relates these volatile compounds to DCA-RQ storage, which uses extremely low $pO_2$ (averages $\leq$ 0.30 kPa for 'Granny Smith' and $\leq$ 0.40 kPa for 'Nicoter') and compares them to DCA-CF (averages 0.40 kPa $O_2$ for 'Granny Smith' and 0.55 kPa $O_2$ for 'Nicoter') (Fig. 1).

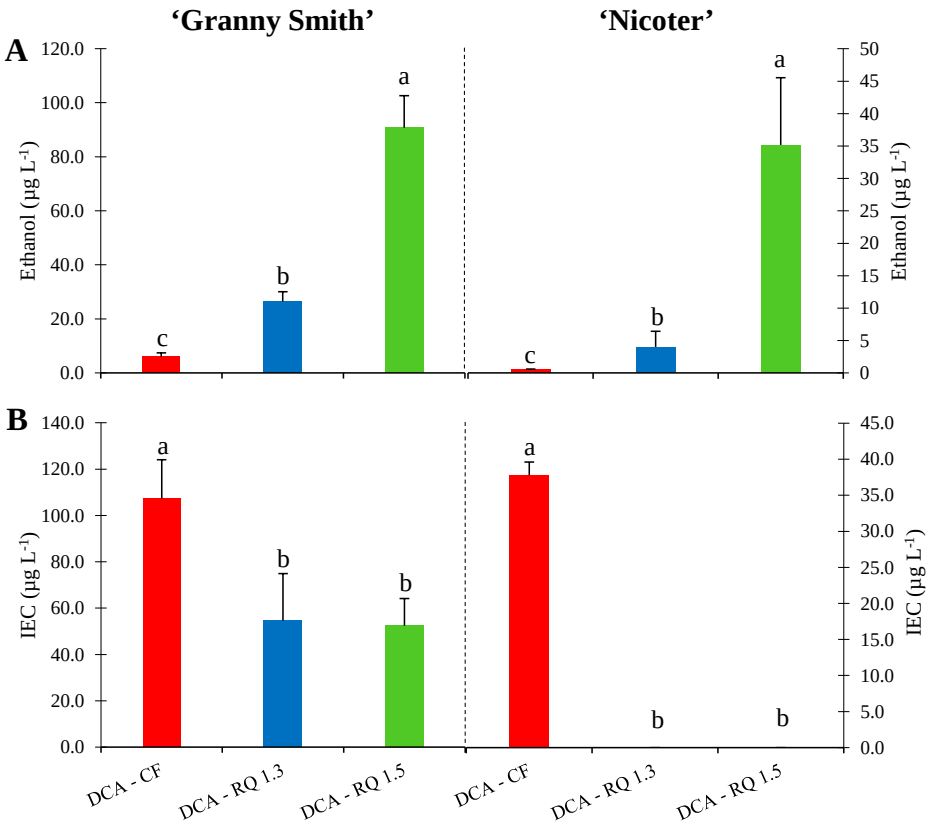



**Fig. 4.** Ethanol concentration (A) and internal ethylene concentration – IEC (B) in 'Granny Smith' and 'Nicoter' apples after 9 months of storage under DCA-CF and DCA-RQ plus 7 d of shelf life at 20 °C. The columns accompanied by the same lowercase letters do not differ statistically between storage conditions by LSD test ($P < 0.05$) for each compound of both cultivars.

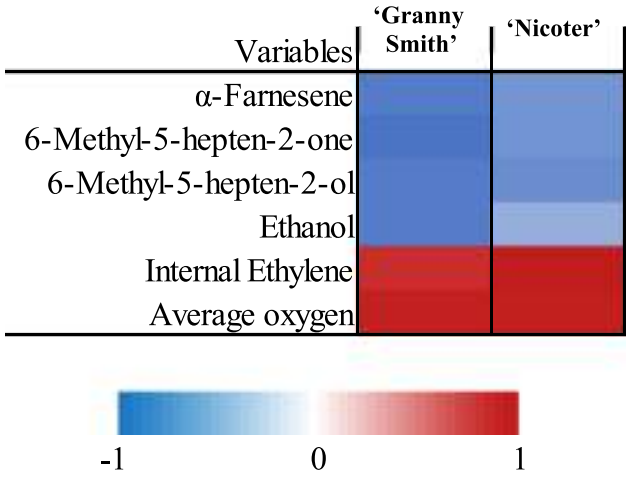



**Fig. 5.** Pearson's Correlation among volatile metabolite, internal ethylene and average oxygen with superficial scald incidence on 'Granny Smith' and 'Nicoter' apples after 9 months of storage under DCA-CF and DCA - RQ plus 7 d of shelf life at 20 °C.

Here, higher concentrations of α-farnesene, MHO, and 6-methyl-5-hepten-2-ol were not associated with SS incidence when 'Granny Smith' and 'Nicoter' apples were stored under DCA-RQ1.3 and DCA-RQ1.5. In the literature, it is reported that these compounds are positively correlated to SS disorder when fruit are stored in RA and CA (Hui et al., 2016). However, an inverse response at ELO partial pressures (DCA-RQ storage) was found in this work. The volatiles mentioned above were negatively correlated with SS when fruit were stored under DCA-RQ, whereas internal ethylene concentration and average oxygen partial pressure had positive correlation (Pearson's Correlation) with SS incidence (Fig. 5). The ultra-low $O_2$ (ULO) pre-treatment (0.2–0.5 kPa $O_2$ and < 0.5 kPa $CO_2$) during 10 d at 20 °C in 'Granny Smith' apple was evaluated, and authors found that this condition suppressed conjugated trienols and internal ethylene production in relation to fruit stored directly at 0 °C in air atmosphere for the same period of time (control fruit), resulting in total control of SS during ripening after 90 and 150 d of storage at 0.5 °C (Zoffoli et al., 2018). However, other mechanisms different from ethylene may be involved in scald development because the same storage conditions (10 d at 20 °C) without ULO had the same level of scald as fruit exposed to pre-treatment ULO (Zoffoli et al., 2018). However, fruit without ULO had considerably higher internal ethylene concentration, and maintained the suppression of conjugated trienol formation (Zoffoli et al., 2018).

The ELO partial pressures employed in DCA-RQ (Fig. 1) resulted in ethanol concentrations 5 to 10-fold higher than DCA-CF and lower internal ethylene concentration (Fig. 4A, 4B), which decreased SS incidence, even with the highest levels of α-farnesene and MHO (Fig. 3A, 3B). Scald incidence was also evaluated in Blanquilla pears stored under a very low $pO_2$ (0.7 kPa –ULO) (Larrigaudière et al., 2019). Despite this $pO_2$ level being much higher than the concentration used in the present experiment, ethylene production and α-farnesene oxidation products were partially inhibited (CTols) compared to 1-MCP-treated fruit and CA storage. This resulted in very low SS incidence in ULO treated pears, which was comparable to 1-MCP-treated fruit (Larrigaudière et al., 2019). Furthermore, Larrigaudière et al. (2019) reported that ethanol levels increased earlier in ULO treated pears than in fruit stored in CA. This response led the authors to assume that the difference in ethanol accumulation time is likely an important parameter that determines scald sensitivity in ULO-treated pears (Larrigaudière et al., 2019). Wang & Dilley (2000) found a negative correlation between ethanol levels and

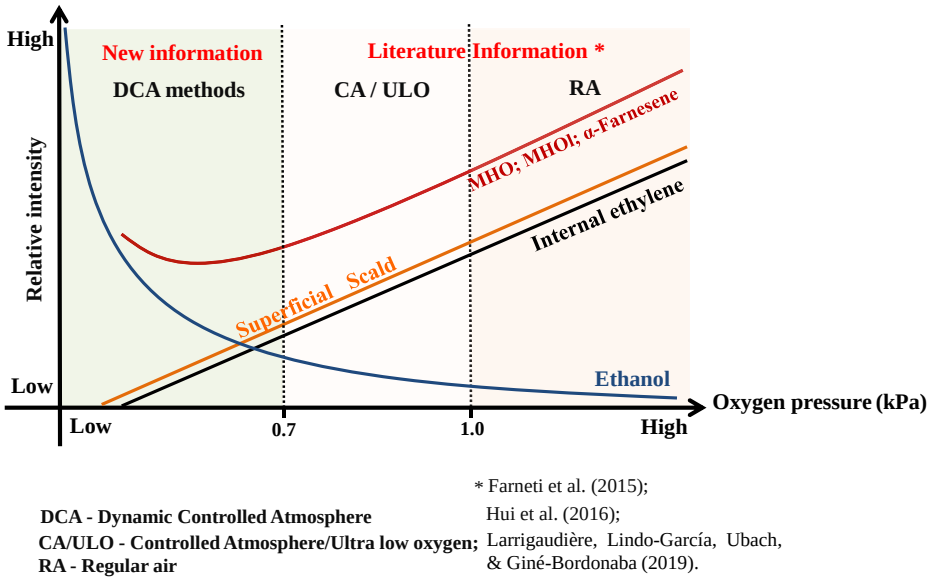



**Fig. 6.** Relationship between metabolites and superficial scald using CA/ULO, RA, and DCA (extremely low oxygen) methods under different $O_2$ concentrations, were MHO: 6-Methyl-5-hepten-2-one; MHOl: 6-Methyl-5-hepten-2-ol.

SS in apples treated with initial low oxygen content (ILO), suggesting a potential antioxidant role of ethanol in SS control. Scott et al. (1995) described similar results when applying ethanol vapor to 'Granny Smith' apples. Moreover, researchers reported that ethanol vapor administered with CA prevented scald development during four months at 1.0 °C, followed by one month in cold air storage plus one week at 20 °C of shelf life (Chervin et al., 2001).

The results found here indicate that SS incidence in 'Granny Smith' and 'Nicoter' apples is not strictly correlated to higher α-farnesene, MHO, and 6-methyl-5-hepten-2-ol concentrations. The higher ethanol concentration, which is due to anaerobic metabolism, was more prevalent in the DCA-RQ1.5 condition. This may have reduced SS incidence even with the presence of high α-farnesene, MHO, and 6-methyl-5-hepten-2-ol concentrations. In another research with 'Granny Smith' apples, the application of a low-oxygen ($LO_2$) atmosphere of 0.5 kPa for 10 d at 20 °C prior to cold storage at 0 °C was effective in preventing SS symptoms compared to those stored in RA as control treatment (Pesis et al., 2012). These authors demonstrated that the fruit had a nice green peel color and SS symptoms were not detected after 6 months of storage at 0 °C plus 7 d at 20 °C. In addition, they reported that $LO_2$-pretreated apples remained healthier due to reduced ethylene production and reduced α-farnesene oxidation product formation, which appear as reactive oxygen species (ROS) during cold storage and enhance peel damage in 'Granny Smith' apples (Pesis et al., 2012). According to Pesis et al. (2007) and Pesis et al. (2010), endogenous ethanol levels are known to increase when apples are stored under low oxygen levels and likely contribute to reduced SS development. This may be explained by the high free radical removal capacity conferred by ethanol, in order to hinder the cold-induced accumulation of ROS (Chanjirakul et al., 2007). A similar response may have occurred with 'Granny Smith' apple stored in DCA-RQ1.5, since this fruit had approximately 4-fold lower percentage of scald, and more than 10-fold higher ethanol concentration compared to DCA-CF. Thus, the ethanol possibly acted as an antioxidant, resulting in a smaller presence of ROS, inhibiting the browning of tissues and the incidence of scald disorder. The accumulation of ROS was much lower when fruit were submitted to previous treatment with low $pO_2$, ethanol vapour, and 1-MCP, followed by refrigerated storage at 0 °C plus 7 d at 20 °C of shelf life than in control fruit (imediatally stored at 0 °C) preventing SS (Sabban-Amin et al., 2011). A recent study reported the evolution of antioxidant capacity, phytochemical content, and ROS in the peel of DCA-CF stored 'Granny Smith' apples (Mditshwa et al., 2017a). According to these authors, fruit stored in DCA-CF (0.5 kPa $O_2$ + 1.0 kPa $CO_2$) were characterized by higher antioxidant capacity, ascorbic acid, and total phenolic concentration compared to regular atmosphere as the control treatment (RA). On the other hand, lipid peroxidation and ROS were significantly lower in fruit stored in this treatment.

DCA-RQ is a promising storage technique that is currently recommended for apple storage, while DCA-CF is more widely used in commercial storage. Both techniques have shown to retain better fruit quality without additional chemical treatments compared to conventional CA storage. In DCA-RQ storage, the higher RQ results in lower $pO_2$, which may induce more ethanol accumulation in the fruit tissue. This shift of aerobic to anaerobic metabolism affects the dynamics of many compounds related to SS in apples, as reported in this study. In order to facilitate the understanding of this dynamics, was performed a schematic representation (Fig. 6) that shows the trend of each compound evaluated here, comparing them with data from literature in RA or CA and, the new issues presented in the present study, the interaction between VCs and SS under DCA methods. In this figure it is possible to observe that at $pO_2$ employed in controlled atmospheres covering the range above 0.7 kPa (CA/ULO and RA) there is a higher incidence of surperficial scald, MHO, MHOl internal ethylene, and α-farnesene, but with low ethanol emission. As the $pO_2$ decreases, these metabolites are reduced, until a point of inflection (<0.4 kPa downwards) where there are higher MHO, MHOl and α-farnesene emissions, however the surface scald tends to zero when DCA is employed using extremely low concentrations (<0.25). At the same time, there is a high concentration of ethanol due to anaerobic metabolism.

## 4. Conclusion

The present study demonstrated, for the first time, that α-farnesene, MHO, and 6-methyl-5-hepten-2-ol accumulation are inversely correlated to superficial scald when 'Granny Smith' and 'Nicoter' apples when stored under DCA-RQ with an oxygen partial pressure below 0.4 kPa. This is likely a result of higher ethanol accumulation due to anaerobic metabolism when fruit were stored in DCA-RQ. Moreover, the action of ethylene is lower but not completely suppressed at ELO conditions. Therefore, the results suggest that SS is more correlated to IEC and higher presence of oxygen in the storage chamber than the accumulation of α-farnesene, MHO, and 6-methyl-5-hepten-2-ol, even when fruit were

stored under ELO partial pressures. Storage under DCA-RQ was more efficient than DCA-CF in controlling SS after long-term storage (9 months) plus 7 d of shelf life for both apple cultivars.

## CRediT authorship contribution statement

**Jossiê Zamperetti Donadel:** Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Validation, Writing – review & editing. **Fabio Rodrigo Thewes:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. **Luana Ferreira dos Santos:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. **Erani Eliseu Schultz:** Methodology, Formal analysis, Writing – review & editing. **Magno Roberto Pasquetti Berghetti:** Methodology, Formal analysis, Writing – review & editing. **Vagner Ludwig:** Methodology, Formal analysis, Writing – review & editing. **Juliana Mesadri:** Methodology, Formal analysis, Writing – review & editing. **Bruna Klein:** Methodology, Formal analysis, Writing – review & editing. **Flavio Roberto Thewes:** Methodology, Formal analysis, Writing – review & editing. **Suele Fernanda Prediger Schmidt:** Methodology, Formal analysis, Writing – review & editing. **Vanderlei Both:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. **Auri Brackmann:** Conceptualization, Methodology, Resources, Writing – review & editing. **Daniel Alexandre Neuwald:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – review & editing. **Roger Wagner:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing – review & editing, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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# Higher $CO_2$ during controlled atmosphere storage of unshelled 'Barton' pecans or carnauba wax coating: Effect on the quality after long-term storage at two temperatures

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ABSTRACT

Pecan nuts, a healthy food, have shown an increased demand for consumption. Therefore, there must be a certain level of care to avoid quality losses, which are primarily influenced by storage conditions and time. This study evaluates the effects of long-term controlled atmosphere (CA) storage with low $O_2$ partial pressure ($pO_2$ – 2 kPa), combined or not with high $CO_2$ ($pCO_2$ – 40 or 80 kPa), carnauba wax coating (CW), and ambient atmosphere (AMB; control) at 10 and 20 °C, on unshelled 'Barton' pecan nut quality. Color, water activity ($A_w$), moisture content (MC), and oxidation markers, such as peroxide value (PV), acidity value (AV), TBARS, and volatile compounds (VC) were evaluated. Storage up to twelve months at 10 °C and with CA (regardless of the temperature) ensured higher luminosity and color parameter b* ("golden") and a lower a* parameter ("reddish"). The MC ranged from 2.8 to 3.6%, irrespective of storage conditions, for up to twelve months, which is suitable. The AMB at 10 °C ensured lower AV, TBARS, and PV compared to the CW treatment. Furthermore, CA with low $pO_2$ and high $pCO_2$, even at 20 °C, guaranteed lower AV, TBARS, and PV. Storage at 20 °C increased characteristic VCs of lipid oxidation (aldehydes, acids, alcohols, ketones, lactones, and esters, especially with CW coating). However, all conditions at 10 °C and with $pCO_2$ (even associated at 20 °C) reduced the presence of these VCs. The CW used as a coating on the pecans did not show satisfactory results and should not be recommended for pecan storage at these applied conditions. Our findings showed that low $pO_2$ and high $pCO_2$ maintain better pecan quality than $O_2$ ambient (20 kPa), even at temperatures above refrigeration (20 °C). Nevertheless, there were no significant differences between 40 and 80 kPa $CO_2$.

## 1. Introduction

The pecan nut [*Carya illinoinensis* (Wanghen) K. Koch] is a dry fruit and one of the most important nuts in commercial production and human consumption (INC, Internacional, 2018). In southern Brazil, pecan crops have been produced in Rio Grande do Sul State for several decades, with favorable climate conditions and annual production of ~ 4,000 tons, which is over 50% of Brazilian production (Fronza et al., 2018; Lesina, 2020; ABNC, 2021). The 'Barton' pecan is the most produced in Rio Grande do Sul State and has a fat content of about 60%, with roughly 90% unsaturated fatty acids (UFA). The fatty acid profile presents oleic (C18:1n9; ~63%), linoleic (C18:2n6; ~23%), and linolenic acids (C18:3n3; ~1%) as major compounds; which make pecan nuts a valuable food (Ribeiro et al., 2020a). Hence, given its high lipid and unsaturated composition, pecan storage studies are paramount due to its susceptibility to oxidative deterioration.

Consuming oils and products containing hydroperoxides can cause food toxicity and induce various diseases, such as cancer, atherosclerosis, and Alzheimer's disease (Kanazawa et al., 2002; Gotoh & Wada, 2006; Corsinovi et al., 2011). Furthermore, improper storage conditions favor microbial growth and jeopardize pecan quality as they are susceptible to fungal development (mold) (Ribeiro et al., 2021), which is often related to potential toxin producers.

Controlled atmosphere (CA) storage prolongs the shelf life of various

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fruits and nuts because it efficiently reduces biochemical activities, including oxidative reactions (Brackmann et al., 2001; Messina et al., 2015; Ribeiro et al., 2020a, 2020b, 2021, 2022), and researchers have investigated CA techniques with pecans (Ribeiro et al., 2020b, 2021, 2022; Thewes et al., 2021). These studies have reported improvements in the quality of whole pecans for up to twelve months under low $pO_2$ storage conditions and 6 months under low $pO_2$ associated with high $pCO_2$ for unshelled nuts (Ribeiro et al., 2020b, 2022; Thewes et al., 2021).

Ribeiro et al. (2020b) observed that changes in pecans due to oxidative reactions, such as browning, peroxide, and acidity values, were minimized when $pO_2$ was reduced (1 and 3 kPa), despite a higher storage temperature (20 °C). Thewes et al. (2021) evaluated the effect of the moisture content (MC) associated with low $pO_2$ in 'Barton' and 'Jackson' pecan storage; this study evidenced that treatment with 6% MC + 2 kPa $O_2$ presented the highest hue and luminosity values (i.e., lighter color) after eight and twelve months of storage. However, when the nuts were stored with 6% MC + 20.9 kPa $O_2$, the highest internal browning incidence, severity, and respiration rates were observed. In line with atmosphere modification, studies have shown that high $CO_2$ levels act directly on the respiration rate, color parameters, firmness, and volatile compound production (Maskan & Karataş, 1999; Zhang & Watkins, 2005). Ribeiro et al. (2022) reported that low $pO_2$ (2 kPa) associated with high $pCO_2$ levels (15 kPa) also led to lower acidity value (AV), and the color parameter b* (yellow) remained high in treatments with CA until six months of storage of shelled 'Barton' pecans, even at a storage temperature of 20 °C. Furthermore, the CA with 15 kPa $CO_2$ ensured the absence and/or low contamination of *Aspergillus* sp. and *Penicillium* sp. after two months of storage and *Cladosporium* sp. up to 4 months of storage at 10 °C (Ribeiro et al., 2021). Thus, these studies showed positive trends in using high $pCO_2$ during storage in CA of shelled pecans; this effect applied to pecan nuts must be better investigated in prolonged storage and unshelled pecans. To the best of our knowledge, no studies have tested long-term storage of whole pecans using CA with high $CO_2$ (>15 kPa) and low $pO_2$, as well as their efficiency at different temperatures, including ambient temperature conditions (20 °C).

Fruit coatings are also valuable in providing additional protection to intact or fresh-cut fruit by forming a semipermeable barrier that reduces the water vapor permeability and inhibits microbial adherence and growth (Motamedi et al., 2018; Marín et al., 2021). Lipid-based coatings derived from natural sources, such as carnauba wax, can create a barrier against water vapor diffusion and provide gloss for fresh fruits, increasing the shelf-life and quality maintenance during storage (Ncama et al., 2018); delayed browning and tissue softening are one of the most notable benefits (Miranda et al., 2022).

Given the above, this study sought to evaluate the effects of low $pO_2$ (2 kPa), isolated or combined with high $pCO_2$ (40 and 80 kPa $CO_2$), and carnauba wax coating at different storage temperatures (10 and 20 °C) on 'Barton' pecans for up to twelve months and evaluate the physicochemical quality and volatile profile.

## 2. Material and methods

### 2.1. Sample origination and pre-treatment

'Barton' pecans were obtained from a commercial orchard in Cachoeira do Sul (30°02′21"S, 52°53′38"W), Rio Grande do Sul State, Brazil, in 2018 harvest season. Approximately 30.0 kg of pecan nuts were collected at full harvest (may month) and transported to the Post-Harvest Research Center at the Federal University of Santa Maria. The drying was performed by placing the pecan nuts in thin layers of perforated plastic fruit crates under ambient conditions (10–20 °C) and 60% relative humidity for 10 days, until the kernels reaching near to 3% of moisture content. Nuts with signs of insect attack, visible fungal growth, breakages, or poorly developed-looking nuts were discarded. The nuts were stored in shell.

### 2.2. Storage conditions

The pecans were stored according to Ribeiro et al. (2020b), with modifications. About 3.0 kg of unshelled pecans were placed in airtight 20-liter containers adapted to allow air to circulate through two hoses to an external gas analyzer. Different $pO_2$ and/or $pCO_2$ conditions were adjusted in each container. The containers of each atmospheric condition treatment were placed inside cold rooms, with different temperatures (20 and 10 °C). For each temperature, was stored a container with pecans at [1] ambient air (AMB), [2] covered with carnauba wax (CW), [3] in controlled atmosphere (CA) with 2.0 kPa $O_2$ + 0.04 kPa $CO_2$ ($2pO_2$), [4] 2.0 kPa $O_2$ + 40 kPa $CO_2$ ($40pCO_2$), and [5] 2.0 kPa $O_2$ + 80 kPa $CO_2$ ($80pCO_2$) (For better explanations see Supplementary Material 1).

During the entire storage period, the temperature control in cold rooms was carried out by electronic thermostats and checked daily with a mercury thermometer located inside the chambers. Relative humidity was kept below 50%. The reduction of $pO_2$ inside containers was obtained by flushing with $N_2$, that was produced by a generator using PSA (Pressure Swing Adsorption) principle. In experimental container with high $pCO_2$, this condition was obtained by flushing them with $CO_2$ from high-pressure cylinders (99.9%; White Martins, Brazil). To monitor and correct the gas composition, two hoses connected the experimental containers to a gas analyzer (Siemens®, Ultramat 23, Germany). A pump and electronic valves allowed the air to circulate from the storage containers to the gas analyzer. Determinations were made twice a week (Supplementary material 2). Treatments AMB and CW were not airtight closed, in order to allow gases exchange with the surrounding atmosphere. The storage was carried out for twelve months, and the analyses were performed at the initial time (after drying, time zero), six and twelve months. For each sampling point, 1.40 kg of nuts were removed for analysis.

#### 2.2.1. Wax application

In treatments with coating film, Aruá carnauba wax was applied (concentration of 15%). This product was prepared on a tray and the pecans remained in contact with the wax for approximately 1 min, under manual agitation, until a film was formed around their shell. Afterwards, they were placed in thin layers in punctured plastic boxes, remaining for 48 h in an environment with air circulation to allow the drying of the applied product. After drying, they were placed in 20 L plastic containers at different temperatures (10 and 20 °C), where they remained throughout the storage period.

### 2.3. Quality analyses

#### 2.3.1. Sample preparation for chemical analysis

To carry out the evaluations, the pecans were peeled and ground in a knife mill (Marconi 630/1, São Paulo, Brazil) before each analysis. The oil was obtained according to the Hara & Radin (1978) method with modifications according to Ribeiro et al. (2020a).

#### 2.3.2. Color analysis

The analysis was performed according to Ribeiro et al. (2020b). After peeling, the kernel color evaluation of the pecan was carried out with a colorimeter (Konica Minolta CR-310, Osaka, Japan), obtaining values of the coordinates L* (measurable in terms of white to black intensity), a* (measurable in terms of intensity from red to green), b* (measurable in terms of yellow and blue intensity), C (chroma, saturation) and H (hue angle).

#### 2.3.3. Moisture content and water activity

The MC of pecan nuts was determined in oven method (AOAC 925.10, 2012), with forced air circulation at a temperature of 105 ± 1 °C

until reaching constant weight. The analyses were performed in triplicate. The water activity ($A_w$) analysis was performed according to Ribeiro et al. (2020b), using directly 2.0 ± 0.1 g each, of ground sample inside the water activity meter (Aqualab Series 4 TEV, USA), performed in triplicate, at 25 ± 1 °C.

### 2.3.4. Acidity value

The determination of AV was carried out using "Cd 3d-63" method of AOCS (2009), with modifications, according to Ribeiro et al. (2020b), using 1 g of pecan oil. The results were expressed in g of oleic acid 100 $g^{-1}$ oil. The analyses were performed in triplicate.

### 2.3.5. Peroxide value

The PV was determined by the International Dairy Federation (IDF) method 74A:1991 (Shanta & Decker, 1994). The results were expressed in meq of $O_2$ $kg^{-1}$ oil. The analyses were performed in triplicate.

### 2.3.6. Lipid oxidation marker - TBARS

The lipid oxidation was determined by the 2-thiobarbituric acid reactive substances (TBARS) assay, as described by Bruna, Ordonez,

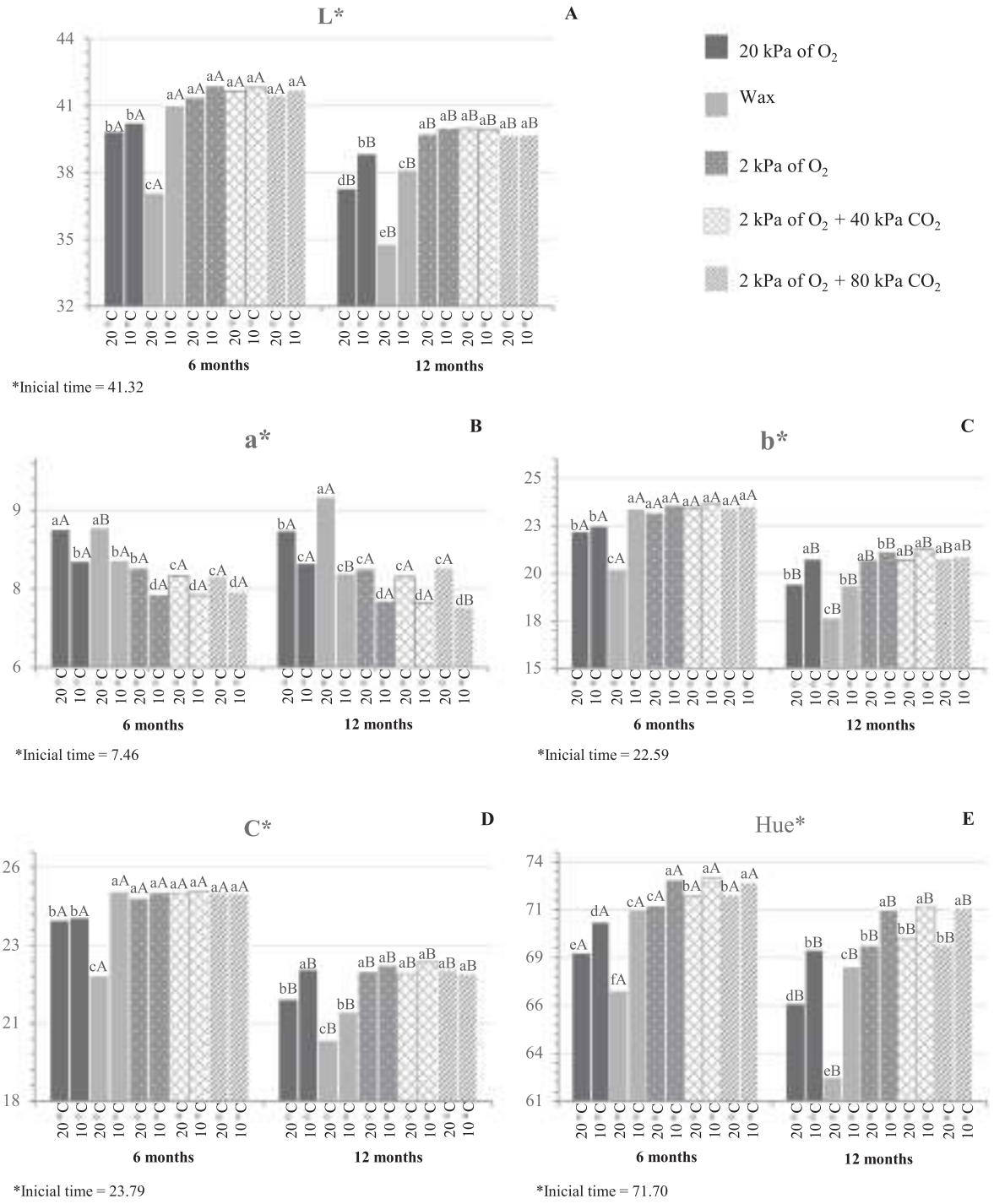



**Fig. 1.** Color parameters of the unshelled Barton pecan nut after six and twelve months of storage with different $pO_2$, $pCO_2$, carnauba wax and temperatures. The mean values followed by the same lowercase letter within each assessed period, and upper case at columns, of the same storage condition did not differ according to the Scott Knott test ($p < 0.05$).

Fernández, Herranz, and Hoz (2001). The results were expressed in mg of malonaldehyde $kg^{-1}$, and were determined in triplicate at six and twelve months of storage.

#### 2.3.7. Volatile compounds analyses

Volatile compounds were extracted by solid phase microextraction technique applied to the headspace (HS-SPME) of vials containing samples, and determined by gas chromatography coupled to a mass spectrometer (Shimadzu GC/MS QP2010 Plus; Shimadzu Corporation, Tokyo, JP) according to Ribeiro et al. (2020a).

#### 2.3.8. Statistical analysis

Data were submitted to analysis of variance (ANOVA) and the means adjusted by the least squares were compared by Scott Knott test with 5% significance by Sisvar Software (Version 5.6, Build 86). In addition, a Principal Component Analysis (PCA) were performed for an exploratory analysis using Pirouette 3.11 software (Infometrix, Woodinville, USA), and heatmap analysis and hierarchical cluster analysis (HCA) were carried out in the Metaboanalyst platform (Xia Lab @McGill, Quebec, Canada) to grouping variables and samples. For both analysis, data matrix was preprocessed by auto-scaling for each variable to obtain the same weight (mean = 0 and variance = 1) (Ribeiro et al., 2000).

## 3. Results and discussion

### 3.1. Color

Color is an important parameter related to storage quality control because brown pigments increase with the progress of oxidation reactions and reduce consumer acceptance (Pannico et al., 2015; Lima et al., 2020; Ribeiro et al., 2020a). The luminosity (L*) of pecan kernels at harvest (initial time) was 41.32 (Fig. 1A), and it decreased under all conditions at the end of the storage period (twelve months). After six months, the lowest L* value was in CW-20, indicating a darker color, although the CW-10 did not differ from treatments under CA. After twelve months, the L* value decreased, albeit the nuts stored under CA maintained the highest values compared to AMB and CW. CW-20 had the lowest L* value, while nuts stored without CA (AMB and CW) at 10 °C presented higher values than at 20 °C ($p < 0.05$). In general, the L* value of kernels tended to decrease during the storage period (Lima et al., 2020; Ribeiro et al., 2020b). According to Ribeiro et al. (2020b), unshelled pecans at six months of storage under ambient conditions (20 kPa) and 20 °C presented the lowest L* value compared to reduced $pO_2$ conditions (1 and 3 kPa). The highest L* values were also found in 'Barton' and 'Jackson' pecans stored under low $O_2$ conditions (4% MC + 2 kPa $O_2$) after twelve months of storage (Thewes et al., 2021).

AMB-20 and CW-20 showed the highest values of a* after six months (Fig. 1B), indicating the highest red coloration in the nuts. Nevertheless, when subjected to 10 °C, these conditions without CA ensured lower a* values, which did not differ ($p > 0.05$) from $2pO_2$ at 20 °C. Lower values of this parameter were maintained in the $2pO_2$, $40pCO_2$, and $80pCO_2$ treatments at 10 °C, followed by treatments with high $CO_2$ at 20 °C. At the 12-month evaluation, AMB-20 and CW-20 still maintained a more intense red color. The treatments $2pO_2$, $40pCO_2$, and $80pCO_2$ at 10 °C maintained lower a* values even after twelve months of storage. By comparing the sixth and twelfth months, the red tone parameter did not show a significant difference ($p > 0.05$) except for treatments $80pCO_2$-10, which decreased, and CW-20, which increased. Robards et al. (1999) considered that one of the main darkening factors is an oxidative enzymatic process, which involves an initial enzymatic oxidation of lipids and phenolic compounds, and pecan nuts are rich in both compounds (Ribeiro et al., 2020a). Rudolph et al. (1992) evaluated the oxidative stability of pecan oil and observed that color changes are associated with $O_2$ effects as carotenoid and tocopherol concentrations decreased and the oil color changed from yellow to reddish.

A significant reduction in b* value (yellow color) (Fig. 1C) was found from six to twelve months of storage under all conditions. In the sixth month of evaluation, higher b* values were maintained with CA (regardless of the temperature) and CW-10 treatments. This parameter decreased with AMB atmosphere in both temperatures, and CW-20 presented the lowest b* value, indicating loss of the characteristic yellow color of the pecan kernel. AMB-10 did not differ ($p > 0.05$) from treatments under CA with higher b* values at the twelfth month; the coating treatments (CW-20) once again presented the lowest b* in this evaluation. Moreover, AMB-10, CW-10, and CA treatments (irrespective of atmospheric composition and temperature) showed lower a* and higher b* values at the end of storage. In addition, the parameter C (saturation) (Fig. 1D) showed the same trend as the b* parameter. The treatments $2pO_2$, $40pCO_2$, and $80pCO_2$ at 10 °C preserved higher H values (hue/tonality) (Fig. 1E) than treatments at 20 °C and without CA, indicating an additional effect of low temperature and atmosphere modification during long-term storage.

According to Ribeiro et al. (2011), green coffee beans (*Coffea arabica* L.) were stored (twelve months) in three packaging conditions: big hermetic bags (two layers of high-density polyethylene and a layer of PVC) with 60 kPa $pCO_2$, big hermetic bags without $CO_2$, and jute sacks (control). The coffee beans packed in big hermetic bags (with or without $CO_2$) exhibited an intensification of the beans' green coloration, stable moisture, and the sensory analysis of coffee beans stored under high $pCO_2$ yielded the best values. Holladay et al. (1979) studied the quality of raw and roasted peanuts and pecans sealed in plastic pouches with atmospheric air (20 kPa of $O_2$) and $CO_2$ (92 kPa) for long-term storage. They found that the flavor and color of the samples stored in the $CO_2$ atmosphere were better than those in air systems. Additionally, Maskan & Karataş (1999) showed that the color of samples stored (pistachio nuts) in higher $pCO_2$ atmospheres was better than those of the control stored in atmospheric systems. Thus, according to the color parameters results, lower temperatures and different controlled atmosphere of $pO_2$ and $pCO_2$ contribute to maintaining the desirable attributes for nuts to have a good appearance, although the carnauba coating was ineffective and depreciated the pecan color at 20 °C.

### 3.2. Moisture and water activity

Pecans can be stored in shells or shelled; however, when stored in shells, the shell acts as an efficient protection barrier, inhibiting moisture exchange between the kernel and the environment. A high MC influences deterioration by lipid hydrolysis and oxidation, which results in organoleptic alteration and microbial development (mold) (Ajith et al., 2014; Costa et al., 2016). The 'Barton' pecans used in this study were stored with an MC of 2.74% and $A_w$ of 0.54, which is appropriate for storing nuts according to the FDA (2017), as these values should be below 6% and 0.7, respectively.

In the sixth month, the MC ranged from 2.65 to 3.57%, and the highest value was in CW-20 (Fig. 2A), followed by AMB (at 10 and 20 °C) and $80pCO_2$-10. Coincidentally, the pecans stored in CW-20 showed higher a* values, inferring that high MC stimulates browning/reddish reactions. The treatments $40pCO_2$ and $80pCO_2$ at 20 °C did not differ ($p > 0.05$) from $40pCO_2$-10 MC. After twelve months, the treatments presented values between 2.81 and 3.01%. The $2pO_2$ treatment showed an increase in MC compared to six months of storage; nonetheless, most treatments did not differ in MC except for CW-10 and $80pCO_2$-10, with lower values.

The initial water activity ($A_w$) and post-storage evaluations showed values within the recommendation for storing nuts (< 0.7) (FDA, 2017). $A_w$ is a thermodynamic measure dependent on water content (moisture), temperature, and the relationship between water and solute; it enables one to determine the water available for different reactions (chemical, enzymatic, degradation-associated) or microorganism metabolism. In the sixth month, the $A_w$ values ranged from 0.52 to 0.63 (Fig. 2B), and the CW-20 treatment showed the highest $A_w$ value. In the twelve-month evaluation, lower $A_w$ values were observed in treatments subjected to

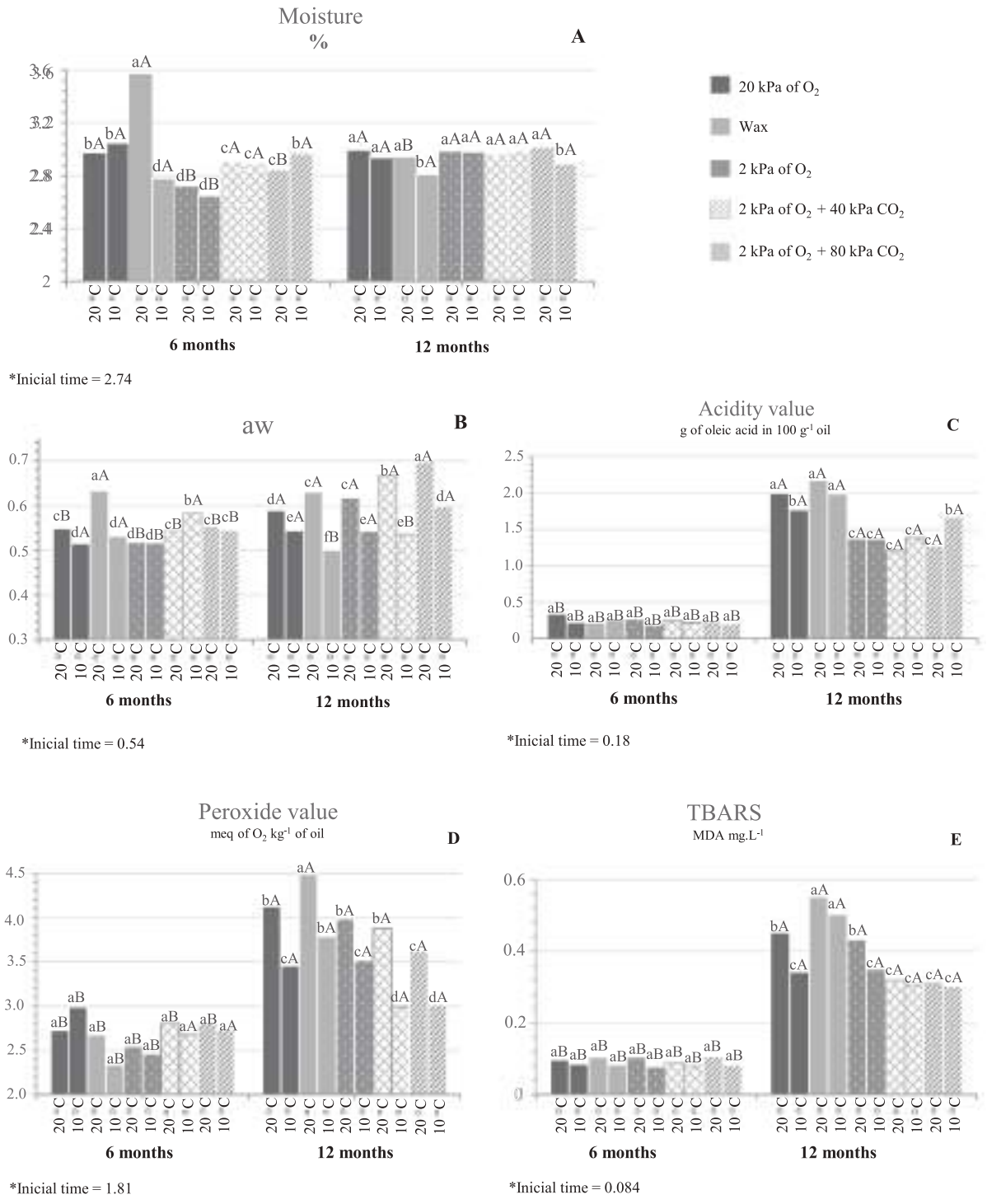



**Fig. 2.** Moisture content (A), water activity (B), Acidity value (C), Peroxide value (D), and TBARS (E) of the unshelled Barton pecan nut after six and twelve months of storage with different $pO_2$, $pCO_2$, carnauba wax and temperatures. Lowercase letters indicate the statistic among treatments in each analyzed period and uppercase letters indicate the statistic difference between the two evaluated period for each condition, according to the Scott Knott test ($p < 0.05$).

10 °C and increased in treatments subjected to 20 °C, especially $pCO_2$ treatments.

Nonetheless, the previous drying, shell maintenance, and treatments efficiently maintained the MC and $A_w$ values recommended for storage. Thus, the lower temperature (10 °C) maintained, the lower $A_w$ values, regardless of the treatments. In addition, we observed a higher $A_w$ in the 40$pCO_2$-20 and 80$pCO_2$-20 treatments in the twelfth month of storage; therefore, as the temperature increases to 20 °C, the interaction of $CO_2$ gas decreases, allowing greater availability of water-free molecules (Maskan & Karataş, 1999). Nevertheless, there is still a gap in knowledge related to the mechanism of action of $CO_2$ in MC and $A_w$, mainly in dry fruit storage.

### 3.3. Acidity value, peroxide value, and TBARS

The pecans' AV, PV, and TBARS at harvest were 0.18 g oleic acid 100 $g^{-1}$ oil, 1.81 meq $O_2$ $kg^{-1}$ oil, and 0.084 MDA mg $L^{-1}$, respectively. No difference between treatments was detected in the evaluation performed

at six months ($p > 0.05$). However, these three quality parameters showed significant increases ($p < 0.05$) in their values after twelve months of storage. The oil acidity from different samples depended on the storage time, which helps explain the low value and absence of difference between treatments at the evaluation of six months.

After twelve months, higher AVs were observed in the treatments with CW (irrespective of the temperature), followed by AMB-20, AMB-10, and 80p$CO_2$-10 (Fig. 2C). The other CA conditions resulted in lower acidity and did not differ ($p > 0.05$) from each other, even at 20 °C. Ribeiro et al. (2020b) also observed that unshelled pecans stored in CA for twelve months with low p$O_2$ (1 kPa and 3 kPa) presented lower AV compared to treatments without CA, with no difference ($p > 0.05$) between the temperatures (10 and 20 °C). In addition, pistachios stored in environments with 98 kPa $CO_2$ and 2 kPa of $O_2$ showed no difference ($p > 0.05$) in AV (<0.6 g oleic acid $100^{-1}$ oil) at 10 and 20 °C, although the nuts had lower values than ambient conditions (20 kPa $O_2$; 1.1 g oleic acid $100^{-1}$ oil) (Maskan & Karataş, 1999).

Interestingly, the 80p$CO_2$-10 treatment presented a higher AV than at 20 °C because lower temperatures are expected to reduce the reactions. At lower temperatures, $CO_2$ interacts more with water (Maskan & Karataş, 1999), forming $HCO^{3-}$. Thus, there is a possibility that a high $CO_2$ concentration, as in the treatment with 80 kPa, has the lowest effectivity on the samples at 10 °C since the presence of $HCO^{3-}$ influences nut pH, acting in the mechanisms of biochemical reactions (Church & Parsons, 1995). The results evidence that the mandatory effect of preventing hydrolysis reactions are reduced p$O_2$, increased $CO_2$, and lower temperatures, avoiding acidity increases for storage in periods that surpass six months. According to Resolution RDC No. 270 (ANVISA, 2005) for olive oil, which has a lipid profile similar to pecan oil (Ribeiro et al., 2020b), extra virgin, virgin, and refined olive oil should not exceed 0.3 g oleic acid 100 $g^{-1}$ oil, 0.8 g oleic acid 100 $g^{-1}$, and 2.0 g oleic acid 100 $g^{-1}$, respectively, to guarantee product quality. Hence, oxidative rancidity is a potential threat to kernel stability due to large amounts of UFAs in pecans (~90%; mainly oleic and linoleic acids).

Peroxide value is a representative parameter that measures oxidation in oils throughout the primary oxidation products, the hydroperoxides of fatty acids (ROOH) (Longobardi et al., 2021). The hydroperoxides quickly decompose into alcohols, aldehydes, ketones, and other secondary oxidation products. Thus, these products make edible oils less acceptable or unacceptable to consumers because of their off-flavors (Mas-Bargues et al., 2021). The limit of PV for acceptance varies from product to product depending on the nature of the oil present, although 20–30 meq $O_2$ $kg^{-1}$ are critical values (Evranuz, 1993). According to Brazilian legislation, refined oils must have a peroxide index near zero, with a limit established for crude oils of 10 meq $O_2$ $kg^{-1}$ of oil (Brasil, 2005).

The CA storage of nuts is notorious and positive regarding lower PV maintenance (Maskan & Karataş, 1999; Ribeiro et al., 2020b, 2022; Thewes et al., 2021). In the pecans evaluated in this study (Fig. 2D), conditions with high p$CO_2$, especially 40 and 80p$CO_2$, at 10 °C, maintained lower PV. The 80p$CO_2$-20 treatment presented a PV value similar to the 2p$O_2$-10 treatment, which may be a positive response for storage with reduced energy consumption with cooling systems. Furthermore, treatments with wax coating were less effective in maintaining lower PV than in an ambient atmosphere.

In other studies, unshelled pecans did not show differences ($p > 0.05$) in PV when stored for six months at 20 °C (1.75 meq of $O_2$ $kg^{-1}$) or 10 °C (1.85 meq of $O_2$ $kg^{-1}$) under low p$O_2$ (1 kPa) (Ribeiro et al., 2020b). Thewes et al. (2021) verified that pecans stored at lower moisture content and low p$O_2$ (4%; 2 kPa $O_2$) had remarkably low PV (0.4 meq of $O_2$ $kg^{-1}$) after eight and twelve months of storage at 20 °C, highlighting the effects of low p$O_2$ in maintaining low PV for up to twelve months. This highlights the effectiveness of reducing oxygen concentrations, delaying peroxide compound formation even at higher temperatures (20 °C). Haq et al. (2013) evaluated the effects of different edible coatings (carboxymethyl cellulose, *Gum Cordia* - *Cordia myxa*) on the storage of pine nuts for four months at 35 °C. The PV increased significantly during storage in all the samples; however, this increase was significantly low in coated pine nuts. Nevertheless, this study obtained an opposing result with wax-coated pecans.

Our findings revealed that high p$CO_2$, when associated with low p$O_2$, helps maintain lower PV. Maskan & Karataş (1999) compared the storage of pistachios under ambient conditions, air saturated with salt solutions ($MgCl_2{\cdot}6H_2O$, $CaCl_2{\cdot}6H_2O$, and $KF{\cdot}2H_2O$), with p$CO_2$ of 98 kPa of $CO_2$ at 10, 20, and 30 °C, and the PV results showed no differences ($p > 0.05$) for 98 kPa of $CO_2$ at 10 and 20 °C. According to these authors, $CO_2$ acts as a barrier at the oil/gas interface and reduces the oxygen uptake rate. It could be inferred that $CO_2$ has an antioxidant effect on the lipid oxidation of nuts.

In this study, the TBARS technique was performed, measuring the interaction of substances reactive to thiobarbituric acid by colorimetry, calculated and expressed in MDA mg $L^{-1}$ (Fig. 2E). The results obtained after twelve months indicated lower MDA values in the pecans stored at 10 °C and in the 40 and 80p$CO_2$ treatments at 20 °C, showing the potential effectiveness of using CA with high $CO_2$, even without refrigeration. Wax coating induced higher TBARS values, indicating low effectiveness against MDA production. In contrast, Haq et al. (2013) found that pine nuts coated with carboxymethyl cellulose and *Gum Cordia* had consistent TBARS values with the PV results, with lower values in the coated samples (<2 mg $L^{-1}$) than the control (>8 mg $L^{-1}$). According to Casagrande et al. (2019), shelled whole Brazil nuts stored for 30 days in different packaging presented the lowest TBARS value with storage in opaque plastic at room temperature (0.064 mg $L^{-1}$), while the highest value was in transparent glass stored in a refrigerator (0.094 mg $L^{-1}$).

Malondialdehyde (MDA) is one of the main biomarkers of lipid peroxidation (Nair et al., 2008). Malondialdehyde originates from polyunsaturated fatty acids (PUFA) when a carbon–carbon double bond is attacked by a free radical, resulting in the formation of unsaturated lipid radical with $H_2O$ release. All PUFAs are susceptible to oxidation, although they do not react similarly. Linolenic acid (C18:3n3), with three cis double bonds, may give rise to 2 MDA molecules. Linoleic acid (C18:2n6), with two cis double bonds, may give rise to 1 MDA molecule, while oleic acid (C18:1n9), with one cis double bond, is not a precursor of this molecule (Mas-Bargues et al., 2021). Therefore, from the oxidation of these fatty acids (present in pecans), MDA can be measured to predict information about the food conservation status condition.

### 3.4. Volatile compounds

In this study, 68 chromatographic peaks were detected and classified into distinct chemical classes: alcohols (13), aldehydes (11), acids (11), ketones (11), esters (10), lactones (6), cyclic alcohols (2), terpenes (2), sulfur (1), and furan (1) (Table 1). The volatile compounds in pecans mainly come from the metabolism of lipids, amino acids, sugars, and protein degradation (Ghidurus et al., 2010; Valdés et al., 2015). The main compounds that generally characterize oil/nut odor are aldehydes (Erten & Cadwallader, 2017), although ketones and alcohols also make up the flavor with notes of mushroom, fat, buttery, and herbs (E-2-octenone, 2-octanone, hexanol, heptanol, octanol, and nonanol) (Maarse, 1991; Acree & Arn, 2004).

The storage temperature significantly influenced the proportion of compound profiles and their respective areas, and pecans' VCs are known to generally increase with storage at higher temperatures (Ribeiro et al., 2020b). The highest peak area values (PAV) were detected at 20 °C, and the conditions that stimulated volatile compound production in the pecans were AMB and CW at 20 °C (mostly acids, alcohols, aldehydes, and esters), followed by 2p$O_2$-20 (acids and alcohols). In contrast, even at 20 °C, treatments with high p$CO_2$ ensured lower PAV or the absence of several compounds. In this sense, $O_2$ availability, associated with a temperature of 20 °C, stimulated VC formation, while high p$CO_2$ prevented the increase and/or production.

**Table 1**
Volatile compounds (peak area value × $10^6$) of 'Barton' pecan at harvest and after storage with shell, coated with carnauba wax, controlled atmosphere conditions and temperatures, during 6 and 12 months.

| | | | | 20 °C | | | | | 10 °C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Compound** | **Months** | **20 kPa $O_2$** | **Wax** | **2 kPa $O_2$** | **2 kPa $O_2$ + 40 kPa $CO_2$** | **2 kPa $O_2$ + 80 kPa $CO_2$** | **20 kPa $O_2$** | **Wax** | **2 kPa $O_2$** | **2 kPa $O_2$ + 40 kPa $CO_2$** | **2 kPa $O_2$ + 80 kPa $CO_2$** |
| Acetic acid | 6 | 14.81 ± 3.93$^{bB}$ | 109.98 ± 2.35$^{aB}$ | 21 ± 1.52$^{bB}$ | 18.65 ± 1.5$^{bA}$ | 20.16 ± 1.08$^{bA}$ | 19.47 ± 5.65$^{bA}$ | 15.52 ± 1.72$^{bA}$ | 21.52 ± 2.7$^{bA}$ | 14.76 ± 1.61$^{bA}$ | 17.02 ± 1.13$^{bA}$ |
| (12.7 ± 3.87) | 12 | 29.17 ± 0.66$^{cA}$ | 140.79 ± 27.63$^{aA}$ | 42.94 ± 3.18$^{bA}$ | 30.2 ± 13.34$^{cA}$ | 12.56 ± 0.95 $^{dB}$ | 8.47 ± 1.05 $^{dB}$ | 9.54 ± 0.49$^{dA}$ | 8.07 ± 1 $^{dB}$ | 10.04 ± 0.38$^{dA}$ | 7.85 ± 1.05 $^{dB}$ |
| Propionic acid | 6 | 0.18 ± 0.03$^{bA}$ | 0.19 ± 0.04$^{bA}$ | 0.18 ± 0.02$^{bA}$ | 0.13 ± 0.02$^{cA}$ | 0.16 ± 0$^{bA}$ | 0.18 ± 0.04$^{cA}$ | 0.1 ± 0.01$^{bA}$ | 0.2 ± 0.02$^{bA}$ | 0.2 ± 0.03$^{bA}$ | 0.36 ± 0.17$^{aA}$ |
| (nd) | 12 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| Butanoic acid | 6 | 0.07 ± 0.01$^{bA}$ | 0.33 ± 0.05$^{aB}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 0.52 ± 0.04$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| Pentanoic acid | 6 | 0.17 ± 0.04$^{aB}$ | 0.18 ± 0.03$^{aB}$ | 0.14 ± 0.01$^{bB}$ | 0.08 ± 0.01$^{cA}$ | 0.12 ± 0.02$^{bA}$ | 0.14 ± 0.02$^{bA}$ | 0.04 ± 0.01$^{cA}$ | 0.11 ± 0.06$^{bA}$ | 0.06 ± 0.01$^{cA}$ | 0.12 ± 0.01$^{bA}$ |
| (nd) | 12 | 0.52 ± 0.06$^{aA}$ | 0.78 ± 0.07$^{aA}$ | 0.24 ± 0.05$^{cA}$ | nd | nd | nd | nd | nd | nd | nd |
| Hexanoic acid | 6 | 1.21 ± 0.35$^{aB}$ | 1.4 ± 0.23$^{aB}$ | 1.13 ± 0.08$^{aB}$ | 0.47 ± 0.03$^{bA}$ | 0.64 ± 0.21$^{bA}$ | 0.94 ± 0.21$^{aA}$ | 0.92 ± 0.11$^{aA}$ | 0.69 ± 0.18$^{bA}$ | 0.35 ± 0.07$^{dA}$ | 0.71 ± 0.1$^{bA}$ |
| (0.38 ± 0.19) | 12 | 4.05 ± 0.86$^{bA}$ | 6.49 ± 0.75$^{aA}$ | 2.12 ± 0.45$^{cA}$ | 0.73 ± 0.14$^{dA}$ | 0.31 ± 0.03$^{dA}$ | 0.49 ± 0.16$^{dA}$ | 0.38 ± 0.09 $^{dB}$ | 0.37 ± 0.01$^{dA}$ | 0.44 ± 0.08$^{dA}$ | 0.34 ± 0.1$^{dA}$ |
| Heptanoic acid | 6 | 0.58 ± 0.15$^{aB}$ | 0.38 ± 0.08$^{aB}$ | 0.39 ± 0.03$^{aB}$ | 0.24 ± 0.04$^{bA}$ | 0.35 ± 0.08$^{aA}$ | 0.44 ± 0.07$^{aA}$ | 0.42 ± 0.07$^{aA}$ | 0.42 ± 0.14$^{aA}$ | 0.26 ± 0.03$^{bA}$ | 0.43 ± 0.05$^{aA}$ |
| (0.3 ± 0.09) | 12 | 1.16 ± 0.16$^{aA}$ | 1.1 ± 0.12$^{aA}$ | 0.67 ± 0.12$^{bA}$ | 0.29 ± 0.11$^{cA}$ | nd | 0.24 ± 0.07$^{cB}$ | 0.09 ± 0.01$^{cB}$ | 0.15 ± 0$^{cB}$ | 0.14 ± 0.04$^{cA}$ | nd |
| Octanoic acid | 6 | 1.41 ± 0.3$^{aB}$ | 1.11 ± 0.41$^{aB}$ | 0.97 ± 0.11$^{aB}$ | 0.71 ± 0.09$^{bA}$ | 1.05 ± 0.28$^{aA}$ | 1.17 ± 0.2$^{aA}$ | 1.14 ± 0.18$^{aA}$ | 1.4 ± 0.55$^{aA}$ | 0.63 ± 0.05$^{bA}$ | 1.3 ± 0.11$^{aA}$ |
| (0.84 ± 0.35) | 12 | 3.17 ± 0.39$^{aA}$ | 2.72 ± 0.33$^{bA}$ | 2.03 ± 0.46$^{cA}$ | 1.11 ± 0.19$^{dA}$ | 0.39 ± 0.04$^{eB}$ | 0.61 ± 0.22$^{eB}$ | 0.27 ± 0.09$^{eB}$ | 0.35 ± 0.12$^{eB}$ | 0.49 ± 0.18$^{eA}$ | 0.29 ± 0.09$^{eB}$ |
| Nonanoic acid | 6 | 1.88 ± 0.54$^{bA}$ | 1.8 ± 0.9$^{bA}$ | 1.07 ± 0.2$^{bA}$ | 1.32 ± 0.21$^{bA}$ | 1.64 ± 0.27$^{bA}$ | 1.82 ± 0.3$^{bA}$ | 1.66 ± 0.31$^{bA}$ | 3.19 ± 1$^{aA}$ | 0.96 ± 0.14$^{bA}$ | 2.43 ± 0.54$^{aA}$ |
| (1.75 ± 0.71) | 12 | 1.77 ± 0.48$^{aA}$ | 1.69 ± 0.21$^{aA}$ | 1.5 ± 0.48$^{aA}$ | 1.09 ± 0.62$^{aA}$ | 0.26 ± 0.08$^{bB}$ | 1.21 ± 0.49$^{aA}$ | 0.43 ± 0.24$^{bB}$ | 0.56 ± 0.22$^{bB}$ | 1.08 ± 0.7$^{aA}$ | 0.36 ± 0.15$^{bB}$ |
| Decanoic acid | 6 | 1.37 ± 0.79$^{aA}$ | 1.25 ± 0.84$^{bA}$ | 1.63 ± 0.63$^{aA}$ | 1.04 ± 0.09$^{bA}$ | 1.19 ± 0.33$^{bA}$ | 0.99 ± 0.13$^{bA}$ | 1.22 ± 0.19$^{bA}$ | 1.76 ± 0.22$^{aA}$ | 0.59 ± 0.02$^{bA}$ | 1.05 ± 0.16$^{bA}$ |
| (0.96 ± 0.04) | 12 | 1.15 ± 0.23$^{aA}$ | 0.58 ± 0.11$^{bB}$ | 0.5 ± 0.06$^{bB}$ | 0.4 ± 0.1$^{bB}$ | 0.13 ± 0.03$^{bB}$ | 0.23 ± 0.12$^{bB}$ | 0.15 ± 0.01$^{bB}$ | 0.16 ± 0$^{bB}$ | 0.25 ± 0.1$^{bA}$ | nd |
| Dodecanoic acid | 6 | 2.56 ± 1.31$^{aA}$ | 2.87 ± 1.7$^{aA}$ | 3.86 ± 2.57$^{aA}$ | 1.13 ± 0.07$^{bA}$ | 1.4 ± 0.58$^{bA}$ | 1.3 ± 0.59$^{bA}$ | 1.15 ± 0.29$^{bA}$ | 0.81 ± 0.06$^{bA}$ | 0.97 ± 0.14$^{bA}$ | 1.38 ± 0.84$^{bA}$ |
| (0.87 ± 0.08) | 12 | 0.94 ± 0.67$^{aB}$ | 1.01 ± 0.29$^{aB}$ | 0.98 ± 0.22$^{aB}$ | 0.55 ± 0.06$^{aA}$ | 0.4 ± 0.07$^{aA}$ | 0.34 ± 0.05$^{aA}$ | 0.3 ± 0.05$^{aA}$ | 0.29 ± 0.03$^{aA}$ | 0.33 ± 0.11$^{aA}$ | 0.22 ± 0.05$^{aA}$ |
| Tetradecanoic acid | 6 | 1.5 ± 0.24$^{aB}$ | 1.91 ± 1.24$^{aA}$ | 2.34 ± 1.65$^{aA}$ | 1.37 ± 0.4$^{aA}$ | 1.21 ± 0.49$^{aA}$ | 1.18 ± 0.09$^{aA}$ | 1.06 ± 0.31$^{aA}$ | 1.33 ± 0.35$^{aA}$ | 0.83 ± 0.26$^{aA}$ | 0.86 ± 0.27$^{aA}$ |
| (1 ± 0.13) | 12 | 3.27 ± 2.58$^{aA}$ | 0.82 ± 0.16$^{bA}$ | 0.53 ± 0.24$^{bB}$ | 0.29 ± 0.1$^{bA}$ | 1.08 ± 0.1$^{bA}$ | 0.76 ± 0.05$^{bA}$ | 0.78 ± 0.2$^{bA}$ | 0.69 ± 0.06$^{bA}$ | 0.82 ± 0.26$^{bA}$ | 0.65 ± 0.03$^{bA}$ |
| Ethanol | 6 | 1.87 ± 0.23$^{eB}$ | 1.75 ± 0.36$^{eB}$ | 3.33 ± 0.1 $^{dB}$ | 3.95 ± 0.17 $^{dB}$ | 11.62 ± 0.54$^{cA}$ | 8.99 ± 4.33$^{cA}$ | 6.69 ± 0.24$^{cA}$ | 9.79 ± 0.54$^{cA}$ | 17.67 ± 0.88$^{bA}$ | 23.34 ± 1.89$^{aA}$ |
| (16.52 ± 4.23) | 12 | 20.13 ± 0.06$^{aA}$ | 9.5 ± 0.51$^{cA}$ | 21.82 ± 0.75$^{aA}$ | 9.37 ± 3.87$^{cA}$ | 4.96 ± 0.3$^{cB}$ | 4.58 ± 0.3$^{cA}$ | 1.91 ± 0.06$^{cA}$ | 5.59 ± 0.29$^{cA}$ | 6.11 ± 0.27$^{cB}$ | 13.28 ± 0.7$^{bB}$ |
| 1-butanol | 6 | 0.24 ± 0.06$^{aB}$ | 0.17 ± 0.06$^{aB}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | 0.8 ± 0.04$^{aA}$ | 1.03 ± 0.18$^{aA}$ | 0.96 ± 0.2$^{aA}$ | 0.92 ± 0.62$^{aA}$ | nd | nd | 0.14 ± 0.01$^{bA}$ | nd | nd | nd |
| 2-Methyl butanol | 6 | 1.13 ± 0.03$^{cA}$ | 1.78 ± 0.03$^{cA}$ | 0.9 ± 0.05$^{aB}$ | 1.29 ± 0.22$^{aA}$ | 1.55 ± 0.08$^{bA}$ | 1.25 ± 0.57$^{cA}$ | 1.4 ± 0.03$^{cA}$ | 1.49 ± 0.22$^{aA}$ | 1.59 ± 0.26$^{aA}$ | 2.05 ± 0.09$^{aA}$ |
| (1.22 ± 0.81) | 12 | 1.75 ± 0.04$^{cA}$ | 3.84 ± 0.56$^{cA}$ | 2.46 ± 0.23$^{aA}$ | 0.59 ± 0.28$^{bA}$ | 0.31 ± 0.05 $^{dB}$ | 0.41 ± 0.15$^{dA}$ | 0.82 ± 0.02$^{dA}$ | 0.47 ± 0.01$^{cB}$ | 0.83 ± 0.1$^{cB}$ | 0.77 ± 0.02$^{cB}$ |
| 3-Methyl butanol | 6 | 1.49 ± 0.23$^{bA}$ | 3.51 ± 0.04$^{bA}$ | 1.29 ± 0.36$^{bA}$ | 1.94 ± 0.12$^{bA}$ | 1.69 ± 0.11$^{aA}$ | 1.85 ± 1.08$^{bA}$ | 1.84 ± 0.12$^{aA}$ | 1.59 ± 0.6$^{bA}$ | 1.58 ± 0.53$^{bA}$ | 2.27 ± 0.18$^{bA}$ |
| (1.18 ± 0.77) | 12 | 2.67 ± 0.11$^{aA}$ | 5.98 ± 0.17$^{aA}$ | 1.2 ± 0.1$^{bA}$ | 1.87 ± 0.79$^{cA}$ | 0.77 ± 0.1$^{cB}$ | 0.75 ± 0.01$^{cB}$ | 1.97 ± 0.09$^{cB}$ | 0.53 ± 0.04$^{cB}$ | 0.61 ± 0.14$^{cB}$ | 0.79 ± 0.03$^{cB}$ |
| Pentanol | 6 | 4.19 ± 0.04$^{aB}$ | 4.03 ± 0.39$^{aB}$ | 2.33 ± 0.24$^{bB}$ | 0.92 ± 0.07$^{cB}$ | 0.73 ± 0.24$^{cA}$ | 1.98 ± 0.53$^{bA}$ | 1.87 ± 0.11$^{bA}$ | 0.54 ± 0.07$^{cA}$ | nd | 0.58 ± 0.01$^{cA}$ |
| (nd) | 12 | 16.5 ± 1.19$^{bA}$ | 21.72 ± 0.79$^{aA}$ | 7.16 ± 1.43$^{cA}$ | 3.44 ± 2.41$^{dA}$ | 0.9 ± 0.06$^{fA}$ | 1.99 ± 0.2$^{eA}$ | 1.43 ± 0.03$^{fA}$ | nd | nd | 0.42 ± 0.04$^{fA}$ |
| 5-Methyl 2-hexanol | 6 | nd | 0.2 ± 0.05$^{aB}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 0.39 ± 0.02$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| Hexanol | 6 | 7.04 ± 0.13$^{aB}$ | 9.09 ± 0.99$^{aB}$ | 6.39 ± 0.15$^{aB}$ | 3 ± 0.28$^{bB}$ | 2.43 ± 0.41$^{bA}$ | 2.96 ± 0.95$^{bA}$ | 1.92 ± 0.35$^{bA}$ | 2.18 ± 0.19$^{bA}$ | 2.62 ± 0.15$^{bA}$ | 2.32 ± 0.17$^{bA}$ |
| (nd) | 12 | 34.41 ± 2.34$^{bA}$ | 63.4 ± 2.86$^{aA}$ | 22.7 ± 3.31$^{cA}$ | 10.2 ± 4.56$^{dA}$ | 3.96 ± 0.31$^{eA}$ | 3.28 ± 0.65$^{eA}$ | 1.53 ± 0.03$^{eA}$ | 1.3 ± 0.12$^{eA}$ | 2.24 ± 0.21$^{eA}$ | 1.37 ± 0.1$^{eA}$ |

(*continued on next page*)

**Table 1** (*continued*)

| | | | | 20 °C | | | | | 10 °C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Compound** | **Months** | **20 kPa $O_2$** | **Wax** | **2 kPa $O_2$** | **2 kPa $O_2$ + 40 kPa $CO_2$** | **2 kPa $O_2$ + 80 kPa $CO_2$** | **20 kPa $O_2$** | **Wax** | **2 kPa $O_2$** | **2 kPa $O_2$ + 40 kPa $CO_2$** | **2 kPa $O_2$ + 80 kPa $CO_2$** |
| 1-Octen-3-ol | 6 | nd | nd | 0.22 ± 0.01$^{aA}$ | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | 0.22 ± 0.06$^{aA}$ | nd | 0.2 ± 0.05$^{aA}$ | 0.06 ± 0.02$^{bA}$ | nd | nd | nd | nd | nd | nd |
| Heptanol | 6 | 3.16 ± 0.07$^{aB}$ | 2.01 ± 0.28$^{bB}$ | 1.47 ± 0.02$^{cB}$ | 0.63 ± 0.09$^{cB}$ | 0.68 ± 0.09$^{cA}$ | 0.86 ± 0.19$^{cB}$ | 0.85 ± 0.12$^{cA}$ | 0.42 ± 0.04$^{cA}$ | 0.69 ± 0.06$^{cA}$ | 0.6 ± 0.06$^{cA}$ |
| (nd) | 12 | 11.7 ± 1.71$^{bA}$ | 13.31 ± 0.35$^{aA}$ | 4.52 ± 0.9$^{cA}$ | 2.05 ± 0.76$^{dA}$ | 0.76 ± 0.09$^{eA}$ | 1.9 ± 0.07$^{dA}$ | 0.44 ± 0.03$^{eA}$ | 0.31 ± 0.06$^{eA}$ | 0.56 ± 0.11$^{eA}$ | 0.46 ± 0.05$^{eA}$ |
| Octanol | 6 | 1.98 ± 0.01$^{aB}$ | 1.44 ± 0.21$^{bB}$ | 1.23 ± 0.08$^{bB}$ | 0.56 ± 0.05$^{cB}$ | 0.64 ± 0.02$^{cA}$ | 0.69 ± 0.13$^{cA}$ | 0.62 ± 0.08$^{cA}$ | 0.74 ± 0.37$^{cA}$ | 0.46 ± 0.12$^{cA}$ | 0.55 ± 0.06$^{cA}$ |
| (nd) | 12 | 6.05 ± 0.88$^{bA}$ | 6.79 ± 0.43$^{aA}$ | 3.72 ± 0.73$^{cA}$ | 1.84 ± 0.67$^{dA}$ | 0.67 ± 0.04$^{eA}$ | 1.02 ± 0.04$^{eA}$ | 0.33 ± 0.06$^{eA}$ | 0.24 ± 0.01$^{eA}$ | 0.53 ± 0.07$^{eA}$ | 0.4 ± 0.07$^{eA}$ |
| Nonanol | 6 | 0.08 ± 0.02$^{bB}$ | 1.58 ± 0.39$^{aB}$ | 0.25 ± 0.02$^{bB}$ | 0.15 ± 0.03$^{bB}$ | 0.21 ± 0.03$^{bA}$ | 0.13 ± 0.04$^{bA}$ | nd | nd | nd | nd |
| (nd) | 12 | 0.84 ± 0.13$^{bA}$ | 3.66 ± 0.42$^{aA}$ | 0.72 ± 0.19$^{bA}$ | 0.46 ± 0.01$^{cA}$ | nd | nd | nd | nd | nd | nd |
| Hexadecanol | 6 | 3.61 ± 0.26$^{aA}$ | 3.69 ± 2.04$^{aA}$ | 4.17 ± 3.18$^{aA}$ | 0.56 ± 0.43$^{bA}$ | 2.29 ± 1.4$^{bA}$ | 1.06 ± 0.1$^{bA}$ | 1.93 ± 1.3$^{bA}$ | 3.89 ± 1.86$^{aA}$ | 1.75 ± 1.13$^{bA}$ | 1.32 ± 0.2$^{bA}$ |
| (1.14 ± 0.42) | 12 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| Nonadecanol | 6 | 1 ± 0.9$^{bA}$ | 2.75 ± 0.57$^{aA}$ | 3.93 ± 3.37$^{aA}$ | 0.36 ± 0.03$^{bA}$ | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| Benzyl alcohol | 6 | 0.36 ± 0.29$^{bA}$ | 0.5 ± 0.1$^{aB}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 1.38 ± 0.08$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| Phenylethyl alcohol | 6 | 0.31 ± 0.03 $^{dB}$ | 1.21 ± 0.09$^{aB}$ | 0.39 ± 0.03$^{dA}$ | 0.31 ± 0.06$^{dA}$ | 0.53 ± 0.03$^{cA}$ | 0.42 ± 0.12$^{dA}$ | 0.67 ± 0.08$^{cA}$ | 0.47 ± 0.06$^{dA}$ | 0.44 ± 0.1$^{dA}$ | 0.86 ± 0.09$^{bA}$ |
| (0.37 ± 0.26) | 12 | 0.95 ± 0.16$^{bA}$ | 2.08 ± 0.36$^{aA}$ | 0.57 ± 0.15$^{cA}$ | 0.38 ± 0.18$^{dA}$ | 0.14 ± 0.01$^{eB}$ | 0.12 ± 0.03$^{eB}$ | 0.32 ± 0.02 $^{dB}$ | 0.07 ± 0.01$^{eB}$ | 0.16 ± 0.03$^{eB}$ | 0.17 ± 0.02$^{eB}$ |
| Ethanal | 6 | 4.1 ± 0.17$^{cA}$ | nd | nd | nd | nd | 2.69 ± 1.23$^{dA}$ | 5.57 ± 0.16$^{bA}$ | 3.72 ± 0.96$^{cA}$ | 3.05 ± 0.81$^{dA}$ | 7.51 ± 2.03$^{aA}$ |
| (6.97 ± 3.94) | 12 | nd | nd | nd | nd | nd | 1.2 ± 0.1$^{aB}$ | 0.16 ± 0.04$^{aB}$ | nd | 0.84 ± 0.01$^{aB}$ | 0.36 ± 0.02$^{aB}$ |
| 2-Methyl propanal | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 0.12 ± 0.02$^{bA}$ | 0.22 ± 0.02$^{aA}$ | 0.21 ± 0.02$^{aA}$ | nd | nd | nd | nd | nd | nd |
| Butanal | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 1.85 ± 0.12$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| 2-Methyl butanal | 6 | 0.6 ± 0.06$^{cA}$ | 0.5 ± 0.04$^{aA}$ | 1.38 ± 0.16$^{cB}$ | 1.42 ± 0.06$^{cA}$ | 0.91 ± 0.07$^{bA}$ | 0.43 ± 0.25$^{cA}$ | 0.52 ± 0.02$^{cA}$ | 1.37 ± 0.04$^{bA}$ | 1.65 ± 0.06$^{bA}$ | 1.55 ± 0.24$^{aA}$ |
| (nd) | 12 | 0.56 ± 0.03$^{cA}$ | 0.48 ± 0.02$^{aA}$ | 1.88 ± 0.03$^{bA}$ | 1.48 ± 0.68$^{cA}$ | 0.4 ± 0.01$^{eB}$ | 0.18 ± 0.01$^{eB}$ | 0.23 ± 0.01 $^{dB}$ | 0.63 ± 0.08$^{eB}$ | 0.56 ± 0.04 $^{dB}$ | 0.71 ± 0.02 $^{dB}$ |
| 3-Methyl butanal | 6 | 0.96 ± 0.02$^{bB}$ | 0.96 ± 0.06$^{aB}$ | 0.97 ± 0.06$^{bA}$ | 0.8 ± 0.02$^{bA}$ | 1.38 ± 0.07$^{bA}$ | 0.81 ± 0.45$^{bA}$ | 1.62 ± 0.05$^{bA}$ | 1.18 ± 0.02$^{bA}$ | 1.01 ± 0.08$^{bA}$ | 0.85 ± 0.58$^{bA}$ |
| (5.87 ± 7.31) | 12 | 1.1 ± 0.09$^{bA}$ | 1.23 ± 0.09$^{aA}$ | 0.83 ± 0.05$^{dA}$ | 0.56 ± 0.25$^{cA}$ | 0.44 ± 0.06 $^{dB}$ | 0.29 ± 0.01 $^{dB}$ | 0.4 ± 0.01$^{cB}$ | 0.27 ± 0.05 $^{dB}$ | 0.33 ± 0.01 $^{dB}$ | 0.36 ± 0.02 $^{dB}$ |
| Hexanal | 6 | 24.34 ± 0.63$^{aB}$ | 20.91 ± 1.44$^{aB}$ | 21.47 ± 0.37$^{aB}$ | 9.21 ± 0.5$^{bB}$ | 4.53 ± 0.24$^{cA}$ | 12.3 ± 4.59$^{bA}$ | 12.54 ± 0.58$^{bA}$ | 6.99 ± 0.33$^{cA}$ | 9.04 ± 2.15$^{bA}$ | 6.16 ± 4.65$^{cA}$ |
| (4.19 ± 4.18) | 12 | 71.78 ± 5.57$^{bA}$ | 98.34 ± 2.81$^{aA}$ | 33.69 ± 3.19$^{cA}$ | 16.76 ± 6.3$^{dA}$ | 7.15 ± 0.01$^{eA}$ | 15.69 ± 1.82$^{dA}$ | 9.47 ± 0.14$^{eA}$ | 6.09 ± 0.66$^{eA}$ | 8.16 ± 0.12$^{eA}$ | 4.03 ± 0.11$^{eA}$ |
| Heptanal | 6 | 3.34 ± 1.94$^{aB}$ | nd | nd | nd | nd | 0.8 ± 0.12$^{bB}$ | nd | nd | nd | nd |
| (0.53 ± 0.04) | 12 | 5.73 ± 0.67$^{aA}$ | 5.85 ± 0.17$^{aA}$ | 1.78 ± 0.23$^{bA}$ | 1.28 ± 0.58$^{bA}$ | nd | 1.77 ± 0.22$^{bA}$ | nd | nd | nd | nd |
| Octanal | 6 | 2.89 ± 0.17$^{aB}$ | nd | 1.47 ± 0.1$^{bA}$ | 0.9 ± 0.06$^{dA}$ | 0.61 ± 0.03$^{dA}$ | 1.1 ± 0.31$^{cA}$ | 0.76 ± 0.1$^{dA}$ | nd | nd | nd |
| (nd) | 12 | 6.26 ± 0.61$^{bA}$ | 9.43 ± 0.38$^{aA}$ | nd | nd | nd | 1.25 ± 0.27$^{cA}$ | 0.46 ± 0.04$^{dA}$ | nd | nd | nd |
| (Z)-2-Heptenal | 6 | 0.12 ± 0.02$^{cB}$ | 0.16 ± 0.03$^{bB}$ | nd | nd | nd | 0.24 ± 0.06$^{aA}$ | nd | nd | nd | nd |
| (nd) | 12 | 0.34 ± 0.01$^{aA}$ | 0.23 ± 0.05$^{bA}$ | nd | nd | nd | nd | 0.12 ± 0.02$^{cA}$ | nd | nd | nd |
| Nonanal | 6 | 1.7 ± 0.16$^{aA}$ | 0.74 ± 0.47$^{bB}$ | nd | nd | nd | 1.79 ± 1.12$^{aA}$ | nd | nd | nd | nd |
| (nd) | 12 | 1 ± 0.16$^{bB}$ | 1.7 ± 0.19$^{aA}$ | 0.35 ± 0.16$^{cA}$ | 0.51 ± 0.3$^{cA}$ | nd | 0.38 ± 0.02$^{cB}$ | nd | nd | nd | nd |
| (E)-2-Decenal | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | 0.26 ± 0.04$^{bA}$ | 0.39 ± 0.01$^{aA}$ | 0.26 ± 0.01$^{bA}$ | nd | nd | nd | nd | nd | nd | nd |
| 2-Propanone | 6 | 2.84 ± 0.18$^{cB}$ | 3.83 ± 0.16$^{cB}$ | 3.27 ± 0.06$^{cA}$ | 3.53 ± 0.1$^{cA}$ | 4.4 ± 0.17$^{cA}$ | 2.85 ± 0.99$^{cA}$ | 4.2 ± 0.22$^{cA}$ | 5.71 ± 0.15$^{bA}$ | 7.35 ± 0.29$^{aA}$ | 3.98 ± 2.44$^{cA}$ |

(*continued on next page*)

**Table 1** (*continued*)

| | | | | 20 °C | | | | | 10 °C | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Compound** | **Months** | **20 kPa $O_2$** | **Wax** | **2 kPa $O_2$** | **2 kPa $O_2$ + 40 kPa $CO_2$** | **2 kPa $O_2$ + 80 kPa $CO_2$** | **20 kPa $O_2$** | **Wax** | **2 kPa $O_2$** | **2 kPa $O_2$ + 40 kPa $CO_2$** | **2 kPa $O_2$ + 80 kPa $CO_2$** |
| (3.71 ± 1.17) | 12 | 9.55 ± 0.3$^{aA}$ | 5.88 ± 0.19$^{bA}$ | 4.23 ± 0.2$^{cA}$ | 2.56 ± 1.04$^{dA}$ | 2.52 ± 0.34 $^{dB}$ | 2.83 ± 0.09$^{dA}$ | 1.88 ± 0.06 $^{dB}$ | 1.65 ± 0.03 $^{dB}$ | 1.95 ± 0.19 $^{dB}$ | 1.75 ± 0.17 $^{dB}$ |
| 2-Butanone | 6 | 1.24 ± 0.1$^{bA}$ | nd | 1.15 ± 0.1$^{bA}$ | 1.08 ± 0.04$^{bA}$ | 1.58 ± 0.08$^{aA}$ | 1.29 ± 0.54$^{bA}$ | 1.27 ± 0.03$^{bA}$ | 1.19 ± 0.05$^{bA}$ | 0.95 ± 0.11$^{bA}$ | 1.41 ± 0.09$^{aA}$ |
| (0.7 ± 0.6) | 12 | 0.72 ± 0.05$^{aB}$ | 0.84 ± 0.03$^{aA}$ | 0.67 ± 0.08$^{aB}$ | 0.9 ± 0.47$^{aA}$ | 0.43 ± 0.02$^{bB}$ | 0.22 ± 0.02$^{bB}$ | 0.39 ± 0.01$^{bB}$ | 0.26 ± 0.04$^{bB}$ | 0.28 ± 0.03$^{bB}$ | 0.25 ± 0.02$^{bB}$ |
| 3-Methyl 2-butanone | 6 | nd | 0.19 ± 0.07$^{aB}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 0.33 ± 0.02$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| 2-Pentanone | 6 | 3.39 ± 0.15$^{aB}$ | 3.2 ± 0.21$^{aB}$ | 2.83 ± 0.1$^{aB}$ | 3.39 ± 0.18$^{aA}$ | 3.19 ± 0.12$^{aA}$ | 2.53 ± 1.07$^{aA}$ | 2.96 ± 0.13$^{aA}$ | 4.37 ± 0.25$^{aA}$ | 1.62 ± 0.23$^{aA}$ | 3.4 ± 2.05$^{aA}$ |
| (2.1 ± 1.73) | 12 | 6.56 ± 0.33$^{bA}$ | 10.09 ± 0.27$^{aA}$ | 5.62 ± 0.38$^{bA}$ | 4.06 ± 2.23$^{cA}$ | 1.97 ± 0.01$^{dA}$ | 1.59 ± 0.11$^{dA}$ | 1.89 ± 0.01$^{dA}$ | 1.2 ± 0.12 $^{dB}$ | 1.73 ± 0.03$^{dA}$ | 1.21 ± 0.05 $^{dB}$ |
| 4-Heptanone | 6 | 0.08 ± 0$^{bA}$ | 0.54 ± 0.04$^{aB}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | 0.23 ± 0.01$^{bA}$ | 1.6 ± 0.51$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| 2-Heptanone | 6 | nd | 1.57 ± 0.23$^{aB}$ | nd | 0.6 ± 0.19$^{bB}$ | 0.54 ± 0.03$^{bA}$ | 0.63 ± 0.05$^{bA}$ | nd | 0.66 ± 0.33$^{bA}$ | nd | 0.84 ± 0.23$^{bA}$ |
| (0.81 ± 0.02) | 12 | 5.36 ± 0.73$^{bA}$ | 6.64 ± 1.38$^{aA}$ | 3.31 ± 0.81$^{cA}$ | 2.11 ± 0.33$^{dA}$ | 0.45 ± 0.01$^{eA}$ | 0.66 ± 0.09$^{eA}$ | 0.17 ± 0.2$^{eA}$ | nd | nd | nd |
| 3-Octanone | 6 | nd | 0.29 ± 0.03$^{bA}$ | nd | nd | nd | 2.36 ± 3.22$^{aA}$ | 0.87 ± 0.04$^{bA}$ | nd | nd | nd |
| (nd) | 12 | 0.41 ± 0.07$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 6-Methyl 5-hepten-2-one | 6 | 0.98 ± 0.03$^{aA}$ | 0.65 ± 0.06$^{bB}$ | nd | nd | nd | 0.97 ± 0.3$^{aA}$ | 0.38 ± 0.07$^{cA}$ | 0.35 ± 0.01$^{cA}$ | 0.25 ± 0.01$^{cA}$ | 0.28 ± 0.02$^{cA}$ |
| (nd) | 12 | 1.07 ± 0.09$^{bA}$ | 1.32 ± 0.05$^{aA}$ | nd | nd | nd | 0.36 ± 0.04$^{cB}$ | 0.28 ± 0.01$^{cA}$ | nd | nd | nd |
| 3-Octen-2-one | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 0.59 ± 0.01$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| 4-Oxoisophorone | 6 | 0.83 ± 0.07$^{aA}$ | 0.07 ± 0.02$^{dA}$ | 0.53 ± 0.08$^{bB}$ | 0.33 ± 0.08$^{cA}$ | 0.5 ± 0.08$^{bA}$ | 0.53 ± 0.25$^{bA}$ | 0.55 ± 0.21$^{bA}$ | 0.75 ± 0.07$^{aA}$ | 0.42 ± 0.21$^{cA}$ | 0.56 ± 0.03$^{bA}$ |
| (nd) | 12 | 0.6 ± 0.11$^{aB}$ | 0.17 ± 0.06$^{cA}$ | 0.73 ± 0.27$^{aA}$ | 0.38 ± 0$^{bA}$ | nd | nd | nd | nd | nd | nd |
| Methyl ethanoate | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | 1.67 ± 0.47$^{aA}$ |
| (2.07 ± 0.61) | 12 | nd | 1.12 ± 0.07$^{aA}$ | 1.06 ± 0.07$^{aA}$ | 0.56 ± 0.3$^{bA}$ | nd | nd | nd | nd | nd | 0.29 ± 0.01$^{cB}$ |
| Ethyl ethanoate | 6 | 1.76 ± 0.13$^{bB}$ | 0.88 ± 0.05$^{bB}$ | 1.65 ± 0.04$^{bB}$ | 1.54 ± 0.11$^{bA}$ | 3.47 ± 0.26$^{aA}$ | 2.08 ± 0.7$^{bA}$ | 3.55 ± 0.1$^{aA}$ | 2.72 ± 0.05$^{aA}$ | 3.47 ± 0.27$^{aA}$ | 3 ± 1.9$^{aA}$ |
| (8.16 ± 3.48) | 12 | 3.76 ± 0.13$^{bA}$ | 2.03 ± 0.05$^{cA}$ | 5.87 ± 0.72$^{aA}$ | 1.59 ± 0.77$^{cA}$ | 1 ± 0.09 $^{dB}$ | 0.79 ± 0.11 $^{dB}$ | 0.52 ± 0.08 $^{dB}$ | 0.71 ± 0.07 $^{dB}$ | 1 ± 0.03 $^{dB}$ | 3.05 ± 0.2$^{bA}$ |
| n-Propyl ethanoate | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (1.13 ± 0.07) | 12 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| Ethyl butanoate | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | nd | 6.57 ± 0.85$^{aA}$ | 3.4 ± 1.47$^{bA}$ | 2.68 ± 0.73$^{cA}$ | 2.16 ± 0.35$^{dA}$ | 0.75 ± 0.1$^{eA}$ | 2.14 ± 0.35$^{dA}$ | 2 ± 0.21$^{dA}$ | 1.99 ± 0.13$^{dA}$ |
| Ethyl 2-methyl butanoate | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 1.36 ± 0.23$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| Ethyl 3-methyl butanoate | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 0.57 ± 0.09$^{bA}$ | 20.26 ± 3.67$^{aA}$ | 18.27 ± 7.4$^{aA}$ | nd | nd | nd | nd | nd | nd |
| 3-Methyl butyl ethanoate | 6 | 0.35 ± 0.34$^{aA}$ | 0.41 ± 0.08$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| Pentyl ethanoate | 6 | nd | 0.08 ± 0.01$^{bB}$ | nd | nd | nd | nd | nd | nd | nd | 0.45 ± 0.25$^{aA}$ |
| (nd) | 12 | nd | 0.73 ± 0.08$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| Ethyl hexanoate | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | 0.42 ± 0.08$^{aA}$ | nd | 0.48 ± 0.15$^{aA}$ | 0.44 ± 0.09$^{aA}$ | nd | nd | nd | nd | nd | nd |
| Hexyl ethanoate | 6 | nd | 0.12 ± 0.02$^{aB}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | 0.74 ± 0.17$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |

(*continued on next page*)

**Table 1** (*continued*)

| Compound | Months | 20 °C | | | | | 10 °C | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 20 kPa $O_2$ | Wax | 2 kPa $O_2$ | 2 kPa $O_2$ + 40 kPa $CO_2$ | 2 kPa $O_2$ + 80 kPa $CO_2$ | 20 kPa $O_2$ | Wax | 2 kPa $O_2$ | 2 kPa $O_2$ + 40 kPa $CO_2$ | 2 kPa $O_2$ + 80 kPa $CO_2$ |
| 2-pentyl furan | 6 | 0.3 ± 0.08$^{aB}$ | 0.27 ± 0.01$^{aB}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | 1 ± 0.24$^{bA}$ | 1.29 ± 0.53$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| 4.4-Dimethyl butyrolactone | 6 | 7.56 ± 0.07$^{cB}$ | 4.25 ± 0.35$^{dA}$ | 6.76 ± 0.55$^{eA}$ | 13.4 ± 0.55$^{aA}$ | 10.96 ± 0.32$^{bA}$ | 9.3 ± 2.94$^{bA}$ | 10.8 ± 0.32$^{cA}$ | 7.81 ± 0.73$^{cA}$ | 8.37 ± 1.23$^{cA}$ | 13.89 ± 0.16$^{aA}$ |
| (10.43 ± 3.06) | 12 | 11.63 ± 0.76$^{aA}$ | 6.61 ± 2.08$^{cA}$ | 8.71 ± 1.44$^{bA}$ | 11.97 ± 4.6$^{aA}$ | 4.32 ± 0.2 $^{dB}$ | 4.36 ± 0.52 $^{dB}$ | 7.6 ± 0.46$^{cB}$ | 4.01 ± 0.41 $^{dB}$ | 7.2 ± 0.73$^{cA}$ | 4.77 ± 0.17 $^{dB}$ |
| 5,5-Dimethyl-2 (5H)-furanone | 6 | 0.5 ± 0.05$^{aA}$ | 0.45 ± 0.04$^{bA}$ | 0.62 ± 0.02$^{aA}$ | 0.63 ± 0.05$^{aA}$ | 0.56 ± 0.09$^{aA}$ | 0.38 ± 0.21$^{bA}$ | 0.35 ± 0.03$^{bA}$ | 0.35 ± 0.05$^{bA}$ | 0.43 ± 0.11$^{bA}$ | 0.44 ± 0.02$^{bA}$ |
| (0.39 ± 0.05) | 12 | 0.53 ± 0.11$^{aA}$ | 0.48 ± 0.06$^{aA}$ | 0.57 ± 0.08$^{aA}$ | 0.51 ± 0.02$^{aA}$ | 0.25 ± 0.04$^{bB}$ | 0.15 ± 0.02$^{bB}$ | 0.24 ± 0.02$^{bA}$ | 0.22 ± 0.01$^{bB}$ | 0.22 ± 0.02$^{bB}$ | 0.2 ± 0.04$^{bB}$ |
| Butyrolactone | 6 | 0.39 ± 0.04$^{aA}$ | 0.31 ± 0.05$^{aB}$ | 0.24 ± 0.04$^{aA}$ | 0.2 ± 0.06$^{aA}$ | 0.3 ± 0.07$^{aA}$ | nd | nd | nd | nd | nd |
| (nd) | 12 | 0.6 ± 0.06$^{bA}$ | 3.99 ± 5.16$^{aA}$ | 0.65 ± 0.07$^{bA}$ | 0.41 ± 0.14$^{bA}$ | 0.28 ± 0.01$^{bA}$ | nd | nd | nd | nd | nd |
| γ-Caprolactone | 6 | 0.21 ± 0.04$^{aB}$ | 0.21 ± 0$^{aB}$ | 0.13 ± 0.01$^{aB}$ | 0.16 ± 0.03$^{aA}$ | 0.19 ± 0.05$^{aA}$ | 0.19 ± 0.05$^{aA}$ | nd | nd | nd | nd |
| (nd) | 12 | 0.85 ± 0.11$^{aA}$ | 0.69 ± 0.4$^{aA}$ | 0.32 ± 0.08$^{bA}$ | nd | nd | nd | 0.05 ± 0.01$^{cA}$ | nd | nd | nd |
| γ-Octalactone | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | 0.39 ± 0.09$^{bA}$ | 0.5 ± 0.03$^{aA}$ | nd | nd | nd | nd | nd | nd | nd | nd |
| δ-Octalactone | 6 | 0.55 ± 0.17$^{bA}$ | nd | 0.31 ± 0.03$^{cA}$ | 0.33 ± 0.04$^{cA}$ | 0.58 ± 0.13$^{bA}$ | 0.85 ± 0.28$^{aA}$ | 0.58 ± 0.29$^{bA}$ | 0.53 ± 0.03$^{bA}$ | 0.51 ± 0.15$^{bA}$ | 0.67 ± 0.24$^{bA}$ |
| (0.19 ± 0.24) | 12 | 0.34 ± 0.09$^{aA}$ | nd | 0.35 ± 0.09$^{aA}$ | 0.31 ± 0.02$^{aA}$ | nd | nd | nd | 0.14 ± 0.06$^{bB}$ | 0.17 ± 0.02$^{bB}$ | 0.11 ± 0.01$^{bB}$ |
| Dimethyl sulfide | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| (nd) | 12 | nd | nd | 0.32 ± 0.05$^{cA}$ | 0.72 ± 0.31$^{aA}$ | 0.5 ± 0.04$^{bA}$ | nd | nd | nd | 0.3 ± 0.01$^{cA}$ | 0.14 ± 0.01$^{dA}$ |
| Limonene (isomer) | 6 | 2.48 ± 1.46$^{cB}$ | 2.88 ± 0.16$^{cB}$ | 3.73 ± 0.36$^{cB}$ | 1.22 ± 0.65$^{cB}$ | 2.01 ± 1.77$^{cA}$ | 9.39 ± 3.36$^{bA}$ | 21.42 ± 1.15$^{aA}$ | 1.75 ± 0.83$^{cA}$ | 2.58 ± 0.52$^{cA}$ | 2.47 ± 0.52$^{cA}$ |
| (1.93 ± 0.42) | 12 | 11.47 ± 2.02$^{bA}$ | 8.97 ± 0.86$^{bA}$ | 14.25 ± 2.26$^{aA}$ | 11.27 ± 4.8$^{bA}$ | 3.54 ± 0.18$^{cA}$ | 6.55 ± 0.67$^{cB}$ | 13.41 ± 0.55$^{aB}$ | 3.72 ± 0.31$^{cA}$ | 4.74 ± 1.65$^{cA}$ | 4.53 ± 0.66$^{cA}$ |
| Sabinene | 6 | nd | nd | nd | nd | nd | nd | nd | nd | nd | 0.74 ± 0.07$^{aA}$ |
| (nd) | 12 | 3.69 ± 1.24$^{bA}$ | 2.26 ± 0.26$^{dA}$ | 4.99 ± 0.92$^{aA}$ | 2.73 ± 1.08$^{cA}$ | 0.69 ± 0.05$^{eA}$ | 0.89 ± 0.06$^{eA}$ | 0.56 ± 0.01$^{eA}$ | 0.93 ± 0.06$^{eA}$ | 1.03 ± 0.11$^{eA}$ | 0.94 ± 0.08$^{eA}$ |

*Mean followed by the same lowercase letter within each assessed period (in the same line), and by the same upper case letter by comparing different storage periods, did not differ according to the Scott Knott test ($p < 0.05$).

However, some treatments at 10 °C showed high PAV of certain compounds after six months, and their values decreased after twelve months, such as C8 and C10 acids in 2p$O_2$-10, ethanol in 40p$CO_2$-10 and 80p$CO_2$-10, 4,4-dimethyl butyrolactone, and ethanal in 80p$CO_2$-10, 2-propanone in 40p$CO_2$-10, and limonene in CW-10, for example.

Aldehydes, acids, and alcohols were abundant in nuts stored in AMB-20, CW-20, and 2p$O_2$-20, although the treatments 40p$CO_2$-20, 80p$CO_2$-20, and conditions at 10 °C contributed to maintaining lower PAVs. The aldehydes 2-methyl butanal, 3-methyl butanal, and hexanal were detected in all treatments and periods analyzed. At twelve months, 2-methyl butanal and 3-methyl butanal had lower PAVs in 80p$CO_2$-20 and all conditions at 10 °C compared to the previous period. The AMB-20, CW-20, and 2p$O_2$-20 treatments showed higher PAV, either after six and twelve months of C5, C6 acids and alcohols, hexanal, and after twelve months of C7-C9 alcohols, and aldehydes, heptanoic and octanoic acids, (E)-2-decenal, ethanol, butanol, and 5-methyl 2-hexanol. These VCs are strongly correlated with higher PVs; consequently, a higher concentration of compounds from oxidation, such as hexanal, heptanal, octanal, and (E)-2-heptenal, C4-C8 acids, and C4-C9 alcohols, occurs (Maarse, 1991).

Hexanal PAV increased from six to twelve months with storage at 20 °C except for 80p$CO_2$-20. Storage at 10 °C reduced hexanal content and did not differ between the six- and twelve-month evaluations. The treatment 80p$CO_2$-20 and under CA conditions at 10 °C, the aldehydes heptanal, (Z)-2-heptenal, and nonanal, present in the other treatments, were not detected. Heptanal and nonanal were also not detected in CW-10, regardless of the evaluation time. Hexanal is the main aldehyde produced during linoleic acid degradation, whereas octanal and nonanal come from oleic acid (Alasalvar, 2005; Maarse, 1991). Hexanal content may increase during storage (Bai et al., 2019; Ghirardello et al., 2013), in addition to being an important marker of oil oxidative stability, which is likely because of the high PVs since peroxides are easily converted into volatile compounds (Valdés et al., 2015). Treatment storage at 40 kPa and 80 kPa of $CO_2$, regardless of temperature, ensured lower PAV of hexanal compared to AMB, CW, and 2p$O_2$-20 ones. This evidence may be related to $CO_2$ action against oxidation (Maskan & Karataş, 1999).

Thewes et al. (2021) found that nuts subjected to low $O_2$ (2 kPa) for twelve months, regardless of MC, had lower hexanal content compared to those under ambient conditions (21 kPa). Ribeiro et al. (2020b) reported that pecan hexanal content was higher at 20 °C than at lower temperatures, regardless of atmospheric conditions (ambient, 3, or 1 kPa). Interestingly, a different study demonstrated that hexanal content increased with time at 10 °C and p$CO_2$-20 (15 kPa of $CO_2$) in the storage of shelled pecans (Ribeiro et al., 2022). Baldwin & Wood (2006) evaluated the hexanal and sensory acceptance of stored 'Desirable' pecans, comparing the ambient condition and many coatings products, and observed that coatings offered higher acceptance rates by panelists concurrently to lower values of hexanal concentration.

Acetic acid, with the highest PAV among the acids, showed the major PAV in CW-20 treatment. Additionally, at twelve months, it decreased in AMB, 2p$O_2$, and 80p$CO_2$ at 10 °C and 80p$CO_2$-20; it did not differ in 40p$CO_2$-20, 40p$CO_2$-10, and CW-10. Acetic acid is commonly found in

almonds and pecans (Maarse, 1991; Xiao et al., 2014; Ribeiro et al., 2020b). Generally, this acid increases during pecan storage and may be associated with fermentation processes (McWilliams & Mackey, 1969; Ribeiro et al., 2020b).

Butanoic and hexanoic acids showed significantly high PAVs in AMB and CW at 20 °C in six and twelve months, respectively. According to Xu et al. (2017), organic acids may be produced through oxidation from their corresponding aldehydes. Conversely, C4 to C9 acids come from the cleavage of fatty acids through lipase action or autooxidation (Maarse, 1991; Wang et al., 2017). In foods, C4 and C5 acids may contribute to cheesy and buttery aromas, while C6 and C8 of cheesy, rancid, and goat odors (Acree & Arn, 2004). A study with shelled pecans showed that the CA with high $pCO_2$ (15 kPa) stimulated C4 to C8 acid production, regardless of the temperature (10 and 20 °C) (Ribeiro et al., 2022). This difference may be related to the shelling process or mainly to the different $pCO_2$ evaluated in both studies, in which the highest $CO_2$ (40 or 80 kPa) was more effective. Ribeiro et al. (2020b) found higher PAVs of acids at 20 °C, regardless of atmospheric conditions.

During storage, the aliphatic alcohols C4–C10 and C12 showed larger PAVs in AMB, CW, and $2pO_2$ at 20 °C. The hexadecanol and nonadecanol were only detected at six months, and the last was only in AMB-20, CW-20, $2pO_2$-20, and $40pCO_2$-20 treatments. According to Wang et al. (2017), aliphatic alcohols are a major class of lipid autoxidation products found in all fat-containing foods, formed by reducing aldehydes and by enzymic reduction of methyl ketones (Valdés et al., 2015). Ribeiro et al. (2022) demonstrated that the concentrations of most alcohols intensified during the storage process, mainly in the $pCO_2$ (15 kPa of $CO_2$) treatment. However, saturated aliphatic alcohols do not significantly contribute to the nut flavors, and they have odor threshold values considerably higher than those of the aldehydes or ketones, which have more organoleptic interest (Maarse, 1991).

The chemical classes ketones, esters, and lactones were also mostly present in the treatments at 20 °C. The methyl ketones C3 to C5 are some of the most significant compounds in the aroma of white mold-ripened cheese (Maarse, 1991; Spanier et al., 2000). In the analyzed pecans, the methyl ketones 2-propanone, 2-pentanone, and 2-heptanone showed higher PAVs in AMB and CW treatments at 20 °C and 2-butanone in the treatments at 20 °C. 2-Butanone generally decreased with prolonged storage and, in the sixth month, was presented in a higher amount with $80pCO_2$, irrespective of storage temperature. These compounds are formed from the decarboxylation of β-keto-acids, while branched-ketones such as 6-methyl-5-hepten-2-one come from carotenoid degradation (Maarse, 1991; Spanier et al., 2000). 4-Heptanone was only detected in AMB and CW at 20 °C and showed higher PAVs in CW-20 after twelve months. 3-Octanone was only detected in treatments without CA, regardless of the temperature and evaluation time, and branched ketones such as 3-methyl butanone were detected only in the CW-20, while 6-methyl 5-hepten-2-one was detected in conditions without CA at 20 °C and treatments at 10 °C. In general, conditions with high $pCO_2$ and at 10 °C tended to maintain lower ketone PAVs. Thewes et al. (2021) showed that pecan storage at 2 kPa of $O_2$, irrespective of the MC, maintained lower PAVs of 2-octanone and 2-heptanone in 'Barton' and 'Jackson' cultivars, respectively, compared to ambient conditions.

Esters may enrich the aroma with fruitier notes. A higher proportion of this class was detected at 20 °C, mainly in CW-20. Esters were detected in lower quantities and mostly under AMB-20 and CW-20 conditions. Ethyl 3-methyl butanoate was detected only at twelve months, with high PAV at $2pO_2$-20 and $40pCO_2$-20, and ethyl butanoate was detected only at this evaluation point and all conditions at 10 °C and CA at 20 °C. Ethyl ethanoate was the only ester detected under all storage conditions and both evaluation points, and the largest PAV was after twelve months in $2pO_2$-20. Ethyl esters are formed in food by bacteria species via esterification (from ethanol and acetate) or a transferase reaction (alcoholysis) from alcohols and acylglycerols when ethanol is available (Liu et al., 2004). In addition, it can also result from esterification reactions of acids with primary alcohols (Maarse, 1991).

Lactones are compounds with odoriferous importance; the 4,4-dimethyl butyrolactone, 5,5-dimethyl-2(5H)-furanone, butyrolactone, and γ-caprolactone showed higher PAVs in the 20 °C treatments. Lactones with over ten carbons may be formed by the lactonization of the corresponding 4- and 5-hydroxy fatty acids, constituents of triglycerides, or may result from lipid oxidation (Watanabe & Sato, 1970). Thus, alternate pathways for UFAs cause the formation of γ or δ-lactones (e.g., decalactone). The odor properties of these lactones are oily, buttery, fatty, and fruity (Acree & Arn, 2004; Wang et al., 2017).

Furthermore, nuts submitted to 20 °C also showed a higher area of furans and terpenes. The 2-pentylfuran compound was detected only in CW-20 and AMB-20, increasing after twelve months. 2-Pentylfuran is an oxidation product from linoleic and other n-6 PUFAs (Valdés, 2015; Durmaz & Gökmen, 2010). This VC was detected only in treatments without CA at 20 °C; nevertheless, it is positively related to pleasant odors such as baked and buttery beans (Maarse, 1991; Acree & Arn, 2004). After twelve months, Sabinene was detected under all conditions and showed higher PAV in $2pO_2$-20. Limonene presented high PAVs in AMB-10 and CW-10 but decreased under these conditions after twelve months of storage. The major source of such compounds is the oxidative decomposition of the carotenoids (Maarse, 1991).

The only sulfur compound found was dimethyl sulfide was detected after twelve months of storage and under CA conditions except for the $2pO_2$-10 treatment. The breakdown of proteins (peptides), microbial metabolism, and enzymatic conversion of sulfur- and nitrogen-containing precursors results in the formation of volatile thiols, sulfides, and disulfides, and normally possess low sensory threshold values (Maarse, 1991). Ribeiro et al. (2020b) found higher PAV of dimethyl sulfide in AMB and $pO_2$ of 3 kPa treatments with refrigeration temperatures (below 10 °C); nevertheless, it was suppressed at all temperatures at 1 kPa of $O_2$.

In this study, the higher PAV production of ketones, aldehydes, alcohols, acids, and lactones in AMB and CW conditions at 20 °C corroborates the PV and TBARS results. This shows that lipid oxidation is the primary mechanism that increases rancidness, including browning and acidity, in pecans during storage (Wang et al., 2017; Ribeiro et al., 2020b).

### 3.5. Exploratory multivariate analysis - PCA and heatmap

The exploratory multivariate analysis was performed by principal component analysis (PCA) (Figs. 3 and 4) to better visualize the main findings in this study. The first three principal components (PC) accounted for 70.72% of the global variation. The main factors of separation among treatments were related to the atmosphere storage condition and storage time. In this way, PC1 (42.16%) (Fig. 3A and 3B) explained CW-20, AMB-20 treatments (sixth and twelfth months), and $2pO_2$-20 (twelfth month), which were positioned in the right quadrants, from the CA treatments (regardless of the temperature), without CA at 10 °C, and the initial time of storage, on the left quadrants of the PCA score graph. In AMB, CW, and $2pO_2$ at 20 °C, the samples were correlated to higher AV, PV, a* parameter, and the highest PAV of the major volatile compounds (acetic acid, C4-C8 acids, C6-C10 aldehydes, C4-C9 alcohols, benzyl alcohol, butyrolactone, octalactone, pentyl furan). Nevertheless, it showed that CA treatments, mainly at 10 °C, were inversely correlated with those cited compounds and positively related with the color parameters L*, b*, H, and C. The CA treatments are located in the upper left region, regardless of the temperature, at six months of storage, which showed a greater relationship with L* and b* parameters, C and Hue angle. The position of the initial time, jointly with CA treatments at six months, indicates that the treatments in CA are effective against the advances of the deterioration by oxidation, maintaining the desirable attributes in this period. An interesting fact is that the treatments with CA, even submitted to 20 °C, allowed the maintenance of this related quality attributes and less association with volatile compounds from oxidation, such as the aldehydes hexanal, octanal, and

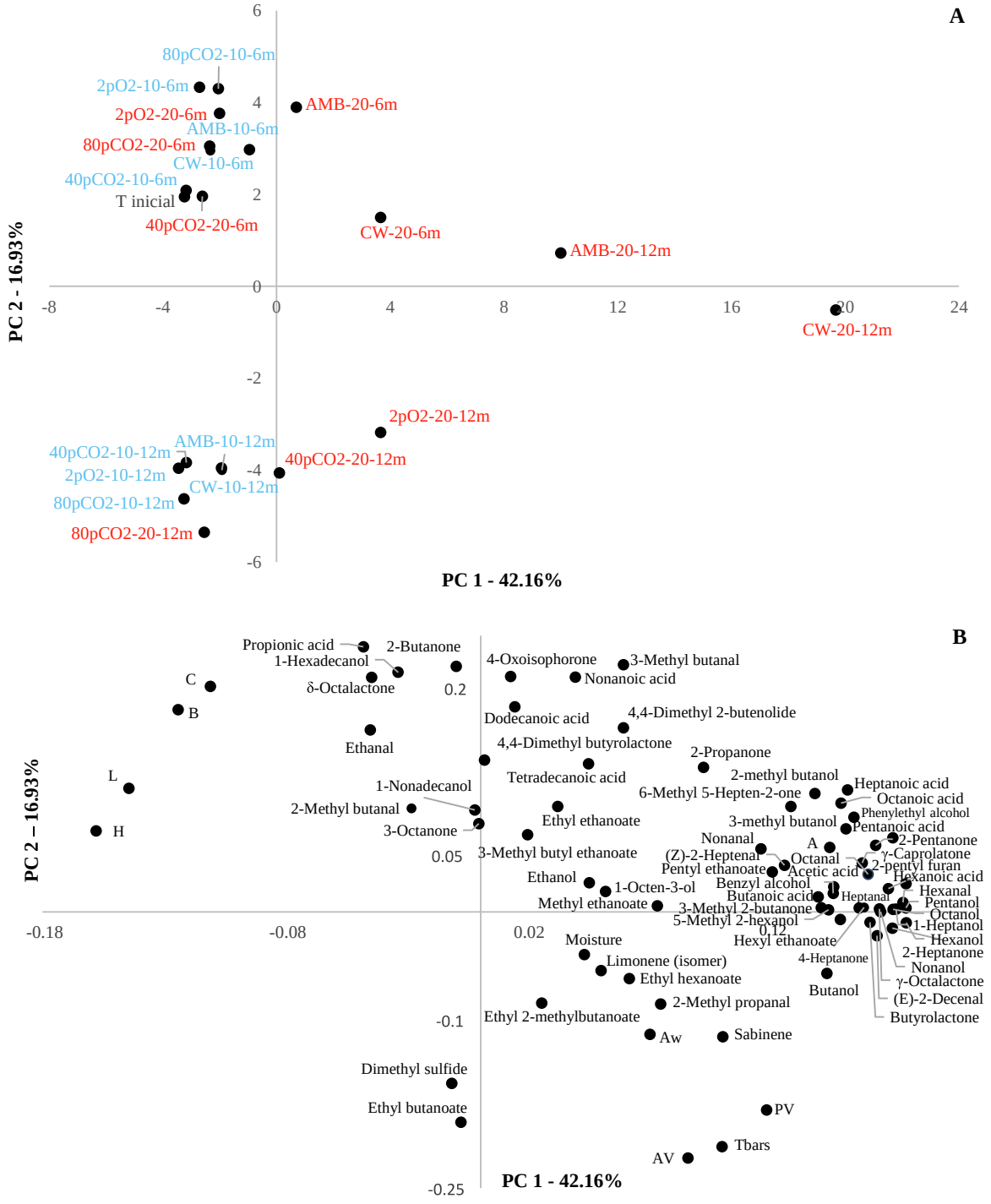



**Fig. 3.** Analysis of principal components of 'Barton' pecan nuts, after storage in different temperatures, oxygen partial pressure, high $CO_2$ and carnauba wax coating. PCA I and II, **(A)** graph of the samples (loadings); **(B)** graph of the variables (scores) (color, moisture content, $A_w$, acidity, peroxides indexes, TBARS and volatile compounds).

nonanal and C4-C8 acids.

The PC2 (16.93%) (Fig. 3A and 3B) separated the sixth month of treatments (upper region) from the twelfth month of storage (lower region), except for AMB-20 at twelve months (upper quadrant). The treatments located in the upper region are correlated with the positive attributes of higher luminosity, color parameters b*, C, and Hue, C3, C9, C10, and C12 acids, butanone, oxoisophorone, octalactone, and hexadecanol. In addition, the PC2 also showed a lower relationship between PV, AV, and TBARS (in the sixth month) with the CA treatments and more association with CW-20 (in the twelfth month). With a lower explanation power, PC3 (11.63%) mainly showed the difference between the treatments $2pO_2$-20–12 m from CW-20–6 m and the influence of time among treatments in CA (Figs. 4A and 4B). It also distinguished the CW-20 treatment from the others due to its higher value of volatile compounds (dodecanoic acid, hexadecanol, nonadecanol, 3-methyl butyl ethanoate), the parameter a*, and MC.

In order to better understand the data obtained, the heatmap provides a good view of relationships and groupings (Supplementary Material 3). The color-coding of correlations, with different colors for positive and negative correlations, is the underlying principle in a

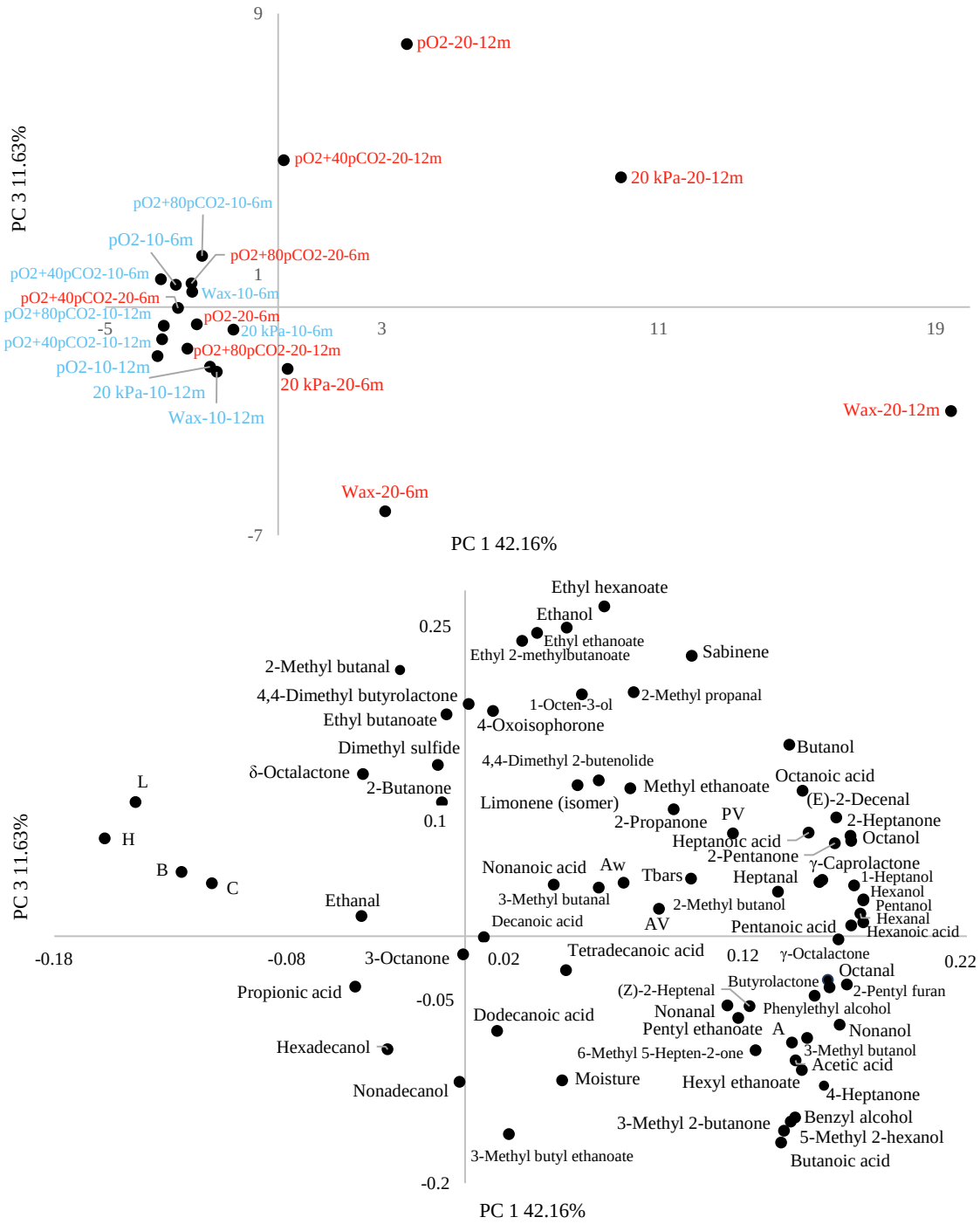



**Fig. 4.** Analysis of principal components of 'Barton' pecan nuts, after storage in different temperatures, oxygen partial pressure, and high $CO_2$ and carnauba wax coating. PCA I and III, **(A)** graph of the samples (loadings); **(B)** graph of the variables (scores) (color, moisture content, $A_w$, acidity, peroxides indexes, TBARS and volatile compounds).

heatmap; a more saturated color indicates a higher correlation value (Reimann et al., 2017). The heatmap presents larger cluster groupings due to the evaluation period (initial time, six and twelve months).

The initial time grouping with the treatments that use high $pCO_2$ (even at 20 °C) and CA at 10 °C in six months may indicate a positive result because these treatments contribute to maintaining the initial quality. At six months, a group of samples was formed in CA, indicating a lower occurrence of aldehydes, ketones, and lower AV and PV values, although it showed a more minor relationship in CW-20. CW-20 showed a greater relationship with aldehydes (C4-C10), alcohols (C5-C9), branched alcohols, reddish color (a*), AV, and PV, and this fact can be confirmed in PC1 (Fig. 3).

The other large grouping for the twelve months occurs due to the general increase in AVs, PVs, TBARS, and VCs. Notably, $2pO_2$-20 formed clusters with $40pCO_2$-20 and AMB-20 treatments due to butanol, sabinene, ethanol, 1-octen-3-ol, and ethyl hexanoate, which can be seen in PC3 (Fig. 4). Through the heatmap, we can also observe the grouping of treatments in CA, which maintains lower correlations with the formation of volatiles characteristic of rancidity (e.g., hexanal, heptanal, octanal, pentanoic, hexanoic, and octanoic acids) even at 20 °C and after twelve months of storage. Lastly, it is clear whether the CW and AMB treatments (without CA) at 20 °C present greater relationships with

several VCs, higher AV, PV, TBARS, and a* values, which can also be seen in the PCA graphs.

## 4. Conclusion

Controlled atmospheric conditions, mainly when combined with high $CO_2$, maintain 'Barton' pecan quality attributes, even when subjected to higher temperatures (20 °C). Hence, it can be considered an effective strategy for pecan storage for up to twelve months, maintaining desirable attributes such as greater luminosity (L*), yellowish color (b*), lower oxidation markers such as acidity, peroxides, and volatile compounds that are characteristic of rancidity, such as hexanal. Regarding the VCs, the high $pCO_2$ of 80 kPa prevented higher hexanal, heptanal, (Z)-2-heptenal, and nonanal (undetected in these treatments) values, which are characteristic off-flavor aldehydes. In addition, 40 kPa of $CO_2$ also protected against increased TBARS and maintained the PAVs of various acids, ketones, and alcohols low from lipid oxidation.

Despite being a promising technique for conserving various stored fruits, the pecans coated with carnauba wax did not show satisfactory results, resulting in more red tones (a*), higher AV and TBARS at both temperatures, and higher PV and VC formation at 20 °C. Therefore, low $pO_2$ and high $pCO_2$ maintain better 'Barton' pecan quality than storage under normal $O_2$ ambient conditions (20 kPa), even at temperatures above refrigeration conditions. Hence, our findings show that even at 20 °C, the association of high $CO_2$ with low $pO_2$ for long-term pecan storage under CA can be recommended.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

The data that has been used is confidential.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112854.

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ORIGINAL PAPER

# Comprehensive study of the volatile profile of Niágara Rosada (*Vitis labrusca*) wines produced from *Brettanomyces anomalus* using GC–FID–MS: a chemical and sensory approach

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## Abstract

There has been a controversy concerning the use of *Brettanomyces* in wines due to its questionable behavior. Some authors reported that this yeast causes adverse effects in wines; other authors reported that this yeast could promote a floral and fruity aroma for wines. In this context, this study analyzed *Brettanomyces anomalus* as alternative yeast for winemaking, providing the relationship between chemical and sensory approaches. Isoamyl acetate (159 $\mu gL^{-1}$), linalyl acetate (20.6 $\mu gL^{-1}$), ethyl butanoate (176 $\mu gL^{-1}$), ethyl hexanoate (29.2 $\mu gL^{-1}$), and ethyl octanoate (6.30 $\mu gL^{-1}$) were related to flavor acceptance, providing a sweet, fruity, and floral profile for the wines. Terpenes (β-myrcene, 20.7 $mgL^{-1}$, and levomenol, 10.3 $\mu gL^{-1}$) and acids, synergistically, drove the aroma and body acceptance. The overall acceptance was related to carbonilic compounds (nonanal) giving a citrus/green scent and benzene compounds, the latter contributing synergistically. This study showed the relevance of the use of *Brettanomyces* to improve the *Vitis labrusca* wine quality, resulting in sweet, fruity, and floral aroma, features very appreciated by Brazilian consumers.

**Keywords** Wine · Non-*Vitis vinifera* · Niágara Rosada · *Brettanomyces* · Volatile compounds · Sensory acceptance

## Introduction

A significant number of factors, such as the grape sanitary conditions, the chemical and mineral composition of the soil, the correct application of the winemaking procedure, and even the *terroir*, determine the wine quality. All these factors can be correlated with the wine's chemical properties, influencing its sensory quality. Some of these chemicals can interact with each other producing alternative and complex chemical compounds, which might influence the sensory wine profile [1].

Alternative winemaking processes can be responsible for several changes in the wine's chemical profile. Several authors have already reported the application of different winemaking procedures, such as the use of alternative yeasts for alcoholic fermentation, showing their potential by providing relevant differences in the chemical composition and sensory acceptance. Schumaker et al. [2] studied the influence of Shiraz wine composition and *Brettanomyces* metabolites on consumer perception. They reported a decrease in fruity aroma and high *Brett* character levels. Also, they mentioned the low overall acceptance by consumers when these metabolites are present.

Guichard et al. [3] studied the effect of *Brettanomyces* on sensory evaluation and volatile quantitation in different French ciders. The authors have reported a decrease in fruitiness due to the ester degradation, assuming that this yeast has a membrane or intracellular acetyl esterase activity. In this context, the assessment of the wine chemical profile produced from alternative winemaking becomes relevant,

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since it provides information regarding its sensory profile, allowing the correlation between both data groups [4].

Besides all the alternative winemaking procedures that can be applied to improve the wine quality, a vital part of the winemaking procedure is alcoholic fermentation. *Saccharomyces cerevisiae* is a widely known yeast that produces several different beverages, such as beers, ciders, meads, and wines [5]. However, the application of *Saccharomyces* in alcoholic fermentation is not exclusive, since recent studies reported the potential of alternative yeasts, such as *Dekkera/Brettanomyces*, showing their potential in fermentations procedures [6, 7].

There is a controversial discussion concerning the *Dekkera/Brettanomyces* "hero/villain" effect on alcoholic beverages. It can be considered a wine spoilage agent due to the lack of sanitary conditions along with the winemaking procedure, as well as yeast that can produce definite aroma and flavor effects in an alcoholic beverage. *Brettanomyces/Dekkera* (general term *Brettanomyces*) is a widely known type of yeast spoilage on wines, producing unpleasant sensory features known as the *Brett* character [8]. Alternatively, this yeast is being used in beers to provide a particular flavor and aroma complexity [9].

*Brettanomyces* produces a set of different volatile compounds such as volatile phenols: 4-ethyl phenol (4-EP), 4-ethyl guaiacol (4-EG), 4-vinyl phenol (4-VP), 4-vinyl guaiacol (4-VG); acids: isovaleric acid, acetic acid and some esters, such as ethyl acetate, among others, providing a typical sensory feature for the beverage. In general, *Brettanomyces* genus is considered a villain for the wine industry, since it provides mousy, medicinal, wet wool, burnt plastic, or horse/sweat smells/scents mainly caused by the high concentration of volatile phenols, as reported by Smith and Divol (2016) [10].

The synthesis of volatile phenols mentioned above depends on the fermentation medium and the concentration of their precursors. For example, *Brettanomyces* contamination is frequently reported in red wines, where the extraction of the volatile phenols' precursors from the grape skin is more intense, since the red wine fermentation is performed with the presence of the grape skins. In contrast, the role of these compounds in overall wine quality is entirely subjective, since the rare reports describing the pleasant effects of these compounds in wine. The metabolic pathway resulting in the synthesis of the volatile phenols involves the presence of the vinyl phenol reductase in addition to the phenyl acrylic acid decarboxylase, an enzyme produced by *Brettanomyces*. The reduction of the 4-VP to 4-EP and 4-VG to 4-EG caused the unpleasant wine off-flavors. The physiological role of these enzymes is still unknown [5].

In contrast, some *Brettanomyces* species, especially *Brettanomyces anomalus*, also known as *Brettanomyces clausenii* or *Dekkera anomala*, have already been considered as a potential yeast that could enhance the floral and fruity flavor producing higher levels of ethyl esters such as ethyl acetate, ethyl decanoate, ethyl caprylate, and ethyl lactate [11].

This work aimed at evaluating the effect of *Brettanomyces* as alternative yeast for Niágara Rosada (*Vitis labrusca*) winemaking, focusing on the chemical, volatile profiles, and sensory acceptance. The manuscript also aimed at assessing the relationship between the volatile profile and sensory acceptance to find relevant sensory acceptance drivers.

## Materials and methods

### *Brettanomyces* growth

Vigvatten Brewery located in Catanduva city (21° 08′ 16″ South, 48° 58′ 22″ West), Northwest region of São Paulo State, Brazil provided the *Brettanomyces anomalus* yeast. The *Brettanomyces anomalus* used in Vigvatten Brewery was provided by Whitelabs (code WLP 645), and the yeast used in this project was isolated from a fermentation process using WLP 645 yeast. The growth of the *Brettanomyces* colonies followed an adaptation of Rodrigues et al. (2001) [12], which consists in inoculating the *Brettanomyces* in Petri Plates containing Potato Dextrose Agar medium (PDA) with bromocresol green indicator to assess the specific colony formation.

There is some information about the *Brettanomyces* behavior in this medium, i.e., this yeast does not degrade the bromocresol green indicator, generating distinctive morphologic greenish colonies, even among themselves, resulting in a relatively easy yeast to identify. Aiming at comparing the *Brettanomyces* colonies, a pure culture of dry *Saccharomyces cerevisiae* plate was also inoculated for the control treatment. After 72 h, the plate inoculated with *Brettanomyces* presented distinctive greenish smooth round colonies, while the *Saccharomyces* plate presented only whitish round colonies.

After this procedure, the Petri Plate containing *Brettanomyces anomalus* was re-inoculated in a primary culture media YPD, 1% (weight/volume) of yeast extract, 2% (weight/volume) of peptone and 2% (weight/volume) of glucose [13] and incubated for 72 h at 25 °C. The colonies were counted using the Neubauer chamber method and for cell viability, colored with methylene blue, after reaching $10^7$ CFU.mL$^{-1}$ [14]. An aliquot of the prepared inoculum was separated for the wine elaboration process.

### Wine samples and micro-vinification

The Niágara Rosada (*Vitis labrusca*) grapes (5 kg for each treatment) were purchased from a local market in the São José do Rio Preto city (20° 49′ 13″ South, 49°22′ 47″

West) in the Northwest region of São Paulo, Brazil. The winemaking method reported by De Castilhos et al. [15] produced both *Saccharomyces* wine (SW) and *Brettanomyces* wine (BW). The grapes were placed into 10-L fermentation flasks, and the winemaking consisted of the grape de-stemming followed by a gentle manual crushing to extract the grape-wine must. Both the SW and BW wines were treated with 15 g of potassium metabisulfite for each 100 kg of grapes as a selective antimicrobial agent.

The alcoholic fermentation was carried out with the inoculation of active dried *Saccharomyces cerevisiae* yeasts in the proportion of 10 g for each 100 L of the must. Aiming at standardizing the alcoholic fermentation procedure, a solution containing this mentioned proportion of *Saccharomyces cerevisiae* Y904 (Amazon Group®) was elaborated to determine the $CFUmL^{-1}$ using the Neubauer chamber. This solution was the standard reference for the required volume of *Brettanomyces* solution that was added to the fermentation flask for standardizing both the SW and BW winemaking procedures. For both the winemaking treatments, the concentration of yeasts was above $10^7$ $CFUmL^{-1}$.

After the inoculation of the yeasts, the grape musts were fermented for 8 days, pumping twice a day. After this fermentation time, both SW and BW wines were dejuiced and chaptalized to 13% (v/v). The wines were racked three times between 10-day intervals. After the second racking, the wines were submitted to malolactic fermentation by the inoculation of *Oenococcus oeni* lactic acid bacterium (Laffort®), controlled by Thin-Layer Chromatography (TLC) [16]. After the third racking, the wines were transferred to a refrigerated ambient (1–3 ℃), allowing the tartrate stabilization. The wines were then bottled and left for 60 days to stabilize. All the winemaking trials were carried out in three repetitions as follows: SW1, SW2, and SW3 for *Saccharomyces* wines and BW1, BW2, and BW3 for *Brettanomyces* wines.

## Conventional enological parameters

The following enological parameters were carried out: alcoholic content (% v/v) (ALC) using a digital densimeter (Anton Paar®) [17], reducing sugar (RSG) by the Lane–Eynon method using a Redutec determiner (Tecnal TE0861®) [17], total (TAC) and volatile (VAC) acidity using a pH meter, titration and a distiller (Tecnal TE0363®) [17], dry extract (EXT) using porcelain capsules and thermostatic bath at 100 °C [17], total phenolic content (PHEN) using an absorbance spectrophotometer at 765 nm [18], and CIELab color indexes using absorbance spectrophotometer at 450, 520, 570, and 630 nm. CIELab parameters were determined using MSCV 7.1 software [19].

## Sensory acceptance

The sensory acceptance was carried out at the Sensory Analysis Laboratory of the Food Technology and Engineering Department of the São Paulo State University. A panel of 91 untrained consumers (the panel data are detailed in Sect. 3.3) assessed the six samples of wine acceptance for appearance, aroma, body, flavor attributes, and overall acceptance, using a nine-point verbal hedonic scale (1 = disliked immensely, 5 = neither liked nor disliked, and 9 = liked immensely). Also, a five-point hedonic scale (1 = definitely will not buy it, 5 = definitely would buy it) assessed the purchase intention [20, 21]. A pre-questionnaire for characterizing the panelists was delivered for the consumers to collect their age, gender, and frequency of wine consumption. The frequency of wine consumption was determined using the annual wine consumption per person (1.73 L/person/year). Very low wine consumption was considered as less than one glass of wine per month; a meager wine consumption was considered as one glass of wine per month. Also, more than one up to two glasses of wine was considered as a moderate wine consumption; more than two up to four glasses of wine a high wine consumption; and more than four glasses of wine, a very high wine consumption.

The sensory acceptance test was carried out on individual booths at room temperature (23–25 ℃) under white lights. The samples were presented in 30 mL transparent wine glasses containing 15 mL of the sample at 18 ℃ with a glass of water for mouth rinsing between the samples assessment. For this analysis, the complete block experimental design was used, i.e., all the consumers assessed all the samples. The samples were presented in a monadic way, i.e., one sample at a time, in a randomized order, coded with three random digits to avoid carry-over effects [22].

The Ethics in Research Committee of the Institute of Biosciences, Humanities, and Exact Sciences, São Paulo State University approved the Ethical Issues regarding sensory acceptance (process n. 2.447.651).

## Analysis of the volatile compounds

The headspace solid-phase microextraction (HS-SPME) technique extracted the wines' volatile compounds, according to Bernardi et al.'s [23] method. An aliquot of 2.0 mL of wine was transferred to a 20 mL glass vial followed by the addition of 600 mg of NaCl. Divinylbenzene/carboxen/polydimethylsiloxane fiber (DVB/Car/PDMS, 50/30 µm × 20 mm, Supelco, Bellefonte, USA) was used for extraction. The volatile compounds' extractions were performed at 35 ± 0.2 °C in a water bath for 45 min, under stirring. Before exposure of the fiber to headspace, the flask containing the samples was maintained at the same temperature as extraction for 5 min,

to balance the temperature and vapor pressure of analytes. The insertion of the fiber into the injection port of a gas chromatograph thermally desorbed the volatile compounds. Samples were analyzed in triplicate.

A gas chromatograph equipped with a flame ionization detector (GC–FID; Varian Star 3400 CX, CA, USA) determined the volatile compounds. The injector operated in the splitless mode for 1 min with an SPME liner (0.75 mm i.d.). The injector and FID temperature were set to 250 °C. The carrier gas was hydrogen at an initial flow rate of 2 $mLmin^{-1}$ (constant pressure of 10 psi). A ZB-WAX plus capillary column (30 m × 0.25 m × 0.25 µm film thickness; Phenomenex, Palo Alto, CA, USA) was used for volatile compounds separation. The oven temperature was held at 35 °C for 2 min and raised to 80 °C at 2 °C/min, then programmed for raise until 150 °C at $4°Cmin^{-1}$, and then to 230 °C at $8°Cmin^{-1}$ and held at this temperature for 5 min. For sample quantitation, the internal pattern allowed the volatile compounds quantitation, using 3-octanol (Sigma-Aldrich, Germany) at a concentration of 1.03 $\mu gL^{-1}$.

A Shimadzu QP2010 Plus gas chromatography coupled with a mass spectrometer (GC–MS) identified the volatile compounds. GC conditions were the same as those used for the GC–FID analysis with a polar column. Helium was the carrier gas at an initial flow rate of 2.0 $mLmin^{-1}$. MS was operated in electron ionization (EI) mode at 70 eV, scanning from 35 to 350 m/z. The volatile compounds were identified by comparison to experimental mass spectra to those of commercial spectra databases (NIST 14) and by comparing the experimental retention index (RI) with the literature.

### Data analysis

The results were expressed as mean values ± standard deviation and Student's *t* test for the enological parameters, and sensory acceptance was performed at $P < 0.05$, aiming at observing significant differences between the wines produced from *Saccharomyces cerevisiae* and *Brettanomyces anomalus*. The Pearson correlation test at $P < 0.05$ allowed the correlation between sensory acceptance and the frequency of consumption. Both the statistical tests mentioned above were performed using the Minitab 17 software (Minitab Inc.). The sensometric approach was applied to determine which chemical group of volatile compounds contributed as a sensory acceptance driver for *Brettanomyces* wine by the application of Factor Analysis, using the Principal Component Analysis (PCA) as extractor methodology and Varimax normalized as rotation factor. This multivariate approach was performed using Statistica 12 software (StatSoft Inc.).

## Results and discussion

### Grape and winemaking parameters

Different Niágara Rosada juices were used for SW and BW winemaking. For SW, Niágara Rosada juice presented 7.11 $gL^{-1}$ of titratable acidity and pH 3.26; for BW, the grape juice presented 6.79 $gL^{-1}$ of titratable acidity and pH 3.30. The wines were properly chaptalized by the insertion of 26.3 and 42.2 g of sucrose per liter of the must for SW and BW, respectively, since the grapes presented at the beginning of the winemaking 20.5 ºBrix (17.4 ºBabo) and 18.6 ºBrix (15.8 ºBabo). The result obtained for winemaking yield (L of wine per Kg of grapes) for both SW and BW samples was approximately 52% (2.6 L) and 60% (3.0 L), respectively.

### Conventional enological parameters

The use of different yeasts significantly influenced the alcohol content (Table 1), showing that *Brettanomyces* wine (BW) obtained a higher alcohol degree. A possible explanation for this result was reported by Steensels et al. [5], who explained that *Brettanomyces* could degrade alternative carbohydrate substrates instead of glucose and sucrose, such as fructose, maltose, galactose, lactose, and mannose. Also, grapes have, in their composition, many

**Table 1** Conventional enological parameters (means ± standard deviation) for *Saccharomyces* wine (SW) and *Brettanomyces* wine (BW)

| Chemical properties[1] | Wines[2] | | *P* value[3] |
|---|---|---|---|
| | SW | BW | |
| ALC (%v/v) | 13.6 ± 0.21 | 15.7 ± 0.25 | **0.001** |
| RSG ($gL^{-1}$) | 1.11 ± 0.09 | 1.11 ± 0.12 | 1.000 |
| TAC ($gL^{-1}$) | 5.57 ± 0.19 | 5.79 ± 0.33 | 0.398 |
| VAC ($gL^{-1}$) | 0.65 ± 0.09 | 0.69 ± 0.12 | 0.677 |
| PHEN ($mgL^{-1}$) | 668 ± 41.6 | 694 ± 19.5 | 0.397 |
| EXT ($gL^{-1}$) | 25.6 ± 0.97 | 30.5 ± 2.28 | 0.075 |
| L* | 79.8 ± 1.00 | 82.9 ± 0.29 | **0.036** |
| C* | 28.5 ± 2.74 | 17.1 ± 0.19 | **0.019** |
| h* | 79.7 ± 10.8 | 74.2 ± 2.64 | 0.486 |
| a* | 8.57 ± 0.18 | 4.63 ± 0.74 | 0.839 |
| b* | 27.7 ± 2.03 | 16.4 ± 0.33 | **0.011** |
| Color intensity | 1.11 ± 0.05 | 0.81 ± 0.02 | **0.014** |
| Tonality | 1.94 ± 0.04 | 1.70 ± 0.01 | **0.011** |

[1]ALC: alcoholic content; RSG: reducing sugars; TAC: total acidity; VAC: volatile acidity. PHEN: total phenolic content; EXT: dry extract; L*: luminosity; C*: chroma; h*: hue angle; a*: redness; b*: yellowness

[2]SW: *Saccharomyces* wine; BW: *Brettanomyces* wine

[3]*P* values for *t*-Student test with $P < 0.05$. Results in bold indicate the presence of significant differences between means

of these carbohydrates, and *Brettanomyces anomalus* can degrade them as an alternative source of carbon for alcoholic fermentation, as reported by Ribéreau-Gayon et al. [24].

Besides, the sucrose fermentation by the *Brettanomyces* genus depends on the expression of a high-efficiency sucrose transporter (with no homologs in *S. cerevisiae*), and this might be the explanation for the high competitiveness of these yeasts in sucrose-based fermentations [5]. This explanation showed that if the *Brettanomyces* can consume other carbohydrate sources, the resulting ethanol content would be higher than the ethanol content produced by the *Saccharomyces*, since this one only can degrade a limited class of carbohydrates, especially glucose, to produce ethanol. The other enological parameters showed no significant results when the wines were compared ($P > 0.05$).

The alcoholic content of SW (13.6% v/v) and BW (15.7% v/v) reached appropriate values for young wines; however, BW reached a mean value that was higher than the maximum limit for alcoholic content recommended by Brazilian legislation (8.6 to 14.0% v/v) [25]. This result is considered a positive outcome due to the possible use of this alternative yeast to avoid the chaptalization process. Both SW and BW wines were classified as dry wines, since they presented reducing sugars content below 4 $gL^{-1}$, and they were considered safe for drinking, since the volatile acidity for both SW and BW wines resulted below 1.2 $gL^{-1}$ in acetic acid equivalents, according to Brazilian legislation [25].

The wine produced from *Brettanomyces* could be considered a full-bodied wine, since its total dry extract resulted above 30 $gL^{-1}$ ($30.5 \pm 2.28$ $gL^{-1}$). This result mentioned above is probably explained by the higher extraction of compounds existent in the skins and seeds, which were responsible for the enhancement of BW dry extract. The improvement of the wine body is crucial for its quality, since the body is responsible for a pleasant mouth-feel that is very appreciated by consumers and experts in this field [26].

The total phenolic content presented values ranging from 668 to 694 $mgL^{-1}$, corroborating the findings from De Castilhos et al. [20], who reported the total phenolic content of Isabel red wines, another *Vitis labrusca* grape, ranging from 666.7 to 675.7 $mgL^{-1}$. The obtained result also contradicted the findings from Soares et al. (2008) [27], who reported approximately 363.2 $mgL^{-1}$ of the total phenolic content from Niágara Rosada extracts. This relevant result for total phenolic content for Niágara Rosada wines is probably explained by the maceration, a step that co-occurs with the alcoholic fermentation and consists of providing the contact between the pomace and the must during 5–8 days. The phenolic exchange can explain the variations in wine total phenolic content during the maceration, since it is not a simple partition phenomenon, i.e., it is crucial considering the initial concentration of the phenolic compounds in the grape berry and other phenomena that possibly occur in winemaking such as copigmentation [28].

The CIELab results showed that luminosity (L*), chroma (C*), yellowness (b*), color intensity (INT), and tonality (TON) presented significant differences ($P < 0.05$) between the wines produced from *Saccharomyces* and *Brettanomyces*. BW presented high values for luminosity, and the other color parameters presented higher values for SW. *Brettanomyces* provided a light-colored wine, i.e., the luminosity from BW (average 82.9 on a scale of 0–100; 0-black; 100-white) was significantly higher when compared to *Saccharomyces* wine (average 79.8 on a scale of 0–100; 0-black; 100-white). SW showed higher values for chroma (average 28.5 for SW and 17.1 for BW), yellowness (average 27.7 for SW, and 16.4 for BW), color intensity (average 1.11 for SW, and 0.81 for BW), and tonality (average 1.94 for SW and 1.70 for BW).

Anthocyanins and vinyl phenolic adducts are responsible for providing the wine color. *Brettanomyces* metabolism can modify the wine color, since it can convert these vinyl phenols to ethyl derivatives, losing its attractive wine color significantly, decreasing the color intensity and tonality parameters, showing a possible explanation for the lower color parameters for BW [29].

## Sensory acceptance

Ninety-one untrained consumers (47 women, 51.6%, and 44 men, 48.4%) assessed the wine acceptance. The average age of the panelists was 22.2 years, with a standard deviation of 5.98 (minimum of 18.0 and maximum of 61.0 years). Of the total number of consumers who evaluated the wine samples, 27 (29.7%) reported that they enjoy wine, and 56 (61.5%) reported that they immensely enjoy wine, resulting in more than 90% of the total number of consumers. Besides, of the total number of panelists, 24 (26.4%) reported very low consumption of wine, 19 (20.9%) reported a meager consumption, 19 (20.9%) reported a moderate consumption, 21 (23.1%) reported a high consumption, and 8 (8.7%) reported very high consumption of wine.

The consumers' frequency of wine consumption was transformed into scores as follows: (1) meager consumption, (2) low consumption, (3) moderate consumption, (4) high consumption, and (5) very high consumption. In most cases, there were no significant correlations between the wine frequency consumption and the sensory acceptance attributes, except for flavor in both SW ($r = 0.209$; $P = 0.046$) and BW ($r = 0.214$; $P = 0.041$) samples. These results showed a weak and positive correlation, which indicates that the higher the wine frequency consumption of the consumers, the higher the flavor acceptance score.

The results showed that the wines produced from *Brettanomyces* yeast did not significantly differ from the wines

produced from *Saccharomyces* (Table 2, P > 0.05 for all sensory attributes). There has been a controversy about the real sensory effect of *Brettanomyces* in wines. Several studies reported undesirable effects that *Brettanomyces* provides in wines, causing negative aroma and flavor effects [30]. On the other way, some studies accounted for its beneficial effects causing pleasant floral and fruity flavors for beers and wines [5, 31].

The results of the sensory acceptance showed a discrete and non-significant preference for the *Brettanomyces* wine (Table 2), presenting slighter higher means when compared with the *Saccharomyces* wine. In the comment session available in the assessment questionnaire, the panelists showed a slight preference for the color of *Brettanomyces* wine, reporting that BW seemed wine-alike than the SW sample.

We applied a sensometric approach to enrich the results obtained and reinforce the data discussion, aiming at observing correlations between the conventional enological parameters and the sensory attributes that were not observed in the univariate statistical approach. Considering that wine chemistry depends on the several reactions that can occur during all the steps applied to transform grapes in wine [26] and its direct correlation with sensory wine acceptance; the relationship between wine chemistry and sensory wine acceptance is very feasible to understand the chemical phenomena that are responsible for sensory wine acceptance [20].

## Volatile profile for the *Brettanomyces* wines

A total of 51 volatile compounds from eight different chemical groups—acetates, esters, carbonilic compounds, acids, alcohols, terpenes, benzene compounds, volatile phenols, and amides—were identified for *Brettanomyces* wines (Table 3). Four volatile compounds, responsible for the Brett character, known as 4-vinyl phenol (4-VP), 4-ethylphenol (4-EP), 4-vinyl guaiacol (4-VG), and 4-ethyl guaiacol (4-EG), were not identified in Niágara Rosada wines produced from *Brettanomyces anomalus* alternative yeast as observed in the chromatograph generated from the GC–MS equipment (Supplementary material). Both the 4-EP and 4-EG can be considered either positive or negative depending on their concentration in wines. At low concentrations (generally below 620 $\mu g L^{-1}$) [32], these volatile compounds contribute to wine aroma complexity; however, at high concentrations, they can create an unpleasant experience for the consumer [33].

These volatile compounds provide plastic, burnt plastic, phenolic/medicinal, cow manure, barnyard, and horse sweat aroma/flavor, and the synthesis of these off-flavors is related to punctual precursors. These aromas result primarily from the enzymatic action converting ferulic and *p*-coumaric acids into their products, 4-VG, and 4-VP, respectively, by the hydroxycinnamate decarboxylase. Then, these compounds are reduced into 4-EG and 4-EP by the vinyl phenol reductase [34]. It seems that only the *Brettanomyces* yeast can reduce the vinyl phenols to their respective sensory detectable compounds: 4-EP, presenting a medicinal/phenolic aroma, and 4-EG, with a clove spice aroma [33]. In this context, we can assume that the wines produced from the *Brettanomyces anomalus* yeast presented no *Brett* character, which is a positive outcome for this study.

This result mentioned above was astounding and unexpected. Possible explanations were considered as follows:

1. As mentioned above, 4-VG and 4-VP are produced from their respective precursors, ferulic and *p*-coumaric acids, and the absence of these volatile phenols can be explained by the minimal concentration of these acids in the Niágara Rosada grape. Burin et al. [35] studied the bioactive compounds from different grapes from both the *Vitis vinifera* and *Vitis labrusca* grape cultivars. The authors reported that the Niágara Rosada grape presented no concentration of ferulic acid, and a low concentration of *p*-coumaric acid, around 269.5 $\mu g. L^{-1}$, showing significant differences when compared to the concentrations of the acids regarding the other *Vitis vinifera* grapes, which presented higher *p*-coumaric acid concentrations. This fact can explain the absence of these volatile phenols in the Niágara Rosada wines.
2. Two analytical methods were performed to guarantee the veracity of the result regarding the volatile phenols. The ionization patterns of the major ions of these compounds were analyzed, and no detection regarding the volatile phenols was found. In this context, this result can also be possibly explained by the conditions used in the GC method. The GC–FID and GC–MS conditions tried to reproduce the conditions of the consumers' sensory assessment, and the method used a temperature that achieved a proximal temperature of the wine in the consumers' mouth (35 ℃). The possibility to perform methods that are more specific for the identification of

**Table 2** Mean ± standard deviation of the sensory attributes for SW and BW red wines

| Sensory attributes | Wines[1] | | *P* value[2] |
|---|---|---|---|
| | SW | BW | |
| Appearance | 5.50 ± 2.04 | 5.67 ± 2.05 | 0.562 |
| Aroma | 6.80 ± 1.88 | 6.96 ± 1.50 | 0.543 |
| Body | 6.05 ± 1.85 | 6.08 ± 1.74 | 0.934 |
| Flavor | 5.53 ± 2.12 | 5.42 ± 2.17 | 0.730 |
| Overall acceptance | 5.90 ± 1.80 | 5.93 ± 1.73 | 0.900 |
| Purchase intention | 3.01 ± 0.99 | 3.02 ± 1.10 | 0.944 |

[1]SW: *Saccharomyces* wine; BW: *Brettanomyces* wine

[2]*P* values for *t*-Student test with $P < 0.05$

**Table 3** Volatile compounds of Niágara Rosada wine produced from *Brettanomyces anomalus* yeast using GC–FID and GC–MS

| Volatile compounds by chemical groups ($\mu gL^{-1}$) | *Brettanomyces* wine | Odor thresholds ($\mu gL^{-1}$) | References |
|---|---|---|---|
| Acetates | 2.840 ± 80 | | |
| Methyl acetate | 10.1 ± 1.7 | 470,000 | [59] |
| Ethyl acetate | 2.540 ± 90 | 100,000 | [40] |
| Isobutyl acetate | 7.16 ± 0.34 | 1600 | [43] |
| Isoamyl acetate | 159 ± 18 | 30 | [36] |
| Linalyl acetate | 20.6 ± 0.8 | 15* | [44] |
| 2-Phenylethyl acetate | 67.7 ± 9.4 | 250 | [54] |
| Benzyl acetate | 32.9 ± 3.9 | 40* | [44] |
| Esters | 360 ± 5 | | |
| Ethyl butanoate | 176 ± 11 | 20 | [36] |
| Isobutyl cyclohexyl-oxalate | 15.3 ± 1.1 | NF | – |
| Ethyl 2-butenoate | 4.46 ± 0.26 | 4000* | [44] |
| Ethyl hexanoate | 29.2 ± 0.1 | 5 | [36] |
| Ethyl lactate | 54.5 ± 1.7 | 154,600 | [56] |
| Ethyl octanoate | 6.30 ± 0.64 | 2 | [36] |
| Ethyl 3-hydroxy butanoate | 18.1 ± 1.4 | 20,000 | [56] |
| Ethyl decanoate | 37.1 ± 4.5 | 200 | [43] |
| Ethyl benzoate | 16.1 ± 1.4 | 570 | [55] |
| Ethyl benzene propanoate | 3.50 ± 0.74 | 40* | [44] |
| Carbonilic compounds | 180 ± 14 | | |
| Ethoxy ethene | 2.50 ± 0.29 | NF | – |
| Acetaldehyde | 23.8 ± 4.6 | 500 | [45] |
| Acetone | 5.42 ± 0.50 | 476* | [44] |
| 1-(1-Ethoxyethoxy)-propane | 62.3 ± 1.9 | NF | – |
| 2-Pentanone | 34.7 ± 14.7 | 70,000* | [50] |
| 1-(1-Ethoxyethoxy)-butane | 30.8 ± 2.7 | NF | – |
| Acetoin | 4.64 ± 0.29 | 800 | [58] |
| Nonanal | 5.74 ± 0.18 | 1 | [36] |
| Acetophenone | 4.00 ± 0.96 | 10,000* | [44] |
| 1,8-Dimethyl-4-(1-methylethyl)-decanone | 6.75 ± 1.57 | NF | – |
| Acids | 347 ± 19 | | |
| Acetic acid | 295 ± 12 | 1000* | [50] |
| Hexanoic acid | 5.22 ± 0.63 | 420 | [54] |
| Octanoic acid | 36.7 ± 5.3 | 500 | [54] |
| Nonanoic acid | 3.53 ± 0.41 | 10,000* | [44] |
| Alcohols | 8.290 ± 540 | | |
| Isobutyl alcohol | 612 ± 0 | 40,000 | [36] |
| Isoamyl alcohol | 4.450 ± 160 | 30,000 | [36] |
| 1-Hexanol | 237 ± 8 | 2500 | [57] |
| 1-Nonanol | 3.18 ± 0.40 | 310 | [59] |
| 1,2,3-Butanetriol | 8.75 ± 2.14 | NF | – |
| Phenylethyl ethanol | 2,970 ± 380 | 14,000 | [58] |
| Benzene compounds | 167 ± 7 | | |
| Styrene | 34.3 ± 1.2 | 730* | [50] |
| 1-Methyl-4-(1-methylethenyl)-benzene | 112 ± 9 | NF | – |
| Benzaldehyde | 20.2 ± 2.7 | 350 | [45] |
| Volatile compounds by chemical groups ($\mu gL^{-1}$) | *Brettanomyces* wine | Odor thresholds ($\mu gL^{-1}$) | References |
| Terpenes | 125 ± 5.8 | | |
| α-Terpinolene | 5.48 ± 0.65 | 20* | [44] |

**Table 3** (continued)

| Volatile compounds by chemical groups ($\mu gL^{-1}$) | *Brettanomyces* wine | Odor thresholds ($\mu gL^{-1}$) | References |
|---|---|---|---|
| α-Terpinyl acetate | 22.7 ± 1.11 | 2500* | [44] |
| Linalool | 7.94 ± 1.80 | 20 | [60] |
| Hydroxyneomenthol | 15.1 ± 0.8 | 15,000* | [44] |
| 4-terpinenol | 2.64 ± 0.48 | 110 | [58] |
| 4-terpineol | 14.1 ± 1.2 | 25* | [44] |
| Citronellol | 6.97 ± 1.03 | 100 | [36] |
| Cis-geraniol | 20.2 ± 1.1 | 30 | [45] |
| β-myrcene | 20.7 ± 0.8 | 15* | [50] |
| Levomenol | 10.3 ± 1.8 | 0.08* | [53] |
| Amides | 8.47 ± 0.87 | | |
| L-Alanine ethylamide | 8.47 ± 0.87 | NF | – |

*NF* not found

*Threshold determined in water solution

these compounds is real; however, the use of higher temperatures generally used in these methods would not correspond to the ideal situation of the sensory evaluation by the consumer as mentioned above, and this would not be feasible for the study. Also, this condition could result in the formation of a higher concentration of these compounds, since they could increase their synthesis in high temperatures, overestimating their concentration.

The main volatile compounds identified and quantitated in the Niágara Rosada wines produced from *Brettanomyces anomalus* yeast were alcohols (total of 8290 $\mu gL^{-1}$), acetates (total of 2840 $\mu gL^{-1}$), and esters (total of 360 $\mu gL^{-1}$). Two alcohols, isoamyl alcohol (threshold 30,000 $\mu gL^{-1}$) and phenyl ethyl ethanol (threshold 10,000 $\mu gL^{-1}$) [36], presented higher concentrations, 4450 $\mu gL^{-1}$ and 2970 $\mu gL^{-1}$, respectively, when compared to the other alcohols. Both the alcohols presented concentrations in Niágara Rosada wines below their respective thresholds and did not provide a great contribution to wine aroma.

It was possible to observe a discrete concentration of isoamyl acetate (159 $\mu gL^{-1}$), one of the essential esters for fruity wines, in comparison to its precursor isoamyl alcohol (4450 $\mu gL^{-1}$). The reaction between the isoamyl alcohol and acetyl CoA in a reaction catalyzed by acetyltransferase synthesized the isoamyl acetate [37]. A possible explanation for the relevant concentration of isoamyl alcohol relies on the fact that acetyltransferase can act in different situations. Lilly et al. [38] studied the effect of yeast alcohol acetyltransferase on the flavor profile of wines, and they reported that the overexpression of different encoded genes from acetyltransferase can improve or not the synthesis of the esters. Besides, Kong et al. [39] reported that isoamyl alcohol was the most abundant alcohol in the Xiang Pearl wine samples, providing whiskey and nail polish aroma, corroborating the results obtained in this present study for Niágara Rosada wines.

Ethyl acetate (threshold 100,000 $\mu gL^{-1}$) [40] presented a higher concentration (2540 $\mu gL^{-1}$) among the acetates, and it provides a fruity aroma (pleasant) or a 'nail polish remover' aroma (unpleasant) for the wines [41] depending on the concentration. According to Ribéreau-Gayon et al. [24] and Canonico et al. [42], low concentrations of ethyl acetate in wines (concentrations up to 150–160 $mgL^{-1}$) provide a pleasant and soft fruity aroma; however, concentrations above these values were associated with 'nail polish remover' and 'solvent' sensory descriptors.

This volatile compound presented a concentration below its threshold and did not contribute to the wine aroma. Despite the ethyl acetate concentration, isoamyl acetate (threshold 30 $\mu gL^{-1}$) [43] presented a concentration above its threshold (159 $\mu gL^{-1}$), showing its active contribution to the fruity and sweet aroma, providing a banana note for the assessed wines samples [39], which is a feature very appreciated by the Brazilian consumers. Linalyl acetate (threshold 15 $\mu gL^{-1}$) [44] also presented a concentration above its respective threshold (20.6 $\mu gL^{-1}$), providing a fruity (bergamot) and flowery (lavender) aroma for Niágara Rosada wine, with sweet and acrid taste [44].

Ethyl butanoate (threshold 20 $\mu gL^{-1}$) [45] also presented a relevant concentration for the *Brettanomyces* wines (176 $\mu gL^{-1}$), providing a strawberry/apple sweet aroma, actively contributing to the wines aroma profile, since its concentration resulted above its threshold. Also, ethyl hexanoate (29.2 $\mu gL^{-1}$, threshold 5 $\mu gL^{-1}$) and ethyl octanoate (6.30 $\mu gL^{-1}$, threshold 2 $\mu gL^{-1}$) presented concentrations above their respective thresholds and contributed significantly to the sweet, fruity, and pleasant aroma of Niágara Rosada wine. Ethyl hexanoate provided a fruity, green apple, banana, and wine-like aroma, and ethyl octanoate provided

a sweet, caramel, fruity, banana, pear aroma for wines [46, 47]. The other volatile compounds were related to the sensory acceptance attributes to verify possible sensory acceptance drivers for *Brettanomyces* wines.

## Sensometric approach

According to the Factor Analysis results (Fig. 1), factor 1 explained 70.18%, and factor 2 explained 29.82% of the total variance. Factor 1 differentiates three sensory acceptance drivers as follows: appearance, flavor, and overall acceptance, with five different groups of volatile compounds as follows: acetates, esters, carbonilic compounds, alcohols, and benzene compounds. Two groups of variables are explained in Component 1, the first grouped acetates, esters, and alcohols with flavor acceptance, and the second grouped carbonilic compounds, benzene compounds with appearance acceptance, and overall acceptance.

According to the sensometric results, the volatile compounds identified and quantitated in Niágara Rosada wines fermented with *Brettanomyces anomalus* that were strongly correlated with flavor acceptance were acetates: ethyl acetate (2540 $\mu gL^{-1}$, threshold 100,000 $\mu gL^{-1}$), isoamyl acetate (159 $\mu gL^{-1}$, threshold 30 $\mu gL^{-1}$), and linalyl acetate (20.6 $\mu gL^{-1}$, threshold 15 $\mu gL^{-1}$), and esters: ethyl butanoate (176 $\mu gL^{-1}$, threshold 20 $\mu gL^{-1}$), ethyl hexanoate (29.2 $\mu gL^{-1}$, threshold 5 $\mu gL^{-1}$), and ethyl octanoate (6.30 $\mu gL^{-1}$, threshold 2 $\mu gL^{-1}$). Among the alcohols, it was possible to highlight the isoamyl alcohol (4450 $\mu gL^{-1}$, threshold 30,000 $\mu gL^{-1}$) and phenyl ethyl ethanol (2970 $\mu gL^{-1}$, threshold 10,000 $\mu gL^{-1}$) [39, 46].

Of these volatile compounds, isoamyl acetate, linalyl acetate, ethyl butanoate, ethyl hexanoate, and ethyl octanoate can be considered flavor acceptance drivers, since they presented concentrations above their respective thresholds. The alcohols presented concentrations below their respective thresholds and, according to the sensometric approach, they cannot be considered flavor acceptance drivers. However, the alcohols possibly influenced the flavor acceptance, despite the resulting concentration below their threshold, in a synergistic way, i.e., the presence of one compound probably enhanced the perception of another [48].

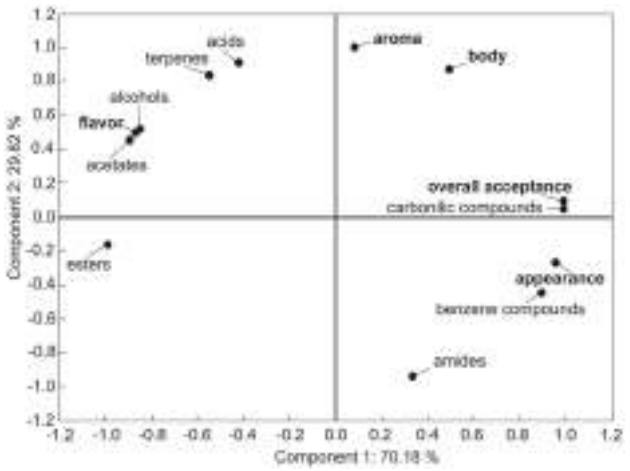



**Fig. 1** Projection of volatile compounds (grouped in their chemical classes) and sensory attributes using Factor Analysis. Sensory acceptance attributes are in bold

These volatile compounds mentioned above are responsible for providing a fruity and sweet flavor for wines [46, 47], which is a feature commonly found in *Vitis labrusca* wines, and Brazilian consumers appreciate it. Steensels et al. [5] reported that *Brettanomyces* could enhance the concentration of volatile compounds that can be appreciated for specific wine tasters, since it might add positive effects enhancing sensory wine complexity, especially in young red wines, corroborating the obtained results. Also, according to the Principal Component Analysis, carbonilic compounds and benzene compounds drove the wine's appearance and overall acceptance. The overall acceptance can influence appearance acceptance, since previous works presented a significant statistical correlation between these two sensory acceptance attributes [20, 49]. Among the carbonilic compounds, 1-(1-ethoxy ethoxy)-propane (no threshold found) showed a higher concentration (62.3 $\mu gL^{-1}$), and it presents an aroma similar to a nut, such as the first acetaldehyde derivatives [44]. 2-Pentanone (threshold 70,000 $\mu gL^{-1}$) [50] was the other carbonilic compound that presented a high concentration (34.7 $\mu gL^{-1}$), and it presents a fruity and ether aroma [51]; however, presented no relevant contribution for wine, since it presented concentration below its threshold.

A carbonilic compound that presented a low concentration (5.74 $\mu gL^{-1}$) but deserved attention was nonanal (threshold 1 $\mu gL^{-1}$) [44], presenting a citrus and green scent note [52]. This compound presented a concentration above its threshold and can be considered a carbonilic compound that has driven the overall acceptance of Niágara Rosada wines.

Among the benzene compounds, styrene (threshold 730 $\mu gL^{-1}$, 34.3 $\mu gL^{-1}$ concentration) [50] and benzaldehyde (threshold 350 $\mu gL^{-1}$, 20.2 $\mu gL^{-1}$ concentration) [45] were the most relevant compounds. According to the obtained results, all these compounds presented no relevance for overall wine acceptance, since all their concentrations were below their respective thresholds. As mentioned above, despite the relevance of the nonanal as a unique overall acceptance driver, the other carbonilic and benzene compounds also contributed, indirectly, to the Niágara Rosada wine's overall acceptance, probably enhancing the sensory features of the other wine volatile compounds synergistically.

Component 2 differentiates two sensory acceptance drivers as follows: aroma and body acceptance, with three different groups of volatile compounds as follows: acids, terpenes, and amides. Two groups of variables explained the PC2, the first grouped acids, and terpenes with aroma and body acceptance; the second was composed only of amides with no sensory acceptance driver.

Among the terpenes found in Niágara Rosada wines, α-terpinyl acetate (22.7 $\mu gL^{-1}$), cis-geraniol (20.2 $\mu gL^{-1}$), and β-myrcene (20.7 $\mu gL^{-1}$) presented the higher concentrations. The α-terpinyl acetate presents herbal, bergamot, lavender, lime, and citrus scent (threshold 2,500 $\mu gL^{-1}$) [44]; cis-geraniol (threshold 30 $\mu gL^{-1}$) [45] presents floral scent, and β-myrcene presents balsamic, and spice scent (threshold 15 $\mu gL^{-1}$) [50]. β-myrcene presented concentration above its threshold, considered one of the aroma acceptance drivers for Niágara Rosada wines. β-Myrcene provides a citrus fruity and slightly minty aroma nuance when the concentration surpasses its threshold [44].

Another important terpene presented a relevant result: levomenol (10.3 $\mu gL^{-}1$; threshold 0.08 $\mu gL^{-1}$), also known as α-bisabolol. It showed a concentration above its threshold, and it provided a light and sweet floral aroma for wine, contributing as a flavor acceptance driver for Niágara Rosada wines produced from *Brettanomyces anomalus* alternative yeast [54].

According to Jackson [49] and De Castilhos et al. [20], acids can strongly influence the wine body's acceptance. Acetic acid (295 $\mu gL^{-1}$, threshold 1000 $\mu gL^{-1}$) [50] and octanoic acid (36.7 $\mu gL^{-1}$, threshold 500 $\mu gL^{-1}$) [54] were the acids that presented the higher concentration; however, these volatile compounds did not contribute for wine body or aroma, since their concentrations resulted below their thresholds. The other acids quantitated also presented concentrations below their respective thresholds, presenting no substantial contribution to wine aroma and body acceptance. Despite this fact, the acids, to a lesser extent, may have contributed along with terpenes to drive the aroma and body acceptance synergistically [48].

## Conclusions

The univariate analysis showed that the use of different yeasts in alcoholic fermentation to produce non-*Vitis vinifera* wines influenced some enological parameters. Due to the lack of significant differences between the wine samples in the univariate sensory acceptance test, a sensometric approach was successfully performed and demonstrated the higher potential of *Brettanomyces* as an alternative yeast for non-*Vitis vinifera* alcoholic fermentation aiming at producing fresh and fruity aroma and flavor for these wines. The *Brettanomyces* wines presented the higher acceptance scores for all assessed sensory attributes, and punctual volatile compounds influenced them. Esters (ethyl butanoate), alcohols (phenylethyl ethanol), and terpenes (levomenol) drove flavor acceptance due to their contribution to the fruity, sweet, and floral aroma. Terpenes (β-myrcene and linalool) drove the aroma and body acceptance. Acids also presented a connection with aroma and body acceptance; however, they presented a weak contribution. Carbonilic compounds (nonanal), which provided a citrus/green scent, and benzene compounds, which presented a weak contribution, drove overall acceptance. This study allowed evaluation of the use of alternative yeasts in the winemaking process, which could guide the wine quality for better acceptance by consumers with unique desires.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00217-023-04342-6.

**Data availability** The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

## Declarations

**Conflict of interest** The authors declare that they have no conflict of interest.

**Compliance with ethics requirements** The Ethics in Research Committee of the Institute of Biosciences, Humanities, and Exact Sciences, São Paulo State University approved the Ethical Issues regarding sensory acceptance (process n. 2.447.651).

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# Tributyrin in the Diet of Lambs and Its Impacts on Energy Metabolism and Oxidative Status in the Liver and Intestine, as Well as the Fatty Acid Profile in Meat

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## Abstract

This study aimed to determine whether adding tributyrin to the diet of lambs impacts health, energy metabolism, ruminal environment, and meat quality. Twelve lambs were used; the control group received a basal diet, while the tributyrin group received a diet with the additive mixed with concentrate (2 g/day/animal). The blood count was used for Leukocyte and lymphocyte counts, which were significantly higher in tributyrin-fed animals than in controls. The activity of the enzymes adenylate kinase and pyruvate kinase was higher in the liver and intestine of the tributyrin group than the controls; cytosolic creatine kinase activity was significantly higher in the intestine of lambs fed tributyrin. Glutathione S-transferase activity in the liver was significantly higher in animals in the tributyrin group. Superoxide dismutase activity was significantly higher in the intestine, with a lower protein carbonyl concentration in the tributyrin group. Bacterial activity through ruminal fluid collection was significantly lower when tributyrin was consumed, unlike the protozoan count, which was significantly higher in animals in the tributyrin group than in the controls. Tributyrin intake caused lower levels of short-chain fatty acids without changing the proportion of volatile fatty acids. The water retention capacity measured using an external compression method was significantly higher in the meat of the tributyrin group. The treatment affected some fatty acids in

the meat, these acids were separated by chromatography where a lower amount of saturated fatty acids and a higher amount of monounsaturated fatty acids in the group that consumed tributyrin. These findings suggest that tributyrin in lamb diet alters blood and rumen environment biomarkers and improves the fatty acid profile of the meat.

## Keywords

Additive, Animal Production, Tributyrin, Fatty Acids, Animal Health

## 1. Introduction

In its pure form, butyrate has an unpleasant odor, with volatile and unstable behavior in the environment [1] to avoid these problems, butyric acid molecules are esterified to mono-, di-, and tributyrates. Compared to butyric acid, tributyrin (TB) is more stable and has less odor, thus allowing a slower release to reach the lower gut and eliminating the possibility of animal consumption rejection. TB encompasses a complex interplay between diet, gut microbiota, energy metabolism, and immunity [2]-[4]. In recent years, it has attracted much attention and is widely used as a food additive in diets to improve the health and performance of farm animals, as it can be metabolized into three free butyric acid molecules in the gastrointestinal tract. In adult sheep, the addition of TB improved the ruminal environment and metabolites, thus resulting in greater activity of fibrolytic bacteria and utilization of dietary nutrients [5] Butyrate recruits innate immunity molecules [6] [7]. In pre-weaned dairy calves, added to milk replacer promoted intestinal development by increasing the intestinal barrier and attenuating the inflammatory response [8] [9].

In practice, TB is used in the production of swine [10], poultry [9] [11], and aquatic organisms [12]-[14] as it is a potent growth promoter with effective antibiotic functions. However, the effects of TB on sheep diets are relatively unknown. In recently weaned lambs, a basal diet with additives of mono-, di-, and tri-glycerols allowed researchers to attribute a positive role to TB in the degradation of ruminal fiber, which combined with other esters of glycerol, increased average daily weight gain, even without an effect on the rumen microbiome. On the other hand [15], evaluated various doses of protected or unprotected TB in the diet of sheep and did not observe a contribution to growth, final weight, average daily gain (ADG), gain:feed ratio, hot carcass yield, or carcass yield. We hypothesize that TB will alter the flora and profile of AVGs in the rumen environment and reach the intestine, where enterocytes will use it, favoring intestinal and animal health. Therefore, this study aimed to determine whether adding TB to the diet of lambs impacts animal health, energy metabolism, rumen environment, and meat quality.

## 2. Materials and Methods

### 2.1. Tributyrin

The TB additive used in this study was a commercial product based on butyric acid glyceride molecules (Villonat®, Feedis, Salto, São Paulo, Brazil).

### 2.2. Animals, Data, and Sample Collection

The experiment was conducted on an Experimental Farm of the Center for Higher Education of the West of the State University of Santa Catarina, in Guatambu (Southern Brazil). FECEO-UDESC at latitude 27˚8'5" South, longitude 52˚47'15" W between March and June 2023.

Twelve castrated male Lacaune lambs (2.5 ± 0.5 months old and 21 ± 2 kg) were housed in 3-$m^2$ wooden pens with individual drinking and feeders for 79 days. The distribution of the stalls in the respective treatments in the installation was sequential to avoid environmental interference. All animals were treated preventively with Ripercol anthelmintic based on 5% levamisole hydrochloride (Ripercol®).

The basal diet was formulated with nutritional requirements for growing lambs, according to NRC 2007. Feed was provided twice daily (08:30 am and 3:30 pm) in a 75:25 concentrate:roughage ratio. The amount of feed provided to the animals was adjusted whenever the animals were weighed, with the total mixed ration (TMR) corresponding to 4% of live weight (**Table 1**).

From the record of the offer and refusal of daily feed, dry matter intake (DMI), feed conversion (weight animal weight in several studies: gain/DMI), and feed efficiency per pen were calculated; for these zootechnical variables, the pen was used as an experimental unit. The additive was adjusted accordingly, ensuring that the animals always ingested 2 g/day/animal defined by the source of tributyrin or butyric acid and the average First, the concentrate was supplied after consumption, and the bulky portion (corn silage) was supplied to guarantee maximum additive consumption.

To evaluate performance, in addition to weighing, weight gain and average daily weight gain were calculated five times on a digital scale (days 1, 15, 35, 45, and 70). The leftovers were weighed before the day's first batch was delivered. Based on this data, feed conversion, and feed efficiency were calculated.

### 2.3. Hematology and Serum Biochemistry

Blood was collected on days 1, 15, 35, 45, and 70 days of the experimental period by puncture of the jugular vein of fasting animals using two types of vacuolated tube: without anticoagulant and with EDTA as anticoagulant; samples were transported refrigerated for serum biochemistry and complete blood counts, respectively.

Blood collected without anticoagulant was centrifuged at 7,000 rpm for 10 min to separate the serum and stored at –20˚C until biochemical assays were performed. Serum activity of aspartate aminotransferase (AST), gamma-glutamyl transferase

**Table 1.** Ingredients and chemical composition of the experimental diet concentrate.

| **Ingredients** (g/kg) | **Composition** | | |
|---|---|---|---|
| Corn meal | 506.00 | | |
| Soybean meal | 246.00 | | |
| Wheat bran | 180.00 | | |
| Mineral core[1] | 47.00 | | |
| Calcitic limestone | 20.00 | | |
| **Chemical composition**[2] | | **Control** | **Tributyrin** |
| **Items** | **Corn silage** | **Concentrate** | **Concentrate** |
| **DM** | 34.00 | 89.64 | 89.87 |
| **MM** | 4.60 | 12.01 | 12.37 |
| **CP** | 8.78 | 22.00 | 22.88 |
| **NDF** | 25.60 | 14.77 | 14.01 |
| **ADF** | 13.70 | 6.66 | 6.22 |
| **EE** | 2.10 | 3.26 | 3.24 |

[1]Nucleus composition: Calcium (min./max.) 175/245 g; Phosphorus (min.) 40 g; Sulfur (min.) 25 g; Magnesium (min.) 12 g; Sodium (max) 100 g. Mineral Matter (max) 980 g; Cobalt (min.) 40 mg; Iron The animals were allocated to two experimental groups: 8 received concentrated feed containing 2 g/day/animal of the TB additive, while another 8 received concentrated feed without the additive. The dose of TB added to the concentrate during its production was based on the concentrate/day consumed. Corn silage and concentrate samples were collected to determine the chemical composition of dry matter (DM), crude protein (CP), ether extract (EE), and ash according to AOAC (2000) (Table 1). (min.) 1,800 mg; Iodine (min.) 80 mg; Manganese (min.) 1,400 mg; Selenium (min.) 12 mg; Zinc (min.) 4,000 mg; Vit. A (min.) 300,000 IU; Vit. D3 (min.) 60,000 IU; Vit. E (min.) 600 IU; Fluoride (max) 600 mg; Monensin sodium (600 mg); [2]DM (dry matter), MM (mineral matter), CP (crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), EE (ether extract). Note: Basal concentrate, used in all groups. The chemical composition of the concentrate from both groups was the same; TB did not change the chemical composition of the concentrate consumed by the lambs.

(GGT), and levels of total protein, albumin, glucose, triglycerides, and cholesterol, calcium, phosphorus were measured on a Zybio EXC-200 model analyzer using commercial kits (Analisa®, Belo Horizonte, Brazil) according to the manufacturer's recommendations. Globulin levels were calculated as total protein – albumin.

Hematologic analyses were performed with whole blood stored in tubes containing EDTA within two hours of collection. An automatic hematologic analyzer measured the erythrocyte count, total leukocyte count, leukocyte differential, hematocrit percentage, and hemoglobin concentration (3-part EQUIP VET 3000®).

## 2.4. Rumen Parameters

Ruminal fluid (50 ml) was collected on days 1, 35, and 70 (two hours after feeding in the morning) through a silicone ororuminal cannula connected to a vacuum pump. Half of the fluid (25 ml) was filtered for pH assessments using a portable digital potentiometer (TESTO 205®) and measuring the functional activity of the

ruminal microbiota using the methylene blue reduction test—MBRT (0.05% solution), as described by [16]. The methylene blue reduction time was measured accordingly. From this filtered portion, 2 mL was used to determine short-chain fatty acids (SCFA), and the proportion of acetic, propionic, butyric, valeric, and isovaleric acids using a gas chromatograph with a flame ionization detector (GC-FID; Varian Star 3600, Palo Alto, USA) and automatic sampler (Varian 8200CX, Palo Alto, USA), according with adjusted detail in **Supplementary Material 1**. The results were expressed as $mmol \cdot L^{-1}$ of each SCFA in ruminal fluid.

An unfiltered aliquot was used in the protozoan counting process using a Sedgewick Rafte chamber, where in one drop of dilution ($10^{-1}$), protozoan microstructures are counted with a 40x objective according to literature [4].

## 2.5. Post-Mortem Analysis

### 2.5.1. Slaughter and Sample Collection

Once the confinement phase (79 days) was completed, the twelve lambs were fasted for 12 hours, and transported to the slaughterhouse following local regulations. After bleeding, skinning, and eviscerating the animals, the liver, jejunum, and longissimus thoracis (LT) muscle were collected for the analyses described below.

### 2.5.2. Energy Metabolism and Oxidative Status in the Liver and Intestine

The intestine was washed in SET buffer (0.32 M sucrose, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4) and homogenized in the same buffer (1:10 w/v) using a Potter tissue homogenizer. The homogenate was centrifuged at 800 × *g* for ten minutes at 4˚C. An aliquot of the supernatant was used to evaluate adenine kinase (AK) activity; the pellet was discarded, and the supernatant was centrifuged again at 10,000 × *g* for 15 min at 4˚C. The cytosol supernatant was collected to determine cytosolic pyruvate kinase (PK) and creatine kinase (CK) activity. The pellet containing mitochondria was washed twice with the same buffer and resuspended in 100 mM Trizma and 15 mM $MgSO_4$ buffer (pH 7.5) to measure mitochondrial CK activity.

CK intestinal activity was conducted based on Hughes's colorimetric method (1962), which estimates creatine levels at a wavelength of 540 nm, as reported in detail by researchers [17]. The results were expressed as nmol of creatine formed/min/mg of protein. Intestine and liver AK activity was measured with an enzymatic assay coupled with hexokinase (HK) and glucose 6-phosphate dehydrogenase [17] [18]. The activity was expressed as pmol of ATP formed/min/mg of protein. Gut and liver PK activity were published in detail by researchers [17]. Activity was expressed as μmol of pyruvate formed/min/mg of protein.

Detection of serum lipid peroxidation was performed indirectly and expressed as serum thiobarbituric acid reactive substance (TBARS), according to [18] Serum glutathione S-transferase (GST) activity was measured using the technique described [19]. Results were expressed as μmol CDNB/min/mg protein.

Superoxide dismutase (SOD) activity was determined according to the

principle of pyrogallol autooxidation, which inhibits the presence of SOD. The variation in optical density was determined kinetically for two minutes at 420 nm, in 10-s intervals, according to the methodology described by researchers [20]. SOD activity was expressed as U SOD/mg protein.

#### 2.5.3. Carcass Characteristics and Meat Quality

Measuring pH with a portable pH meter equipped with a penetrating electrode (Testo® model 205) in carcasses' loins (LT) immediately after evisceration, then individually weighing the carcass components on a digital scale. These were cooled at 4˚C for 24 h to obtain cold carcass weight (CCW); in the cold carcass, pH was also measured. Hot and cold carcass yields were calculated by expressing hot carcass weight (HCW) and CCW as a percentage of empty body weight (EBW), being (EBWZ = slaughter BW – [HCW OR CCW * 100]). Subsequently, the carcasses were ribbed in the twelfth and thirteenth to obtain the LT muscle, and stored at –18˚C until analysis of the chemical composition and fatty acid profile of the meat.

#### 2.5.4. Color and Water Retention Capacity

The LT muscle was used to evaluate meat quality characteristics immediately upon arrival in the laboratory. Color analysis was performed using a portable spectrophotometer model CM-25d (Konika Minolta®) placed on the surface of the muscle to record brightness (L*), redness (a*), and yellowness (b*). All meat quality measurements were made in triplicate for each lamb.

For water retention capacity (WRC), a circular piece of meat (0.05 kg) from LT muscle was weighed on a precision scale and subjected to a rapid absorptive method of external compression, where the meat was pressed between two paper filters and loaded with a 2-kg block for five minutes, as detailed by researchers [21]. The WRC value was expressed as a percentage: WRC (%) = (Final weight – initial weight) × 100.

#### 2.5.5. Chemical Composition and Fatty Acid Profile

The LT muscle samples were thawed to detail the chemical composition of the meat obtained using a near-infrared reflectance spectrometer, model Spectra Star 2600 XT series of Near-Infrared Analyzers (Unity Scientific®).

Fatty acid profiling, intramuscular fat extraction, methylation, and fatty acid profile analysis on LT samples were performed according to literature [22] as follows: at 15 mL polypropylene tube was added 1.5 g of ground muscle, 0.5 mL of distilled water, 5 mL of methanol, and 2.5 mL of chloroform to undergo mechanical agitation for 1 hour and 30 minutes, thus forming a homogeneous solution. Next, 2.5 mL of chloroform and 2.5 mL of 1.5% sodium sulfate ($Na_2SO_4$) solution were added to promote a biphasic solution and undergo mechanical agitation for 30 minutes, then centrifuged for 10 minutes at 2000 rpm. The lipids in the lower phase of the solution were subjected to fat quantification by gravimetric method and fatty acid analysis. For fat, 2.5 mL of the obtained solution was placed in a crucible and subjected to an oven for 45 minutes at 105˚C; the crucible was then

weighed to quantify the fat after evaporation of the chloroform.

The fatty acids (FA) methylation was from a previously obtained solution using quantitative method [21]. In a glass tube with a lid, 0.8 mL of solution in 1 mL of 0.4 M KOH in methanol was added, kept in a water bath at 100˚C for 10 minutes, and cooled to room temperature. Then, 3 mL of 1 M $H_2SO_4$ in methanol was added and kept in a water bath at 100˚C for ten minutes. After cooling, 2 mL of hexane was added, vortexed for two minutes, and centrifuged at 2000 rpm for 10 min. Finally, 1 μL of hexane with fatty acid methyl esters (FAME) was subjected to chromatographic analysis.

A gas chromatograph model TRACE 1310 was used to characterize FAME, equipped with a flame ionization detector (Thermo Scientific) with hydrogen used as a carrier gas at a constant flow of 1.5 mL/min. One microliter of sample was injected into a split-splitless injector, operated in split mode with a 1:10 ratio at 250˚C.

FAME separation used an RT 2560 chromatography column (100 m × 0.25 mm × 0.20 μm film thickness, Restek, USA). The initial oven temperature was programmed at 100˚C for 5 min and increased to 180˚C at an 8˚C/min rate. Then, it increases to 210˚C at a rate of 4˚C /min, and finally up to 240˚C, increasing by 20˚C/min and maintaining for 20 min at isothermal conditions. The detector temperature was kept constant at 250˚C.

Fatty acid values were expressed as a percentage (%) determined by comparing the total amount of FA to the authentic standard (MIX-37, Sigma Aldrich, St. Lois, MO). The percentages of saturated fatty acids (SFA), monounsaturated fatty acids (MIFA), and polyunsaturated fatty acids (PUFA) were calculated.

## 2.6. Statistical Analysis

The design was in a completely randomized model where each pen was considered an experimental unit. Data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene tests, respectively. The complete blood count results, quantification of protozoa in ruminal fluid, and the oxidative status of tissues required transformation to achieve normality and homogeneity and they were then retransformed to the original units for description. All data were analyzed using the SAS "MIXED procedure" (SAS Inst. Inc., Cary, NC, USA; version 9.4), with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed effects test. Growth performance data (exception for PC) were tested for fixed treatment effects using animal (treatment) as a random effect. Body weights, complete blood counts, and serum biochemical data were analyzed as repeated replicates. All data obtained on day 1 for each variable were included as covariates in each analysis. The first-order autoregressive covariance structure was selected according to Akaike's least information criterion. Means were separated using the PDIFF method, which selected a t-test, and all results were expressed as means with standard errors. Between treatments, differences were defined when $P \leq 0.05$ and trends when $P > 0.05$ and $\leq 0.10$.

## 3. Results

### 3.1. Performance

There was no effect of treatment and treatment x day interaction for body weight of lambs (Table 2). TB intake did not enhance weight gain, alter DMI, or improve feed conversion and efficiency (Table 2).

Table 2. Zootechnical performance of lambs fed with tributyrin.

| Items | Treatments | | SEM | *P*-value |
|---|---|---|---|---|
| | Control | Tributyrin | | |
| Weight gain (kg) | | | | |
| Adaptation period (10 days) | 20.2 | 19.8 | 0.36 | 0.86 |
| D1 | 23.9 | 23.8 | 0.33 | 0.94 |
| D15 | 28.6 | 27.7 | 0.35 | 0.92 |
| D35 | 33.9 | 33.2 | 0.35 | 0.95 |
| D53 | 37.4 | 37.9 | 0.46 | 0.97 |
| D70 | 44.1 | 44.2 | 0.41 | 0.97 |
| Days 1 to 70 (trial period) | 20.2 | 20.4 | 0.23 | 0.96 |
| Average daily gain (ADG), kg/day | 0.288 | 0.291 | 0.05 | 0.94 |
| Dry matter intake (DMI). kg/day | 1.37 | 1.38 | 0.11 | 0.96 |
| Feed conversion (DMI/ADG). kg/kg | 4.75 | 4.77 | 0.18 | 0.94 |
| Feed efficiency (ADG/DMI). kg/kg | 0.210 | 0.210 | 0.01 | 0.98 |

### 3.2. Hematology and Serum Biochemistry

Table 3 displays the results of hematologic and biochemical variables. Treatment had a significant effect on leukocyte count (P = 0.045). There was a significant effect of treatment (P = 0.022) and a tendency for treatment x day interaction (P = 0.080) for lymphocyte count, being significantly higher in TB animals compared to control. No significant effect of treatment and treatment x day interaction was observed for monocyte, granulocyte, erythrocyte, platelet counts, or hemoglobin concentration and hematocrit percentage.

There was a trend toward a treatment effect for serum glucose concentration, which was significantly higher in the blood of animals that consumed TB. No significant effect was observed for treatment and treatment x day interaction for AST, GGT, total protein, cholesterol, albumin, globulin, calcium, or phosphorus.

### 3.3. Ruminal Indicators

The results of biomarkers in the rumen environment are displayed in Table 4.

**Table 3.** Serum biochemistry and blood count of lambs fed tributyrin mixed with concentrate.

| Variable[1] | Treatments | | | | |
|---|---|---|---|---|---|
| | | | SEM | *P*-value | |
| **Biochemistry** | **Control** | **Tributyrin** | | **Treat.** | **Treat. × day** |
| AST (U/L) | 95.59 | 94.02 | 2.659 | 0.930 | 0.912 |
| GGT (U /L) | 64.97 | 65.13 | 2.347 | 0.898 | 0.941 |
| Total prot. (g/dL) | 6.48 | 6.55 | 0.088 | 0.923 | 0.881 |
| Cholesterol (mg/dL) | 79.24 | 78.72 | 1.505 | 0.906 | 0.784 |
| Glucose (mg/dL) | 93.97 | 99.4 | 1.065 | **0.091** | 0.141 |
| Albumin (g/dL) | 3.36 | 3.35 | 0.056 | 0.953 | 0.932 |
| Globulin (g/dL) | 3.11 | 3.20 | 0.104 | 0.947 | 0.956 |
| Calcium (mg/dL) | 11.31 | 11.42 | 0.228 | 0.970 | 0.970 |
| Phosphorus (mg/dL) | 10.60 | 10.58 | 0.303 | 0.962 | 0.947 |
| **Hemogram** (μ/L) | | | | | |
| Leukocytes | 7.470[b] | 10.33[a] | 0.951 | **0.045*** | 0.105 |
| Lymphocytes | 5.380[b] | 7.727[a] | 0.316 | **0.022*** | **0.080*** |
| Monocytes | 0.621 | 0.901 | 0.242 | 0.180 | 0.207 |
| Granulocytes | 1.469 | 1.706 | 0.155 | 0.274 | 0.398 |
| Erythrocytes | 10.10 | 10.42 | 0.166 | 0.965 | 0.981 |
| Hemoglobin (g/dL) | 12.44 | 12.61 | 0.240 | 0.950 | 0.963 |
| Hematocrit (%) | 27.83 | 27.54 | 0.240 | 0.951 | 0.968 |
| Platelets | 282.8 | 239.9 | 20.80 | 0.694 | 0.725 |

[1]AST (aspartate aminotransferase), GGT (gamma-glutamyl-transferase). *Within the lines, differ (P ≤ 0.05) or tend to differ (P ≤ 0.10).

**Table 4.** Ruminal parameters of lambs fed tributyrin mixed with concentrate.

| Variáveis[1] | Treatments | | | | |
|---|---|---|---|---|---|
| | **Control** | **Tributyrin** | **EPM** | *P*-value | |
| | | | | **Treatment** | **Treat × Day** |
| pH ruminal fluid | 6.243 | 6.403 | 0.03 | 0.920 | 0.950 |
| MBRT[1] | 92.05[b] | 118.5[a] | 6.21 | **0.031** | 0.128 |
| Protozoans (n°/L) | | | | 0.253 | **0.050*** |
| D1 | 131.7 | 133.5 | 9.66 | | |
| D35 | 100.2 | 136.4 | 9.57 | | |
| D70 | 110.7[b] | 131.4[a] | 4.26 | | |
| SCFA (mnol/L) | | | | **0.02*** | **0.01*** |
| D1 | 83.4 | 83.8 | 0.36 | | |
| D35 | 74.1[a] | 62.8[b] | 0.34 | | |
| D70 | 90.5[a] | 85.1[b] | 0.33 | | |

**Continued**

| | | | | | |
|---|---|---|---|---|---|
| Acetic acid (%) | 59.2 | 59.4 | 0.81 | 0.95 | 0.97 |
| Propionic acid (%) | 28.4 | 27.7 | 0.34 | 0.96 | 0.92 |
| Butyric acid (%) | 8.49 | 8.69 | 0.12 | 0.93 | 0.95 |
| Isovaleric acid (%) | 1.59 | 1.69 | 0.03 | 0.82 | 0.76 |
| Valeric acid (%) | 2.23 | 2.42 | 0.06 | 0.88 | 0.71 |
| Acetic/Propionic | 2.24 | 2.23 | 0.05 | 0.97 | 0.95 |

[1]MBRT (methylene blue reduction test). *Within the lines, differ (P ≤ 0.05) or tend to differ (P ≤ 0.10).

There was no significant effect of treatment for pH; however, there was a significant effect of treatment for MBRT, with a longer time for consumption of methylene blue by bacteria, indirectly indicating lower microbial activity in the rumen environment when lambs consumed TB but without treatment x day interaction for MBRT. There was a significant treatment x day interaction on day 70 (P = 0.50) for protozoa counts in rumen fluid, with the number being significantly higher in lambs that consumed TB.

There was a significant effect of treatment and treatment x day interaction for concentrations of short FA, being significantly lower in lambs that consumed TB on days 35 and 70 (Table 4). We did not find a significant effect of treatment on the proportion of volatile FA called acetic, propionic, butyric, valeric, or isovaleric acids (Table 4; P > 0.05).

### 3.4. Energetic Metabolism and Oxidative Status

Table 5 displays the activity of energy metabolism enzymes and oxidative status in the liver and intestine. Treatment significantly affected AK activity in the liver (P = 0.05) and intestine (P = 0.01) PK activity. AK and PK significantly reduced their activities in the livers and intestines of lambs that consumed TB compared to the control group. In the intestine, there was a significant effect of treatment on cytosolic CK activity (P = 0.02), significantly higher in the jejunum of lambs in the TB group; Mitochondrial CK activity tended to be lower in the jejunum of animals in the TB group (P = 0.08).

There was no effect on protein carbonyl in the liver; however, there was in the intestine. That is, the concentration of this biomarker of protein oxidation was lower in the jejunum of lambs that consumed TB (Table 5). Treatment affected GST activity in the liver (P = 0.01), with the highest activity in lambs in the TB group; however, there was no change in this enzyme in the jejunum of the animals. There was no effect of treatment on SOD activity in the liver; however, in the intestine/jejunum, the activity of this enzyme was higher (P = 0.01) in lambs that consumed TB than control animals. It had no treatment effect for TBARS levels in the liver or intestine.

**Table 5.** Energy metabolism and oxidative status of the liver and intestine of lambs fed with tributyrin mixed with concentrate.

| Variable[1] | Treatments | | | |
|---|---|---|---|---|
| | Control | Tributyrin | SEM | *P-value* |
| **Liver** | | | | |
| AK | 9.98[a] | 7.95[b] | 0.29 | 0.05* |
| PK | 0.56[a] | 0.47[b] | 0.01 | 0.01* |
| TBARS | 1.85 | 1.82 | 0.24 | 0.92 |
| Carbonyl protein | 3.68 | 3.47 | 0.63 | 0.73 |
| GST (U/L) | 152[b] | 274[a] | 5.89 | 0.01* |
| SOD (U/mg) | 12.8 | 14.6 | 0.95 | 0.56 |
| **Jejunum** | | | | |
| AK | 9.29[a] | 6.35[b] | 0.42 | 0.01* |
| PK | 0.86[a] | 0.59[b] | 0.06 | 0.05* |
| (CK-C)[2] (nmol/mg) | 1.85[b] | 2.50[a] | 0.07 | 0.02* |
| (CK-M)[2] (nmol/mg) | 3.92[a] | 3.38[b] | 0.07 | 0.08* |
| TBARS | 2.64 | 2.25 | 0.13 | 0.43 |
| Carbonyl protein | 4.58[a] | 3.27[b] | 0.15 | 0.02* |
| GST (U/L) | 46.3 | 51.2 | 2.84 | 0.55 |
| SOD[1] (U/mg) | 8.56[b] | 11.7[a] | 0.89 | 0.01* |

[1]cytosolic creatine kinase (CK-C), mitochondrial creatine kinase (CK-M), thiobarbituric reactive substances (TBARS), glutathione S-transferase (GST), superoxide dismutase (SOD), adenylate kinase (AK), pyruvate kinase (PK). [2]nmol of creatine formed per min per mg of protein (CK-C e CK-M), nmol of pyruvate formed per min per mg of protein (PK). *Within the lines, differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$).

### 3.5. Carcass and Meat

Carcass results are presented in Table 6. There were no significant differences between treatments in carcass yield or pH in hot and cold carcasses. There were no significant differences between groups for the color characteristics (brightness, red color, and yellow color) or chemical composition (protein, fat, and organic matter) of the meat (Table 7). However, WRC was significantly higher in meat from TB-fed animals (Table 7; $P = 0.050$).

Results of the fatty acid profile in meat are displayed in Table 8. There was a significant effect of the treatment for the sum of SFA ($P = 0.046$) and monounsaturated fatty acids (MUFA) ($P = 0.050$), with lower SFA and higher MUFA in animals in the TB group. In the meat of animals in the TB group, there was a higher proportion of oleic acid ($P = 0.041$) and omega-3 eicosapentaenoic acid ($P = 0.042$), in addition to a tendency toward a higher percentage of linoleic acid ($P = 0.074$). The animals that consumed TB had a lower proportion of palmitic acid ($P = 0.001$) and a tendency toward a lower percentage of palmitoleic acid ($P = 0.091$). The other variables determined in chromatography did not differ between groups and are presented in Table 8.

**Table 6.** Carcass parameters of lambs fed tributyrin mixed with concentrate.

| Variables[1] | Treatment | | | |
|---|---|---|---|---|
| | Control | Tributyrin | EPM | *P*-value* |
| HCW (%. kg) | 47.4 | 47.5 | 0.051 | 0.917 |
| CCW (%. kg) | 43.5 | 44.3 | 0.034 | 0.183 |
| pH HCW | 6.88 | 6.86 | 0.090 | 0.951 |
| pH CCW | 6.21 | 6.47 | 0.314 | 0.804 |

[1]HCW (hot carcass weight), CCW (cold carcass weight). Hot carcass weight (HCW), cold carcass weight (CCW). *Within the lines, differ (P ≤ 0.05) or tend to differ (P ≤ 0.10).

**Table 7.** Meat quality of lambs fed with tributyrin mixed with concentrate.

| Variables[1] | Treatment | | | |
|---|---|---|---|---|
| | Control | Tributyrin | EPM | *P*-value |
| WRC (%) | 84.8[b] | 87.7[a] | 1.562 | **0.050** |
| **Cor** | | | | |
| Luminosity (L) | 35.9 | 34.3 | 0.548 | 0.625 |
| Redness (a) | 10.8 | 9.35 | 0.420 | 0.502 |
| Yellowness (b) | 9.72 | 8.42 | 0.340 | 0.752 |
| **Chemical composition** (%) | | | | |
| Moisture | 71.9 | 72.4 | 0.952 | 0.909 |
| Total protein | 22.2 | 21.6 | 0.812 | 0.892 |
| Total fat | 2.61 | 2.62 | 0.625 | 0.974 |

[1]WRC (water retention capacity). [a-b]Within the lines, unusual subscripts differ (P ≤ 0.05) or tend to differ.

**Table 8.** Fatty acid profile of lamb meat fed with tributyrin mixed with concentrate.

| Variables | Treatment | | | |
|---|---|---|---|---|
| FA (%) | Control | Tributyrin | SEM | P-value |
| C10:0 (Capric) | 0.068 | 0.065 | 0.003 | 0.951 |
| C12:0 (Lauric) | 0.080 | 0.083 | 0.003 | 0.957 |
| C14:0 (Myristic) | 1.937 | 1.766 | 0.067 | 0.428 |
| C14:1 (Myristoleic) | 0.088 | 0.093 | 0.018 | 0.921 |
| C15:0 (Pentadecanoic) | 0.305 | 0.317 | 0.033 | 0.938 |
| C16:0 (Palmitic) | 26.115[a] | 23.902[b] | 0.306 | 0.001* |
| C16:1 (Palmitoleic) | 1.948[a] | 1.605[b] | 0.061 | 0.091* |
| C17:0 (Heptadecanoic) | 1.175 | 1.076 | 0.045 | 0.820 |
| C18:0 (Stearic) | 15.325 | 15.718 | 0.146 | 0.967 |
| C18:1n9t (Elaidic) | 1.381 | 1.393 | 0.091 | 0.983 |
| C18:1n9c (Oleic) | 43.18[b] | 45.02[a] | 0.350 | 0.041* |

**Continued**

| | | | | |
|---|---|---|---|---|
| C18:2n6c (Linoleic) | 4.774[b] | 5.201[a] | 0.177 | 0.074* |
| C20:0 (Arachidic) | 0.132 | 0.115 | 0.003 | 0.873 |
| C18:3n6 (?-Linolenic) | 0.074 | 0.069 | 0.003 | 0.882 |
| C20:1n9 (cis-11-Eicosenoic) | 0.100 | 0.113 | 0.002 | 0.904 |
| C18:3n3 (a-Linolenic) | 0.176 | 0.198 | 0.008 | 0.913 |
| C21:0 (Henicsanoic) | 0.300 | 0.279 | 0.012 | 0.856 |
| C20:2 (cis-11.14-Eicosadienoic) | 0.043 | 0.048 | 0.002 | 0.899 |
| C22:0 (Behenic) | 0.026 | 0.031 | 0.002 | 0.621 |
| C20:3n6 (cis-8.11.14-Eicosatrienoic) | 0.155 | 0.166 | 0.009 | 0.795 |
| C20:4n6 (Arachidonic) | 2.459 | 2.566 | 0.122 | 0.823 |
| C24:0 (Lignoceric) | 0.027 | 0.030 | 0.002 | 0.941 |
| C20:5n3 (cis-5.8.11.14.17-Eicosapentaenoic) | 0.059[b] | 0.072[a] | 0.004 | 0.042* |
| C24:1n9 (Nervonic) | 0.024 | 0.024 | 0.002 | 0.981 |
| C22:6n3 (cis-4.7.10.13.16.1 Docosahexanoic) | 0.042 | 0.042 | 0.003 | 0.978 |
| **Sum (%)**[1] | | | | |
| SFA | 45.49[a] | 43.38[b] | 0.333 | 0.046* |
| MUFA | 46.72[b] | 48.25[a] | 0.335 | 0.050* |
| PUFA | 7.782 | 8.363 | 0.332 | 0.510 |

[1]Saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA). [a-b]Within the lines, unusual subscripts differ ($P \leq 0.05$) or tend to differ.

## 4. Discussion

The addition of TB to the lamb diet had more marked effects on immune system cells, oxidative status, and fatty acid profile in the meat. Therefore, we need to understand the response mechanisms that were accentuated in these variables. SCFAs are believed to have a close relationship between diet, energy metabolism, ruminal and intestinal microbiota, and host immunity. SCFAs are used as energy and signaling for rumen development and immunomodulatory functions [3] [23]. However, its use in animal feed for ruminants is generally in protected form, which increases the costs of the additive, mainly due to the pungent odor that limits consumption in the free form for ruminants. In the present study, when butyric acid was used as TB, this did not limit the consumption of concentrate by the lambs. In another study, adult sheep had reduced food consumption when the additive TB was present [5] the trophic effect of exogenous TB on the gastrointestinal system is age-dependent in ruminants because butyrate stimulates IGF-1 and IGF-2 in young animals for rumen development, resulting in a greater absorptive surface in the rumen and better growth performance [8] [24]; this effect is not necessary for adult animals. The lambs in the present study were three months old when we started the study, although they had already adapted to the palatability

of the concentrate. According to researchers, the various butyric molecules are more effective in this early stage of the ruminants' life having effects on nutrient absorption and promoting animal development in the first months of life [25]; however, this effect does not persist or is no longer different from control animals over time [23] [26] [27]. Growth performance was analyzed in this study as a complementary variable, since our sample size was planned to study mechanisms and pathways of action of BT in lambs without differences between groups, although offering sodium butyrin in the diet of calves in the pre-weaning phase [8] and found an increase in growth performance while found positive modulations in the diversity of the intestinal microbiota [28]. On the other hand, researchers [1] used 0.03% of tributyrin or sodium butyrin per kg of DM consumed and found lower final weight and ADG/DMI of calves than the control. The same authors reported an increase in plasma glucose concentration in both phases of the study, suggesting an improvement in energy metabolism, corroborating our trend for higher glycemia levels.

Researchers who fed lambs with glycerol monolaurate, a medium-chain glyceride, combined with TB (3 g/kg DM) recorded changes in the rumen microbiome, highlighting a greater abundance of the phyla Bacteroidetes and Verrucomicrobia, the first group of bacteria being more generalized in the process. This phenomenon differed from the second group, which had an enzymatic system for breaking down more complex polysaccharides/fibers. Furthermore, these researchers found that this additive could increase the apparent digestibility of NDF [29] and observed an increase in the relative abundance of the phyla Firmicutes, Bacteroidetes, and Fibrobacteres in lambs that consumed TB. In the present study, we did not evaluate microbiota or digestibility but rather rumen bacterial activity, which was lower in the rumen fluid of lambs that consumed TB. Our finding might indicate the antimicrobial effects of the glyceride, a biological property that is well known. This lower bacterial activity may be directly related to the lower levels of volatile FA in the rumen of these lambs. Nevertheless, this is a hypothesis that needs to be tested.

In adult sheep and *in vitro*, researchers observed more significant activity of fibrolytic enzymes (kilase and carboxymethyl cellulase), a higher concentration of total volatile FA, and lower rumen pH in the presence of TB [5]. A class of molecules that links the microbiota and the immune system are volatile FA, and they even play an active role in the innate reactions of cattle [6] [30]. Butyrate requires the mobilization of $Ca^{2+}$ with a particular concentration in plasma to produce cytokines [6]. However, in the present study, there were no changes in serum $Ca^{2+}$ levels; at the evaluated moments, this relationship was not observed. However, there were changes in immune response biomarkers.

According to the literature, the stimulus that TB causes in the animal can alter the immune function of lambs, especially free butyrate fatty acids that are received by G/GPCR proteins (FFA2 AND FFA3) throughout the gastrointestinal tissue, acting on the expression of immunocompetent cells, such as neutrophils, and on

the mobilization of $Ca^{2+}$ in organs such as the liver, small intestine (duodenum), and rumen [1]. These differential effects on immune cell functions at various times can be explained by two independent mechanisms [5]: a) activation of free fatty acid receptors, FFAR2 and FFAR3 [1] and b) inhibition of histone deacetylase (HDAC) [29].

SCFA are agonists mainly of FFA2 and receipt free fatty acids in an order according to the chain length (C4 > C3 > C2), as the length of the carbon chain is fundamental in the interaction of fatty acids and triglycerides with cells of the system immunological [29]. On the other hand, the mechanism in the modulation of butyrate immune responses is related to HDAC inhibition that occurs in macrophages and deserves to be investigated in future studies for TB.

In both tissues (liver and intestine), we observed effects on the oxidative status associated with changes in the activity of ATP metabolism enzymes, *i.e.*, AK, PK, and CK. The increase in cytosolic CK activity in the jejunum demonstrates that TB treatment stimulates ATP production and improves energy homeostasis since it is considered a central controller of cellular energy homeostasis activity through the reversible interconversion of creatine into phosphocreatine [31]. It is essential to report that CK accumulates a large pool of rapidly diffusing phosphocreatine for temporal and spatial buffering of ATP levels. Thus, CK is particularly critical in tissues with large and fluctuating energy demands, such as muscles, jejunum, and brain [17].

AK is involved in energy metabolism and homeostasis of cellular adenine nucleotide ratios in different intracellular compartments through catalyzing the reversible transfer of a γ-phosphate group from ATP to AMP, releasing two molecules of ADP [32] In the present study, the use of tributyrin reduced AK activity, indicating that treatment impairs liver energetic homeostasis, a condition that can be corroborated with reduced PK activity in the same tissue. It is essential to highlight that AK and PK activities were also reduced in jejune, but cytosolic CK activity increased, which can be considered a compensatory mechanism that aims at improving oxidative energy metabolism [18]. It is necessary to highlight a mutual compensatory relationship between CK, AK, and PK to safeguard the cellular energy economy, which contributes to efficient intracellular energetic communication to maintain the balance between cellular ATP use and synthesis [18]. Therefore, the use of TB improves energy metabolism in the jejunum by increasing cytosolic CK, while the reduction in AK and PK activity may be compensatory mechanisms for maintaining homeostasis.

Analyzing the intestinal and hepatic oxidative status, ingesting TB has an antioxidant effect. Other studies mentioned an activation of the activity of antioxidant enzymes combined with a reduction in oxidation reactions; however, the mechanisms involved are not clear; nevertheless, we believe that this phenomenon is related to improved animal health in general, primarily due to the anti-inflammatory effect provided by butyric acid glycerides. Researchers fed sodium butyrate via milk to calves and found that serum glutathione peroxidase activity increased linearly with increasing sodium butyrate levels, while serum maleic dialdehyde

concentration decreased linearly [8]. The researchers also evaluated the activity of CAT and SOD, but with no effect of treatment on both [8], unlike our study, where the activity of intestinal SOD was higher associated with lower protein oxidation in this organ. Furthermore, the present study observed more significant GST activity in the liver, a hepatic antioxidant enzyme critical for the organ's detoxification process.

In carcasses, our findings aligned with those of other authors [15], who evaluated different doses of protected or unprotected TB fed to lambs, *i.e.*, without effects on hot carcass yield and final carcass yield. However, in the present study, carcasses from animals fed with TB suffered fewer cooling losses; this is an excellent result and may be directly related to the antioxidant response of TB, which leads to cell protection. However, this finding can also represent a negative effect if we consider that losing less water can make it difficult to adequately reduce pH, which is necessary for muscle transformation and maturation.

Like other variables, the color, chemical composition, and fatty acid profile of lamb can be influenced by nutrition and production systems [33] [34]. Therefore, many nutritionists have used this knowledge to produce healthier foods, as discussed below. In the present study, eicosapentaenoic (omega n3) and linoleic (omega C18:2n-6) fatty acids were in more significant proportion in the meat of lambs that consumed TB; when added to other unsaturated substances, meat showed a higher proportion of MUFAs, while the levels of SFA were lower, making the meat more sensitive to oxidation but healthier for humans. Several risk factors for cardiovascular diseases are beneficially modified by increased intake of MUFAs, including blood pressure, platelet reactivity, thrombosis, plasma concentrations of triacylglycerols, vascular function, cardiac arrhythmias, blood flow variability, heart rate, and inflammation [35]. Because the VFA profile results were discreet in rumen fluid, with a lack of treatment effect on serum biomarkers of lipid metabolism, we could not determine the mechanism involved in the changes in the fatty acid profile in the meat of lambs that consumed TB.

## 5. Conclusion

Intake of 2 grams of TB/day by lambs did not alter growth performance variables but changed the activity/concentration of blood, ruminal environment, and meat markers. Among our primary conclusions, we highlight the intestinal and hepatic antioxidant activity associated with regulating ATP in the phosphorylation process. Furthermore, TB consumption increases the proportion of unsaturated FA in meat and reduces SFAs.

## Authors' Contributions

Santos S. A. I, Da Silva A.S., and Kessler J.D. contributed to the design and implementation of the research for the analysis of results and preparation of the published work, explicitly writing the initial draft. Klein. B.; Wagner, R.; Silva L.E.L., Silva P.M. and Baldissera M.D. performed laboratory analysis. Brunetto, A. and Deolindo, G.L. participated in the experiment and collected samples and data.

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## Declarations

### Ethics Approval

The project was approved by the ethics committee on the use of animals in research of UDESC (CEUA nº46844151222). The study followed the guidelines of the Brazilian Council for Animal Experimentation, Brazil.

### Consent for Publication

All authors have consented to the publication and presentation of the data in this article.

### Conflicts of Interests

The authors declare that they have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

### Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author.

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## Appendix

**Supplementary Material 1.** Standardization of methods for measuring short-chain fatty acids.

| | Acetic acid | Propionic acid | Butyric acid | Isovaleric acid |
|---|---|---|---|---|
| $R^2$ | 0.9998 | 0.9996 | 0.9998 | 0.9998 |
| Equation | y = 0.011x − 0.0027 | y = 0.0193x − 0.0121 | y = 0.0268x − 0.0118 | y = 0.034 − 0.0051 |
| Linear range (mmol·L$^{-1}$)* | 2.22 - 132.99 | 1.72 - 102.95 | 1.35 - 81.15 | 0.57 - 18.30 |
| LOD (mmol·L$^{-1}$) | 1.11 | 0.86 | 0.68 | 0.29 |
| LOQ (mmol·L$^{-1}$) | 2.22 | 1.72 | 1.35 | 0.57 |
| Accuracy | 96.44 | 95.31 | 92.84 | 93.56 |
| Repeatability (RSD) | 3.31 | 2.54 | 2.14 | 1.80 |

The LR (linear range), LOD (limit of detection), and LOQ (limit of quantification) were expressed as mmol SCFA per L of rumen fluid.

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SHORT COMMUNICATIONS

# The addition of curcumin to the diet of post-weaning dairy calves: effects on ruminal fermentation, immunological, and oxidative responses

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## Abstract

Incorporating Curcumin into animal diets holds significant promise for enhancing both animal health and productivity, with demonstrated positive impacts on antioxidant activity, anti-microbial responses. Therefore, this study aimed to determine whether adding Curcumin to the diet of dairy calves would influence ruminal fermentation, hematologic, immunological, oxidative, and metabolism variables. Fourteen Jersey calves were divided into a control group (GCON) and a treatment group (GTRA). The animals in the GTRA received a diet containing 65.1 mg/kg of dry matter (DM) Curcumin (74% purity) for an experimental period of 90 days. Blood samples were collected on days 0, 15, 45, and 90. Serum levels of total protein and globulins were higher in the GTRA group ($P < 0.05$) than the GCON group. In the GTRA group, there was a reduction in pro-inflammatory cytokines (IL-1ß and IL-6) ($P < 0.05$) and an increase in IL-10 (which acts on anti-inflammatory responses) ($P < 0.05$) when compared to the GCON. There was a significantly higher ($P < 0.05$) concentration of immunoglobulin A (IgA) in the serum of the GTRA than the GCON. A Treatment × Day interaction was observed for haptoglobin levels, which were higher on day 90 in animals that consumed Curcumin than the GCON ($P < 0.05$). The catalase and superoxide dismutase activities were significantly higher ($P < 0.05$) in GTRA, reducing lipid peroxidation when compared to the GCONT. Hematologic variables did not differ significantly between groups. Among the metabolic variables, only urea was higher in the GTRA group when compared to the GCON. Body weight and feed efficiency did not differ between groups (meaning the percentage of apparent digestibility of dry matter, crude protein, and acid detergent fiber (ADF) and neutral detergent fiber (NDF). There was a tendency ($P = 0.09$) for treatment effect and a treatment x day interaction ($P = 0.05$) for levels of short-chain fatty acids in rumen fluid, being lower in animals that consumed curcumin. There was a treatment vs. day interaction ($P < 0.05$) for the concentration of acetate in the rumen fluid (i.e., on day 45, had a reduction in acetate; on day 90, values were higher in the GTRA group when compared to the GCON). We conclude that there was no evidence in the results from this preliminary trial that Curcumin in the diet of dairy calves interfered with feed digestibility. Curcumin may have potential antioxidant, anti-inflammatory, and immune effects that may be desirable for the production system of dairy calves.

**Keywords** Anti-inflammatory · Antioxidants · Curcumin · Dairy calves · Physiology · Rumen fermentation

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## Introduction

Calf rearing is a primary activity on a dairy farm because discarded cows must be replaced; doing so improves the zootechnical indices of the herd because the genetic potential of these females is greater than that of their progenitors (Santos and Lopes, 2014). During the first months of life, the animals are still exposed to a series of problems related to the relative immaturity of the immune system (Cortese, 2009). At this stage, the animals must receive maximum attention in handling, beginning with colostrum ingestion and including the cleaning and hygiene of the sheds and feed (da Costa and Silva, 2011). The post-weaning period poses a challenge for calves as they transition from a liquid diet to consuming solid feed, requiring them to adapt their feeding behavior and digestive processes to meet nutritional needs independently (Glombowsky et al., 2020).

The literature suggests that calf rearing is neglected, as producers understand that this phase represents only a cost for the farm with low return, so they prioritize production animals (da Costa and Silva, 2011). However, accelerating animal growth in the early stages can lower breeding costs (Hoffman et al., 1996). To accomplish this, the supply of nutrients according to the nutritional requirement of each category must be prioritized, including identifying alternatives such as additives to assist in feed efficiency and decrease disease occurrences (Oliveira et al., 2004). Ionophores (especially monensin) are the most used additives in cattle feed, and their effects range from improved feed efficiency to decreased methane production and coccidiostatic effects (Russell and Strobel, 1989). Restrictive impositions on using feed additives such as antibiotics as growth promoters cause the industry to seek natural substitutes with similar effects and ensure the absence of waste (Stradiotti Júnior et al., 2004).

Some natural compounds from plants have therapeutic properties that reduce the appearance of diseases and help maintain health (Pearce and Jin, 2010). These compounds are called phytotherapeutics or phytogenics, and they have antimicrobial (Fukayama et al., 2005), immunological (Sivaram et al., 2004), and antioxidant activities (Jaguezeski et al., 2018). Recent research has demonstrated the beneficial effects of adding Curcumin to the diet of various animal species (Jaguezeski et al., 2018; Glombowsky et al., 2020; da Rosa et al., 2020). Curcumin is extracted from *Curcuma longa* and has shown anticocidial (Cervantes-Valencia et al., 2015; Glombowsky et al., 2020), anti-inflammatory (Szebeni et al., 2019; Peng et al., 2021), antibacterial (Dai et al., 2022; da Rosa et al., 2020) and antioxidant (Jaguezeski et al. 2018; Tian et al., 2023) effects. A study showed that Curcumin in the diet of lactating sheep increased the digestibility of neutral detergent fiber (NDF) (Jaguezeski et al., 2018). Therefore, the justification for using Curcumin in animal diets is based on the biological properties of this additive and literature findings: (a) The antioxidant response, when stimulated, reduces oxidative stress, a biochemical disorder responsible for causing oxidative imbalance; when exacerbated, it interferes in the functionality of cells and consequently in the general health and growth of animals (Trujillo et al., 2013; Jaguezeski et al., 2018); (b) The anti-inflammatory action in additives is desired, as there is a negative correlation between weight gain and inflammatory response, that is, if the animal organism diverts energy to inflammation/inflammatory response it will no longer be used for productive purposes such as growth (Szebeni et al., 2019; Peng et al., 2021; Sureshbabu et al., 2023); (c) as Curcumin increases fiber digestibility, it is believed to influence the ruminal fermentation process, mainly related to the behavior of volatile fatty acids (Jaguezeski et al., 2018).

We hypothesize that this phytogenic compound, when fed to dairy calves in the post-weaning phase, can stimulate the immune and antioxidant system, decrease the risk of disease and cell damage caused by free radicals, promote more significant daily weight gain, and accelerate growth. Therefore, this work aimed to determine whether adding Curcumin to the diet would influence hematologic, immunological, and metabolic variables in dairy calves in the post-weaning phase.

## Materials and methods

### Additive, installation, animals, and experimental procedures

The experimental curcumin was a commercial product extracted from *Curcuma longa* tuber by Jieliang Extract® Biotech Co., Ltd., Hebei, China. The curcumin purity (% ) in product was determined using high performance liquid chromatography (HPLC) following the methodology described by Coradini et al. (2014) with modifications (Jaguezeski et al. 2018; Campigotto et al., 2020). Briefly, commercial product (0.6 g) was diluted in 10 mL of acetonitrile, then the mixture was homogenized using ultrasound (30 min), magnetic stirring (30 min) and centrifugation (30 min at 15.000 rpm). Sample were then filtered (0.45 µm) and injected into the chromatograph. The chromatographic system ($y = 2836198x - 612{,}806$, $R^2 = 1$) consisted of a Shimadzu® (Taichung, Taiwan) CLC-ODS (M) with column of 15 cm x 4.6 mm x 5 µm, pre-column, mobile phase composed of acetonitrile: (70:30 v/v), flow rate of 0.6 mL/min, detection at 427 nm and injection volume of 20 µL (Coradini et al., 2014). As control, 1000 µg/curcumin of Sigma standard (99% purity) was prepared. Briefly, 0.001 g

of Sigma product and commercial curcumin were weighed and diluted in 10 mL of acetonitrile, following the same homogenization procedure and the same chromatographic conditions as above. We found a purity of 74.0% curcumin in the curcuma extract.

The study was performed in the ruminant sector at the experimental farm of the University of the State of Santa Catarina (FECEO/UDESC) in Guatambu, Santa Catarina, Brazil. The animals were housed in a collective pen with 8.65 m² per animal, an individual feeder with a kennel, concrete floor and roof, and a collective *ad libitum* water drinker.

Fourteen weaned females of the Jersey breed were used, with 105 ± 8.0 SE days of life and an average weight of 105 ± 4.80 SE kg. The experimental period lasted 90 d, the first 15 d for the animals to adapt to the environment, diet, and treatment; and days 16 to 90 was the experimental period. The calves were divided into two groups considering body weight (BW) and age to have homogeneous groups: GTRA ($n=7$) animals received 65.1 mg/kg dry matter (DM) Curcumin; and GCON ($n=7$) control animals, received the basal diet without Curcumin.

The basal diet was formulated according to the nutritional requirements of the NRC (2001) for 90 days of experiment and a weight gain of 0.450 kg per day using corn silage and concentrate feeds (Table 1) adjusted every 15 d according to BW and nutritional requirements for age and category. Diets were provided twice daily, 50% in the morning (8:00 am) and 50% in the afternoon (4:00 pm), individually with animals restrained in a kennel. At the time of feeding, the concentrate was introduced first, followed by the silage.

**Table 1** Ingredients and chemical composition of feed Jersey calves subjected to experimentation with the additive curcumin

| Ingredients | g/kg | |
|---|---|---|
| Ground corn | 163.3 | |
| Soybean meal | 186.7 | |
| Wheat bran | 105.0 | |
| Soybean husk | 70.0 | |
| Premix[1] | 58.4 | |
| Corn Silage | 416.6 | |
| Chemical Composition (g/kg) | Corn Silage | Concentrate |
| Dry Matter | 300.6 | 896.3 |
| Crude Protein | 77.2 | 200.0 |
| Ether extract | 21.4 | 23.1 |
| Ash | 36.5 | 64.2 |
| Neutral detergent fiber | 475.1 | 280.9 |
| Acid detergent fiber | 253.8 | 129.9 |

*Note 1* Premix composition: Calcium (max/min): 210/198 g/kg, Phosphorum: 35 g/kg, Magnesium: 14 g/kg Sodium: 70 g/kg, Sulfur: 25 g/kg, Cobalt: 20 g/kg, Copper:588 mg/kg, Iron: 1,200 mg/kg, Iodine: 38 mg/kg, Manganese: 1,500 mg/kg, Selenium: 30 mg/kg, Zinc: 2030 mg/kg Chromium: 10 mg/kg, Vitamin A: 2000,000 IU/kg, Vitamin D: 40,000 IU/kg, Vitamin E: 1,000 mg/kg

The Curcumin was mixed with the concentrate at a rate of 100 mg/kg of DM to achieve the desired level of supplementation (Jaguezeski et al. 2018; Glombowsky et al. 2020; Marcon et al. 2021a, b). Two hours after providing the diet (concentrate and silage), the leftovers were individually weighed and recorded in a spreadsheet to calculate dry matter intake, feed conversion, and feed efficiency.

## Weighing

Before the morning feeding, the animals were weighed using an electronic scale moving bar up to 3000 kg (Digitron, Guarapuava-Paraná, Brazil) on days 0, 15, 30, 45, 60, 75, and 90 to calculate average daily gain.

## Feed consumption and digestibility

From the 15th day until the end of the study, the leftover feed was weighed on a digital scale and recorded in a specific spreadsheet to determine the dry matter intake of each animal to calculate feed conversion and feed efficiency. An amount corresponding to 10% of the weight of the leftovers was stored at − 20 °C for further analysis.

For three consecutive days of the study (Dermauw et al., 2013; Costa e Silva et al., 2015), fecal samples (approximately 150 g per collection) were collected directly from the rectal ampulla on days 88, 89, and 90 (08:00, 12:00, and 16:00), totaling 9 stool samples that were combined into a homogeneous pool. After collection, the samples were frozen, and in the late afternoon of day 90, they were homogenized, forming one pool per animal. Then, the samples were dried in a forced-air ventilation oven (55 °C by 72 °C). The samples were processed in a specific mill (Wiley® Mill, Hoboken, New Jersey, United States) with a 1-mm sieve and stored for later analysis. On the same days as fecal collection, samples of the total diet were collected, homogenized, dried in an oven, and ground using the same methodology as feces.

Analysis of the chemical composition of diets was presented in Table 1, using the same methodology described below for feces and total diet to determine the digestibility coefficients. The pre-dried and ground samples it to be dried at 105 ºC to obtain the dry matter and the mineral material in a muffle at 600 ºC (Silva and Queiroz, 2002). The micro-Kjeldahl method determined the nitrogen content (Method 984.13, AOAC, 1997), which allowed for predicting the crude protein content through mathematical calculation. To determine the NDF content, the samples were placed in Ankom fiber bags (Komarek, 1993) and treated with a neutral detergent solution in a fiber analyzer at 110 °C for 40 min (Senger et al., 2008); for concentrate samples, we included α-amylase (Mertens, 2002). Acid detergent fiber

(ADF) concentrations were determined according to AOAC (1997, method 973.18). Values were obtained using a Near Infrared Reflectance Spectrometer, model Spectra Star 2600 XT series of Near Infrared Analyzers (Unity Scientific®) for ether extract concentrations.

Indigestible neutral detergent fiber (iNDF) was used as an internal marker to calculate apparent feed digestibility (Cochran et al., 1986; Huhtanen et al., 1994). The feed and feces samples were weighed in bags with 16 µm porosity and incubated in the rumen of cattle for 288 h in one fistulated bovine (male, Jersey, and 2.2 years old) fed for 14 days prior to the beginning of incubation, with the same diet provided to the Jersey this study. Then, they were washed with tap water, treated with neutral detergent in an autoclave (Senger et al. 2008), and dried in a forced-air ventilation oven at 55 ºC. Digestibility was calculated as 1 – (iNDF in feed (% of DM)/iNDF in feces (% of DM).

## Samples collection

Blood sample collections were performed on days 1, 15, 45, and 90. Samples were collected in vacuolated tubes: tubes with anticoagulants (EDTA 10%) were used for complete blood count, and tubes without anticoagulants were used for serum. After collection, these samples were sent to the laboratory and centrifuged at 2800 g for 10 min to separate serum. Then, a serum fraction was stored in 1.8-ml Eppendorf microtubes at − 15 ºC.

On days 45 and 90, ruminal fluid samples were collected using an esophageal tube two hours after feeding the animals.

## Rumen fluid: volatile fatty acid profile and pH

To determine the pH of the rumen liquid, we used a Kasvi® bench pH meter immediately after collection; 15 mL of rumen fluid from each calf was filtration and after was stored in a Falcon tube and frozen at − 20 °C for further analysis.

The rumen fluid samples were thawed in a natural environment until they reached a temperature of 5 °C and then were manually homogenized. The tubes were subjected to 30 min of mechanical agitation to help extract the compounds. After these processes, 1-mL aliquots of samples were transferred to polypropylene microtubes (2 mL) and centrifuged for 5 min (12,300 × *g*). Then, 100 µL of the supernatants were removed and transferred to a new microtube containing 100 µL of formic acid (P.A.). The mixture was vortexed for 30 s and centrifuged again for 3 min. After centrifugation, 50 µL of the supernatant of the mixture was transferred to 250 µL tubes, and 100 µL of the 3-octanol internal standard methanolic solution (665 µg/mL) was added.

The short chain fatty acids profile was based on the methodology described by Leal et al. (2020). The samples were injected into a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3400, Woonsocket, RI, USA) and an autosampler (Varian 8100); 1 µL of the extract was injected in 1:10 split mode. The carrier gas used was hydrogen at a constant pressure of 20 psi. The analytes (acetic, propionic, butyric, valeric, and isovaleric acids) were separated on a CP WAX-52CB capillary column (60 m × 0.25 mm; 0.25 µm stationary phase thickness). The initial column temperature was 80 °C for 1 min and increased by 15 °C/min until reaching 120 °C, soon after reaching 230 °C rising to 20 °C/min, where it remained for 1 min. Inlet and detector temperatures were set to 250 °C. The validation of the method included the following parameters: selectivity, linearity, linear range, repeatability, precision, the limit of detection, and the limit of quantification for acetic, propionic, butyric, valeric, and isovaleric acids.

## Blood count and serum biochemistry

Counting erythrocytes and leukocytes and hemoglobin and hematocrit calculations were performed on the automatic counter (ICOUNTER 3D®). For leukocyte differentiation, we used the Romanowsky method (Thrall et al., 2015) on glass slides.

The biochemical analyses were performed on a Bioplus 2000® with commercial kits from Gold Analisa®, following the methodologies recommended by the manufacturer for aspartate aminotransferase (AST), gamma-glutamyltransferase (GGT), total protein, glucose, triglycerides, albumin, and urea. Globulin levels were calculated as the difference between total protein and albumin.

## Cytokines and proteinogram

Serum cytokine levels serum were measured. Interleukin 1β (IL-1β), IL-6, and IL-10 were measured using commercial kits and an enzyme-linked immunosorbent assay device (AwarenessTechnology, Inc., USA; Chem Well; USCN, Wuhan, China).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to Fagliari et al. (1998) using mini-gels (10×10 cm) to determine levels of immunoglobulins A (IgA), heavy chain immunoglobulins (Ig heavy chain), ceruloplasmin and hapatoglobin. The gels were stained with Coomassie blue and photographed to identify and quantify protein fractions using Labimage 1D software (Loccus Biotechnology). A standard containing fractions with molecular weight between 10 and 250 KD (Kaleidoscope - BIORAD) was used as a reference.

## Antioxidant activity

For lipid peroxidation, the FOX methodology was used (Hermes-Lima, 1995; Monserrat et al., 2003), which is based on iron oxidation and measures the amount of lipid peroxides. The result was expressed as nmol/mL. Serum superoxide dismutase (SOD) activity was measured spectrophotometrically (Marklund and Marklund, 1974), and the results were expressed as U SOD/mg of blood protein. For glutathione S-transferase (GST), we used the technique of Mannervik and Guthenberg (1981), and GST activity was measured as the rate of dinitrophenyl-S-glutathione formation at 340 nm in a medium containing 50 mM potassium phosphate, pH 6.5, 1 mM GSH, and 1 mM 1-chloro-2,4-dinitrobenzene as substrate and tissue supernatants (approximately 0.045 mg protein), expressed as U GST/mg blood protein. Glutathione peroxidase (GPx) was measured using tert-butylhydroperoxide as a substrate (Wendel, 1981). Enzyme activity was determined by monitoring the disappearance of NADPH at 340 nm in a medium containing 100 mM potassium phosphate buffer/1 mM EDTA, pH 7.7, 2 mM GSH, 0.1 U/mL GR, 0.4 mM azide, 0.5 mM tert-butyl hydroperoxide, 0.1 mM NADPH, and tissue supernatants (approximately 0.065 mg protein). Results expressed as U GPx/mg protein.

## Statistical analyses

The number of animals required per treatment was previously calculated using the 'POWER procedure' of SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4). The variable selected to calculate the number of animals per treatment was globulin. The estimated serum concentration of globulin in the GCON was 4.00 g/dL; a 15% increase in the Curcumin group (4.60 g/dL) was expected. The standard deviation was 0.35, alpha was 0.05, and power was 80%. The serum concentration in the GCON and the standard deviation were estimated using results from previous experiments of our group.

All data collected in this experiment were analyzed using the 'MIXED procedure' of SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4), with Satterthwaite approximation to determine the denominator degrees of freedom for the test of fixed effects. The average daily gain, dry matter intake, feed conversion, feed efficiency, and digestibility were tested for the fixed effect of treatment using the animal as the random effect. BW and all blood results were analyzed as repeated measures and were tested for fixed effects of treatment, day, and treatment × day, using the animal as a cluster effect. The d 1 results were included as an independent covariate. To generate the average per treatment, the d 1 results were removed from the data set but kept as covariates for these variables, because the animals are not receiving curcumin treatment yet. The first-order autoregressive covariance structure was selected according to the lowest Akaike information criterion. Means were separated using the PDIFF method (Student's t-test), and all results were reported as LSMEANS followed by SEM. Significance was defined when $P \leq 0.05$, and tendency when $P > 0.05$ and $\leq 0.10$.

# Results

## Performance

Performance results are shown in Table 2. There was no treatment effect or interaction between treatment vs. day for BW ($P > 0.05$). There was also no treatment effect for average daily weight gain, feed intake, feed efficiency, or feed conversion ($P > 0.05$). There was a day effect for body weight, which increased in both groups.

**Table 2** Effects of curcumin addition to the diet of Jersey calves on body weight, daily weight gain, feed intake, and apparent nutrient digestibility

| Items | Treatments | | SEM[2] | P-values[1] | | |
|---|---|---|---|---|---|---|
| | GCON: Control | GTRA: Curcumin | | Treat | Day | Treat × Day |
| Initial weight (kg) | 97.0 | 96.3 | 1.30 | 0.96 | NE | NE |
| Final weight (kg) | 159 | 156 | 1.30 | 0.16 | 0.01 | 0.98 |
| Average daily gain (kg/day) | 0.69 | 0.66 | 0.05 | 0.76 | NE | NE |
| Total dry matter intake (kg) | 265 | 265 | 1.43 | 0.96 | NE | NE |
| Dry matter intake (kg/day) | 3.53 | 3.53 | 0.02 | 0.95 | NE | NE |
| Feed conversion (Kg DMI/Kg BW) | 5.16 | 5.28 | 0.13 | 0.55 | NE | NE |
| Feed efficiency (Kg BW/Kg DMI) | 0.19 | 0.19 | 0.005 | 0.53 | NE | NE |
| **Apparent digestibility** | | | | | | |
| Dry matter (DM) | 0.73 | 0.73 | 0.06 | 0.97 | NE | NE |
| Crude protein | 0.79 | 0.78 | 0.09 | 0.93 | NE | NE |
| Neutral detergent fiber (%) | 0.61 | 0.61 | 0.03 | 0.98 | NE | NE |
| Acid detergent fiber (%) | 0.58 | 0.58 | 0.04 | 0.96 | NE | NE |

[1] No effect ($P > 0.05$) or trend ($P > 0.10$) of difference was observed between groups for the performance and digestibility variables. [2]Stander error of the mean. NE (not evaluated)

**Table 3** Effects of curcumin addition to the diet of Jersey calves on ruminal fluid pH and short-chain fatty acid concentrations

| Items | Treatments | | SEM[2] | P-values[1] | | |
|---|---|---|---|---|---|---|
| | GCON: Control | GTRA: Curcumin | | Treat | Day | Treat × Day |
| pH | 6.40 | 6.41 | 0.08 | 0.61 | 0.46 | 0.87 |
| Total SCFA (mmol/L) | 50.9[a] | 46.8[b] | 2.70 | 0.09 | 0.01 | 0.05 |
| Acetic acid (mmol/L) | 34.7 | 32.1 | 2.62 | 0.85 | 0.01 | 0.05 |
| Propionic acid (mmol/L) | 8.17 | 7.56 | 0.68 | 0.19 | 0.04 | 0.07 |
| Butyric acid (mmol/L) | 5.97 | 5.24 | 0.82 | 0.28 | 0.09 | 0.11 |
| Isovaleric acid (mmol/L) | 1.03 | 0.97 | 0.07 | 0.79 | 0.19 | 0.20 |
| Valeric acid (mmol/L) | 1.05 | 0.94 | 0.05 | 0.45 | 0.25 | 0.15 |

[1] Within a row when there are different letters ([a–b]), differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$) by t-test. [2]Stander error of the mean

**Table 4** Effects of curcumin addition to the diet of Jersey calves on erythrocytes, hematocrit, hemoglobin, total leukocytes, neutrophils, lymphocytes, monocytes and eosinophils

| Items | Treatments | | SEM[2] | P-values[1] | | |
|---|---|---|---|---|---|---|
| | GCON: Control | GTRA: Curcumin | | Treat | Day | Treat × Day |
| Erythrocytes (x$10^6$ µL) | 7.40 | 7.60 | 0.25 | 0.54 | 0.01 | 0.16 |
| Hematocrit (%) | 36.3 | 37.9 | 2.96 | 0.80 | 0.01 | 0.08 |
| Hemoglobin (g/dL) | 8.67 | 8.87 | 0.25 | 0.51 | 0.01 | 0.22 |
| Leukocytes (x$10^3$ µL) | 9.79 | 10.5 | 0.45 | 0.26 | 0.01 | 0.10 |
| Neutrophils (x$10^3$ µL) | 1.59 | 2.10 | 0.42 | 0.38 | 0.01 | 0.63 |
| Lymphocytes (x$10^3$ µL) | 7.96 | 8.16 | 0.38 | 0.71 | 0.01 | 0.39 |
| Monocytes (x$10^3$ µL) | 0.22 | 0.22 | 0.08 | 0.99 | 0.82 | 0.95 |
| Eosinophils (x$10^3$ µL) | 0.02 | 0.02 | 0.007 | 0.50 | 0.87 | 0.70 |

[1] Within a row when there are different letters ([a–b]), differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$) by t-test. [2]Stander error of the mean

Knowing the feed consumption, the dose of Curcumin ingested daily was defined as 230 mg/animal/day, and the Curcumin consumption per kg of dry matter ingested was calculated as 65.1 mg/kg DM. Also, knowing the BW, the dose of Curcumin per kg of BW of the calves was determined: 1.82 mg/kg.

## Digestibility

The treatment did not affect the apparent digestibility of dry matter, crude protein, NDF, and ADF ($P > 0.05$; Table 2).

## Acid profile in the rumen liquid

Results of the acid profile in the rumen fluid are shown in Table 3. There was a tendency for treatment effect and treatment vs. day interaction for levels of SCFA in rumen fluid, being lower in animals that consumed curcumin. There was a day effect for both groups, for SCFA, acetic acid, and propionic acid. There was no effect of treatments for acetic, butyric, propionic, valeric, or isovaleric acids. There was an interaction between treatment and day for acetic acid ($P = 0.05$); on day 45, the highest concentration was observed in the GTRA. By contrast, on day 90, the concentration of acetic acid was lower in the group that consumed Curcumin. There was a trend of treatment vs. day interaction at day 90 when the concentration of this acid was lower in the calves of the GTRA.

## Hemogram

Table 4 shows hemogram results. There was no treatment effect ($P > 0.05$) for the erythrogram (erythrocyte count, hematocrit, or hemoglobin) and leukogram (lymphocytes, neutrophils, monocytes, and eosinophils); but there was day effect was verified for both groups for red blood series, as well as leukocytes, lymphocytes and granulocytes. There was an interaction trend between treatment and day for hematocrit percentage ($P = 0.08$) and total leukocyte count ($P = 0.10$). Hematocrit and total leukocytes were higher in the calves that consumed Curcumin on day 90 than the GCON.

## Serum biochemistry

Serum biochemistry/metabolism results are presented in Table 5. There was a day effect and interaction between treatment and day ($P < 0.05$) for albumin levels at day 15 (higher in the GTRA). There was a treatment effect (higher in the GTRA), day, and interaction between treatment and day for total protein and globulin concentration ($P < 0.01$); there was a higher concentration of these two variables in the serum of the animals treated with Curcumin on days 45 and 90. There was an effect of the treatment for urea concentration, which was higher in the GTRA ($P = 0.04$), and a trend of treatment vs. day interaction for this variable on day

**Table 5** Effects of curcumin addition to the diet of Jersey calves on serum biochemistry (mean of albumin, globulin, total protein, glucose, triglycerides, urea, AST and GGT)

| Items | Treatments | | SEM[2] | P-values[1] | | |
|---|---|---|---|---|---|---|
| | GCON: Control | GTRA: Curcumin | | Treat | Day | Treat × Day |
| Albumin (g/dL) | 2.84 | 2.92 | 0.10 | 0.36 | 0.02 | 0.05 |
| Globulin (g/dL) | 3.67[b] | 4.34[a] | 0.21 | 0.01 | 0.01 | 0.01 |
| Total protein (g/dL) | 6.52[b] | 7.26[a] | 0.22 | 0.01 | 0.01 | 0.01 |
| Glucose (mg/dL) | 80.3 | 80.0 | 2.47 | 0.93 | 0.36 | 0.96 |
| Triglycerides (mg/dL) | 29.4 | 26.7 | 2.54 | 0.73 | 0.12 | 0.45 |
| Urea (mg/dL) | 18.7[b] | 23.6[a] | 1.51 | 0.04 | 0.03 | 0.09 |
| AST (mg/dL) | 63.0 | 67.6 | 2.84 | 0.32 | 0.26 | 0.83 |
| GGT (mg/dL) | 17.3[b] | 19.3[a] | 0.70 | 0.05 | 0.38 | 0.58 |

[1] Within a row when there are different letters ([a–b]), differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$) by t-test. [2]Stander error of the mean.[3] Aspartate aminotransferase (AST) and gamma-glutamyltransferase (GGT)

**Table 6** Effects of curcumin addition to the diet of Jersey calves on levels of cytokines (IL-1β, IL-6, and IL-10) and proteinogram (IgA, IGG heavy chain, ceruloplasmin, and haptoglobin)

| Items | Treatments | | SEM[2] | P-values[1] | | |
|---|---|---|---|---|---|---|
| | GCON: Control | GTRA: Curcumin | | Treat | Day | Treat × Day |
| Interleukin 1β (pg/mL) | 29.5[a] | 21.9[b] | 3.82 | 0.01 | 0.01 | 0.01 |
| Interleukin 6 (pg/mL) | 10.8[a] | 6.84[b] | 0.83 | 0.01 | 0.01 | 0.01 |
| Interleukin 10 (pg/mL) | 39.2[a] | 53.6[b] | 5.45 | 0.03 | 0.01 | 0.02 |
| IgA (g/dL) | 0.60 | 0.74 | 0.02 | 0.01 | 0.01 | 0.01 |
| IGG heavy chain (g/dL) | 1.23 | 1.28 | 0.06 | 0.21 | 0.01 | 0.15 |
| Ceruloplasmin (g/dL) | 0.50 | 0.47 | 0.02 | 0.46 | 0.02 | 0.42 |
| Haptoglobin (g/dL) | 0.30 | 0.36 | 0.02 | 0.22 | 0.01 | 0.01 |

[1] Within a row when there are different letters ([a–b]), differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$) by t-test. [2]Stander error of the mean

**Table 7** Effects of curcumin addition to the diet of Jersey calves on levels of lipoperoxidation (LPO) and activities of glutathione S-transferase (GST), superoxide dismutase (SOD), and glutathione peroxidase (GPx)

| Items | Treatments | | SEM[2] | P-values[1] | | |
|---|---|---|---|---|---|---|
| | GCON: Control | GTRA: Curcumin | | Treat | Day | Treat × Day |
| LPO (x10$^3$ nmol/mL) | 5.41[a] | 3.84[b] | 0.84 | 0.01 | 0.01 | 0.05 |
| GST (U GST/mg of protein) | 3.10[b] | 5.90[a] | 0.46 | 0.01 | 0.01 | 0.01 |
| SOD (U SOD/mg of protein) | 11.3[b] | 14.4[a] | 0.76 | 0.05 | 0.01 | 0.01 |
| GPx (U GPx/mg of protein) | 6.24[b] | 9.12[a] | 0.49 | 0.03 | 0.01 | 0.01 |

[1] Within a row when there are different letters ([a–b]), differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$) by t-test. [2]Stander error of the mean

15 of the experiment ($P = 0.09$). GGT activity was higher in the GTRA ($P = 0.05$). We did not find a treatment effect or a treatment vs. day interaction ($P > 0.05$) for AST, glucose, or triglycerides (Table 5).

## Interleukins and proteinogram

Cytokine levels are presented in Table 6. There was a treatment effect, and treatment vs. day interaction for serum concentrations of IL-1, IL-6, and IL-10 ($P < 0.05$). Pro-inflammatory interleukins (IL-1 and IL-6) concentrations were lower; the anti-inflammatory cytokine (IL-10) was higher in the serum of animals that consumed Curcumin. Higher concentration of immunoglobulin A (IgA) in Jersey serum from the GTRA than the GCON ($P < 0.01$; Table 6). Treatment × day interaction, was observed for haptoglobin levels, higher on day 90 in animals that consumed Curcumin ($P < 0.01$). Ceruloplasmin and heavy chain IGG levels did not differ between treatments ($P > 0.05$; Table 6). In both groups, a day effect was observed for immunological variables (Table 6).

## Oxidative status

Biomarkers of oxidative reactions are shown in Table 7. There was treatment effect, day, and treatment vs. day interaction ($P < 0.05$) for lipoperoxidation (LPO), GST, SOD, and GPx. LPO was lower in the animals that consumed Curcumin in general and on day 15. The activities of the three antioxidant enzymes were higher in the GTRA, which reflected a treatment effect and a treatment vs. day interaction for GST, SOD, and GPx.

## Discussion

Several studies showed the positive effects of using Curcumin as an additive in animal feed, particularly productive increments, improvement in the digestibility of nutrients, and better response of the immune and antioxidant systems, resulting in better performance. We monitor several variables related to calf health using blood analysis. Oxidative stress markers and immunological variables were measured, and we recorded performance and rumen health data. Natural additives offer an alternative to chemical additives, often restricted in their use. The literature suggests that we should prioritize the maximum performance of calves in rearing systems, as the age at first calving would be lower, and production costs would be minimized (Fouladgar et al., 2016). Feeding a diet with high energy levels leads to more significant average weight gain; however, diets with high energy levels affect the growth of the mammary gland secretory tissue (Sejrsen and Brolund Larsen, 1977). Marcon et al. (2021a, b) observed higher average daily weight gain in lambs feed with Curcumin without increasing feed intake. Glombowsky et al. (2020) found that calves supplemented with Curcumin had significantly more significant weight gain than control animals. Our study did not observe differences in average daily weight gain, dry matter intake, feed conversion, or feed efficiency.

Increasing ruminant production gains is associated with increasing nutrient digestibility and feed intake (Hashemzadeh-Cigari et al., 2014). Glombowsky et al. (2020) performed in vitro tests and found that Curcumin is a potentiator of digestibility in hay and concentrate after 72 h of incubation; the mechanism needs to be elucidated. In the study, we did not find changes in the digestibility of dry matter, crude protein, NDF, or ADF in animals fed with Curcumin. Changes in nutrient digestibility can also alter the production of short-chain volatile fatty acids produced in the rumen (Tekippe et al., 2013); however, a lower concentration of SCFA in the ruminal fluid of animals that consumed Curcumin was found here. Nevertheless, there were fluctuations in the concentration of ruminal acetic acid; that is, levels were higher (day 45) or lower (day 90) in animals that consumed Curcumin; already, the propionic acid tended to be lower in concentration on day 45. The opposite effect was reported by Tian et al. (2023), who observed that lambs fed a diet containing 300 mg/kg of curcumin showed an increase in the production of ruminal acetate and propionate. Furthermore, the authors also reported an increase in the ruminal bacterial population (*Ruminococcus albus* and *Fibrobacter succinogenes*) which is related to an increase in the activity of enzymes such as pectinase and parboxymethyl cellulase. The difference in the effects of Curcumin between studies may be related to the dose and animal species, but our main hypothesis is related to the ruminal microbiota that directly impacts the production of SCFA, knowing that there is great individual variation, and direct influence of feed and environment; remembering that these microorganisms come from the environment. However, more studies on the subject are necessary to understand the mechanisms and factors involved.

Some studies showed that Curcumin decreases the total leukocyte count and reinforces its anti-inflammatory action (Fu et al., 2014; Jaguezeski et al., 2018). In the present study, we did not find decreases in the total leukocyte count in the GTRA; however, IL-1ß levels were lower in the animals that received Curcumin. IL-1ß activates STAT3, the factor responsible for the transcription of inflammatory cytokines, increasing the inflammatory responses of cells. Ahmed and Gilani (2011) reported that one of the primary effects of Curcuminoids (especially dimethoxy Curcumin) is the reduction of IL-1ß levels. Likewise, IL-6 is produced from responses to infections and tissue injury and has a pathological effect on inflammation (Tanaka et al., 2014). For IL-6, GTRA showed lower values compared to GCON ($P < 0.01$), reinforcing its anti-inflammatory effects. IL-10 plays a crucial role in anti-inflammatory responses and in preventing autoimmune diseases (Saraiva and O'Garra, 2010). Salah et al. (2022) induced inflammatory responses in human intestinal cells and found that although Curcumin did not affect decreasing pro-inflammatory cytokines (IL-1ß, IL-6, and IL-8), there was a considerable increase in IL-10 secretion, as Curcumin induced the transcription of this protein. This finding may explain the increases in IL-10 values in the present study.

A higher concentration of IgA in the serum of Jersey heifers that consumed Curcumin suggests that this additive stimulates the humoral immune response at the intestinal level. Sheep fed with 450 mg and 900 mg Curcumin had higher concentrations of IgA and IgM (Jiang et al., 2019). This positive effect of Curcumin is desirable, but we do not have an explanation of the mechanism involved since the number of lymphocytes did not differ between treatments, which was our hypothesis since B lymphocytes produce immunoglobulins. In addition, we highlight the highest concentration of haptoglobin at the end of the experiment in calves that were fed Curcumin. We believe that the hepatoprotective effect of Curcumin at this dose may be related to the better health of this organ (Fernández Bermejo et al., 2022) responsible for producing this protein. Haptoglobin is a major acute-phase protein in cattle with a significant positive association with bovine respiratory disease in calves (Murata et al., 2004). This acute-phase protein eliminates metabolites (e.g., free hemoglobin) released from cellular degradation, helping to prevent oxidative tissue damage because of its peroxidase activity. Haptoglobin also helps recycle heme residues

for further metabolic processes rather than being utilized by pathogens (Hanthorn et al., 2014; Moisá et al., 2019). Therefore, in addition to the anti-inflammatory effect, Curcumin in ruminant diets can stimulate an immune response.

Curcumin has shown great free radical scavenging power through its antioxidant effects (Deogade and Gathe, 2015). The determining factor for the antioxidant effects of Curcumin lies in its chemical structure, which is formed by two methoxylated phenols bonded to two unsaturated carbonyl groups (Sreejayan and Rao, 2011). Adding Curcumin increases total antioxidants, which are responsible for decreasing oxidative stress. The lipid peroxidation levels caused by free radicals were lower in the GTRA ($P < 0.01$), demonstrating this effect.

The end of the experiment, a tendency towards a higher percentage of hematocrit for Curcumin animals was observed in the current study; but no difference in the number of erythrocytes and hemoglobin concentration; result similar to that described by Glombowsky et al. (2020) when providing curcumin to lactating and weaned calves. In previous studies, researchers verified higher erythrogram biomarkers in dogs that consumed Curcumin (Campigotto et al., 2020), which according to the literature may be attributable to the protective properties of curcumin, increasing erythropoiesis and avoiding oxidative damage (Sayed and El-Kordy, 2014); which may be related to the trend towards higher hematocrit in Jersey.

## Conclusion

We conclude that there was no evidence in the results from this preliminary trial that Curcumin in the diet of dairy calves interfered with feed digestibility. Curcumin may have potential antioxidant, anti-inflammatory, and immune effects that may be desirable for the production system of dairy calves.

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**Author contributions** PV Novakoski and AS Da Silva contributed to the research's design and implementation to analyze the results. R Wagner, GV Kozloski, and M Vedovatto helped develop the project and its execution and financing. MG Vitt and VL Molosse participated in the execution of the experiment and collection of samples and data. ACH Xavier, B Klein, LE Leonardi, and CF Milarch performed the laboratory analysis. All authors discussed the results and contributed to the final manuscript.

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**Data availability** All data and materials used in the experiment are available and ready to be provided.

## Declarations

**Ethical approval** This study was approved by the Ethics Committee of Use of Animals (ECUA) of Universidade do Estado de Santa Catarina (UDESC), protocol number 4985190421, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA/Brazil).

**Consent to participate** All names in the author list were involved in various stages of experimentation or writing.

**Consent for publication** All authors agree to submit the paper for publication in the Tropical Animal Health and Production.

**Conflict of interest** The authors declare no conflicts of interest.

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Article

# Nanocapsules Prepared by Nanoprecipitation Protects Chia Seed Oil (*Salvia hispanica* L.) against Thermal Degradation

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**ABSTRACT:** Chia seed (*Salvia hispanica* L.) is a natural product with high nutritional and medicinal value due to its lipid composition, mainly polyunsaturated fatty acids, which are unstable in the presence of light and heat. Nanotechnology is a prominent field, as nanomaterials can protect substances from degradation. The present study utilized nanocapsules to protect chia seed oil through nanoprecipitation, followed by physicochemical characterization, thermal stability analysis, and *in vitro* cytotoxicity. The identification and quantification of components of the bulk and nanoencapsulated oil were performed by gas chromatography, and nuclear magnetic resonance proved oil encapsulation. Nanocapsules had a mean diameter of 246.23 ± 1.53 nm, a polydispersity index of 0.148 ± 0.08, and a zeta potential of −3.84 ± 0.05 mV. Nanocapsules were not cytotoxic to VERO cells after 48 hof incubation. In conclusion, nanoencapsulation protected chia seed oil against degradation, preserving its constituents to enhance biological efficiency for health purposes.

**KEYWORDS:** *Fatty acids, Chia seed oil nanoencapsulation, Solvent diffusion method, Gas chromatography, Nuclear magnetic resonance, Long-term storage stability*

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## INTRODUCTION

Chia (*Salvia hispanica* L.) is an annual herbaceous plant that belongs to the Lamiaceae family. It is native to southern Mexico and northern Guatemala and is cultivated in several South American countries.[1] Chia seed oil (CSO) is a functional food rich in polyunsaturated fatty acids (PUFAs), especially α-linolenic acid, linoleic acid, tocopherols, phenolic acids, vitamins, and antioxidants.[2] Dietary consumption of food sources containing bioactive compounds such as phenolics, fatty acids, and sterols from chia is associated with a decreased risk of cardiovascular disease, hepatotoxicity,[3] oxidative diseases related to obesity,[4] and damage from type 2 diabetes and hypertension.[5] In the food industry, nanotechnology has emerged to increase the shelf life of bioactive compounds, to create improved storage strategies, and to provide protection against degradation. In this way, nanotechnology also facilitates the incorporation of the compounds as supplements in food.[6] Oils can be incorporated into nanoformulations, especially in nanocapsules, where the oily core is protected by a polymeric coating.[7] The encapsulation of PUFA-rich oils provides a physicochemical barrier against thermal degradation and oxidation-inducing agents, such as oxygen, free radicals, ultraviolet radiation, and volatile compounds. Nanocapsules prevent and/or delays oxidation reactions of the fatty acids, extending the oils bioactivity.[8,9]

In a previous study, nanocapsules containing CSO (P-CSO-NCs) in the form of a hydrogel were produced and physicochemically characterized; they were included in a hydrogel used for skin application.[10] This nanoformulation can reduce the loss of and optimize the use of CSO, allowing it to exert its potential health benefits[2,5] and increasing bioavailability. The present study aimed to further characterize P-CSO-NCs, including the fats present, the morphology, the thermal stability, and the *in vitro* safety of this nanoformulation.

## MATERIALS AND METHODS

**Materials.** Naringin, bromide 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), sorbitane monostearate (Span 80), Pluronic F-127 (MW 12600 g/mol), FAME Mix-37 standards (P/N 47885-U), Dulbecco's Modified Eagle's Medium (DMEM), and acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polysorbate 80 (Tween 80), lactose, and maltodextrin were purchased from Synth (Diadema, SP, Brazil). Ethanol were purchased from J.T. Baker (VWR International Global Exports, Batavia, NY, USA). Capric and caprylic acid triglyceride mix (Crodamol GTCC) was purchased from Alpha Química (Cachoeirinha, RS, Brazil). Bulk CSO was purchased from Seiva Brazilis (CAS 93384-40-8/EINECS 297-250-8; São Paulo, SP, Brazil). Other chemicals and solvents were pharmaceutical grade and used as received.

**Production of P-CSO-NCs.** The nanocapsules were prepared via nanoprecipitation, as described by Balest.[11] An organic phase (0.25 g of CSO, 0.191 g of cetyl palmitate, 0.775 g of Span 80, and 0.25 g of

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Pluronic F-127 in 66.5 mL of ethanol) and an aqueous phase (0.191 g of Tween 80 in 133.25 mL of Milli-Q ultrapure water) were prepared separately and stirred magnetically at 60 °C until solubilization. Afterward, the organic phase was poured onto the aqueous phase, with the aid of a funnel for instant formation of nanocapsules.[12,13] The suspension was incubated at 60 °C under agitation for 10 min in an Ultraturrax homogenizer at 8000 rpm to homogenize the particles.[14] Then, to eliminate solvent and excess water, the suspension was placed in a rotary evaporator until reaching a final volume of 25 mL, in which the CSO concentration was 10 mg/mL. Blank nanocapsules (P-NCs) were produced in the same way, but CSO was replaced by Crodamol GTCC due to its inert character.[15] Both formulations were prepared in three batches to determine the physicochemical and thermal stability characteristics.

**Physicochemical Characterization of Nanocapsules.** *Determination of the Refractive Index.* The refractive index of the nanocapsules was determined in a graduated-scale refractometer through an optical system. First, the equipment was calibrated with distilled water. Then, a drop of the sample was placed in the equipment, where the boundary line between a light and a dark part was observed through the focusing eyepiece. After this adjustment, the scale, which corresponds to the refractive index of the sample, was verified.

*Evaluation of the Particle Size Distribution.* The nanocapsule suspensions were analyzed with a Microtrac instrument, which uses laser diffraction to determine the particle size. The equipment was zeroed with distilled water; then, the samples were placed directly into the recirculator. The parameters required for analysis were: a refractive index of 1.34 (verified according to the above-mentioned methodology) and spherical and transparent particles. The results are expressed as the mean of three independent readings.

*The Mean Particle Diameter, Polydispersity Index (PDI), and Zeta Potential.* The mean diameter and PDI were measured by dynamic light scattering with a Zetasizer analyzer. The samples were diluted in ultrapure water (500 times, v/v). The zeta potential was determined using electrophoresis, with samples diluted in 10 mM NaCl (500 times, v/v). The results are expressed as the mean of three readings from three different batches of suspensions.

*Determination of the Suspension pH.* The pH was determined in a potentiometer previously calibrated with pH 4.0 and 7.0 buffer solutions. The results are expressed as the mean of three readings from three different batches of suspensions.

**Structural Analysis.** *Fatty Acid Determination by Gas Chromatography.* The structural analyses of bulk CSO and P-CSO-NCs were performed in two steps by gas chromatography: extraction and derivatization of acylglycerols and then analysis of the fatty acid profile. For extraction and derivatization of acylglycerols, CSO was extracted from the nanocapsules and weighed in test tubes that were incubated at 50 °C for 15 min in a heating block. Afterward, 5 mL of acetonitrile and 5 g of NaCl were added, and the test tubes were centrifuged for 5 min. Subsequently, 2 mL of hexane was added, and homogenization was performed by vortexing for 1 min before centrifuging again for phase separation. Acylglycerols were derivatized according to the AOCS Ce 2-66 method,[16] with adaptations described by Chen et al.[17] The organic phase containing the acylglycerols was transferred to 2 mL microtubes and was dried at 40 °C with a nitrogen flow. For the CSO samples, 10 mg each of Span 80, Tween 80, and cetyl palmitate were weighed into 2 mL microtubes. For all samples, 100 $\mu$L of a methanolic solution of potassium hydroxide (2 M), 100 $\mu$L of internal standard C23:0 (methyl tricosanoate) at a concentration of 5 mg/mL, and 1 mL of hexane were added. Afterward, the tubes were vortexed vigorously for 1 min and centrifuged for 3 min at 13000 rpm for phase separation. Finally, the upper fraction containing the fatty acid methyl esters diluted in hexane was removed for further chromatographic analysis.

For analysis of the fatty acid profile, the fatty acid methyl esters were analyzed by injecting 1 $\mu$L into a gas chromatograph equipped with a flame ionization detector. The injector temperature was set to 250 °C in split mode with a split ratio of 30:1. The carrier gas was hydrogen, used at a constant pressure of 15 psi. The fatty acid methyl esters were separated in a ZBWAX capillary column (60 m × 0.25 mm; 0.25 $\mu$m film thickness). The initial column temperature was 50 °C for 0.5 min, which was increased to 180 °C at a rate of 15 °C/min, remaining at 180 °C but at an increased rate of 20 °C/min, and then to 230 °C at 10 °C/min, maintained for 4 min. The detector was kept at a constant 250 °C. The fatty acid methyl esters were identified by comparing analyte retention times with FAME Mix-37 standards. The results are expressed as mass of fatty acid/mass of sample by using internal standard PI and considering the carbon chain length and ester-to-acid factors via flame ionization detection.[18]

*Identification of Linoleic Acid in Nanocapsules by Nuclear Magnetic Resonance (NMR).* Proton ($^1$H) and carbon ($^{13}$C) NMR spectra were obtained using a Bruker DPX spectrometer operating at a frequency of 400 MHz. Chemical shifts ($\delta$) are expressed in parts per million (ppm) with respect to tetramethylsilane (TMS) and dimethyl sulfoxide (DMSO-$d_6$) as solvents, with multiplicity in parentheses (s = singlet, d = doublet, t = triplet, qua = quartet, chi = quintet, sex = sextet, m = multiplet, dd = double doublet, sl = long singlet); the number of hydrogens was deduced from the relative integral and the coupling constant (J) is expressed in hertz (Hz). The NMR analysis of P-NCs and P-CSO-NCs was performed by the spray-drying technique, with lactose as a drying adjuvant at a concentration of 10%, as adapted from Hoffmeister et al.[19] Lactose was previously dispersed in the nanocapsule suspensions and kept under magnetic stirring for 20 min. The parameters recorded in the drying equipment were as follows: inlet temperature, 100 °C; sample flow, 0.21 L/h; air flow, 4.5 L/min; blower control, 3.81 kgf/cm$^3$; and atomizing nozzle no. 2 (1 mm).

*Scanning Electron Microscopy (SEM).* SEM was used for morphological characterization of the nanocapsule suspensions. The samples subjected to microscopy were dried by the spray-drying technique.[19] Lactose or maltodextrin was used as a drying adjuvant at a concentration of 10%, according to a previously described methodology. After drying, the P-CSO-NC and P-NC samples (with lactose or maltodextrin) were prepared for microscopy. For this endeavor, the nanocapsule samples were placed on carbon tape and then covered with a thin layer of gold (necessary due to the composition's lack of a conductive character). A sample of the same formulation as the nanocapsule suspension was removed before spray drying and tested by placing two drops of this suspension containing the nanocapsules (P-NCs and P-CSO-NCs) on the carbon tape, which was incubated for approximately 50 min. Then, the remaining moisture was removed by vacuum spraying.

*Stability of the Formulations.* The thermal stability of the nanoformulations was assessed by monitoring several physicochemical parameters (the mean particle diameter, the PDI, the zeta potential, and pH) for 120 days, following the methods described above. In brief, P-NCs and P-CSO-NCs (in triplicate) were prepared and incubated at different temperatures protected from light: 4 ± 2 °C (refrigerated); 25 ± 2 °C (cabinet); and 40 ± 2 °C (climate chamber). Analyses were performed on the day of production ($t$ = 0) and then at 7, 15, 30, 60, 90, and 120 days. The results are expressed as the mean of measurements from three batches each day.

**Cell Culture.** African green monkey kidney fibroblasts (VERO, ATCC CCL-81) were grown in 75 cm$^2$ bottles in DMEM supplemented with 10% inactivated fetal bovine serum and 1% penicillin/streptomycin (100 IU/50 $\mu$g/mL) to prevent bacterial contamination. The culture bottles were incubated at 37 °C and 5% $CO_2$. Once confluent, the cells were seeded at a density of 1 × 10$^4$ cells/well in 96-well culture plates. Twenty-four hours later, after the cells had adhered, they were treated with the nanocapsule suspensions, in triplicate, on three different days. The concentrations for cytotoxicity analysis were 10, 20, 30, 50, 70, and 90 $\mu$g/mL. For the analysis of P-NCs, the volume used was equivalent to the 90 $\mu$g/mL concentration of P-CSO-NCs. For analysis of free CSO, a stock solution was prepared with 10 mg of CSO solubilized in 1 mL of absolute ethanol. Then, dilutions were prepared for the same concentrations described above, with a maximum ethanol concentration of 0.01%. The cells were incubated with P-NCs, P-CSO-NCs, or free CSO for 24 or 48 h, in two independent plates in a cell culture incubator at 37 °C and 5% $CO_2$. The positive control (cell death indicator) was 100 mM hydrogen peroxide ($H_2O_2$). For the survival control, wells contained only cells in culture


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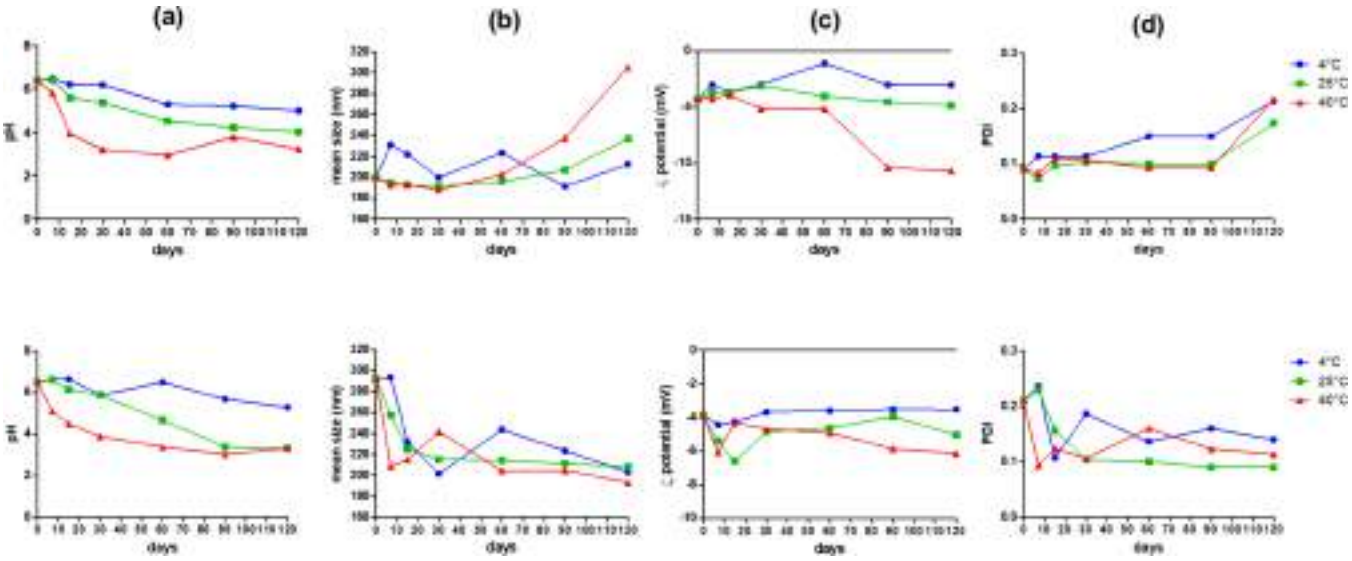



**Figure 1.** Physicochemical properties of the blank nanocapsules (P-NCs, top graphs) and the nanocapsules containing chia seed oil (P-CSO-NCs, bottom graphs): (a) pH, (b) mean diameter, (c) zeta potential, and (d) the polydispersity index—when stored at 4, 25, and 40 °C for 120 days. Each symbol represents three independent batches; $p < 0.05$.

medium. Ethanol was also a control due to its use to solubilize the free CSO.

*Assessment of Cytotoxicity.* After the incubation period, VERO cell viability was evaluated by the colorimetric method proposed by Mosmann.[20] In brief, 20 $\mu$L (5 mg/mL) of MTT was added to each well of the culture plate after incubation for 24 or 48 h. After reduction of MTT for 4 h by viable cell dehydrogenases, the supernatant was removed from the wells and the formazan crystals produced were solubilized in 200 $\mu$L of DMSO-$d_6$. The absorbance at 570 nm was measured using an enzyme-linked immunosorbent assay (ELISA) reader. The experiment was performed in triplicate, and the results are expressed as percentage viability using control cells (no treatment) as a comparison.[21]

**Statistical Analysis.** The data are expressed as the mean and standard deviation. They were subjected to the Shapiro−Wilk test to determine whether they followed a normal distribution. Statistical analyses for parametric data were performed by one-way analysis of variance (ANOVA), followed by Dunnett's complementary test. In the stability study, two-way ANOVA was applied, with temperature and time as the factors, followed by the Bonferroni test. For all tests, $p < 0.05$ was considered to be statistically significant. The data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

## RESULTS

The P-NCs and P-CSO-NCs had a particle size distribution in the nanometer: 198 ± 0.01 nm and 231 ± 0.02 nm, respectively. The mean diameter of the P-NCs was 205 ± 2.92 nm and the PDI was 0.08 ± 0.01. For the P-CSO-NCs, the mean diameter and PDI were 246 ± 1.53 nm and 0.148 ± 0.08, respectively. The initial pH was 6.40 ± 0.09 for the P-NCs and 6.51 ± 0.12 for the P-CSO-NCs, and the zeta potential was −4.31 ± 1.54 mV for the P-NCs and −3.84 ± 0.05 mV for the P-CSO-NCs. The pH of the P-NCs and P-CSO-NCs was similar, demonstrating that the addition of CSO did not influence the pH.

Figure 1 shows the measurements of the physicochemical properties over time. Analysis of pH, the zeta potential, and the PDI indicated a significant temperature × storage time interaction ($p < 0.0001$). The mean diameter of the P-NCs and P-CSO-NCs was similar over the 120-day period. The pH of the P-NCs changed over the 120-day storage period. There was an increase beginning at day 20 in the suspensions stored at 4 °C and a decrease starting from day 15 in those stored at 40 °C compared with those stored at 25 °C. For the P-CSO-NCs, the pH increased starting on day 60 when stored at 4 °C and decreased beginning on day 7 when stored at 40 °C compared with those stored at 25 °C, returning to the initial value on day 60. The zeta potential of the P-NCs decreased on day 60 when stored at 4 °C and increased from day 90 when stored at 40 °C compared with the suspensions stored at 25 °C. For the P-CSO-NCs, the zeta potential decreased on days 15 and 120 when stored at 4 °C; however, when stored at 40 °C, the zeta potential decreased on day 15 and increased on day 90 when compared with the suspensions stored at 25 °C. Finally, the P-NCs showed no change in the PDI, but for the P-CSO-NCs, the PDI increased on days 30 and 90 when stored at 4 °C, and on day 60 when stored at 40 °C.

From the chromatogram of the P-CSO-NCs (Figure 2a), linoleic acid was identified at 15.5 min, similarly to bulk CSO. Other fatty acids were also identified in the P-CSO-NC chromatogram, corresponding to the components of the nanocapsule formulation. Given that linoleic acid is also present in Span 80 (a component of the nanocapsule formulation), the quantification of this acid after CSO extraction from the nanocapsule was a result of derivatization of both CSO and the nanocapsule component. Therefore, it was not possible to quantify the linoleic acid present only in CSO extracted from the P-CSO-NCs. As can be seen in Figure 2b, several fatty acids were identified in the bulk CSO, including palmitic (C16), oleic (18:1, n-9), linoleic (C18:2, n-6) and linolenic (C18:3, n-6) acids at 11.5, 14.7, 15.5, and 17.0 min, respectively.

$^1$H and $^{13}$C NMR was used to assess the integrity of the molecules involved in the structure of the nanocapsules and to confirm the encapsulation of CSO (Figure 3). The $^1$H NMR spectrum of the bulk CSO shows characteristic fatty acid signals. A few differences can be seen in the displacements of some signals—some are of higher or lower intensity—and there are also overlapping signals. These differences are partly due to the structural similarity of the fatty acids present in the bulk CSO, but especially because it is a bioactive substance that has several constituents, mainly palmitic, linoleic, oleic, and linolenic acids


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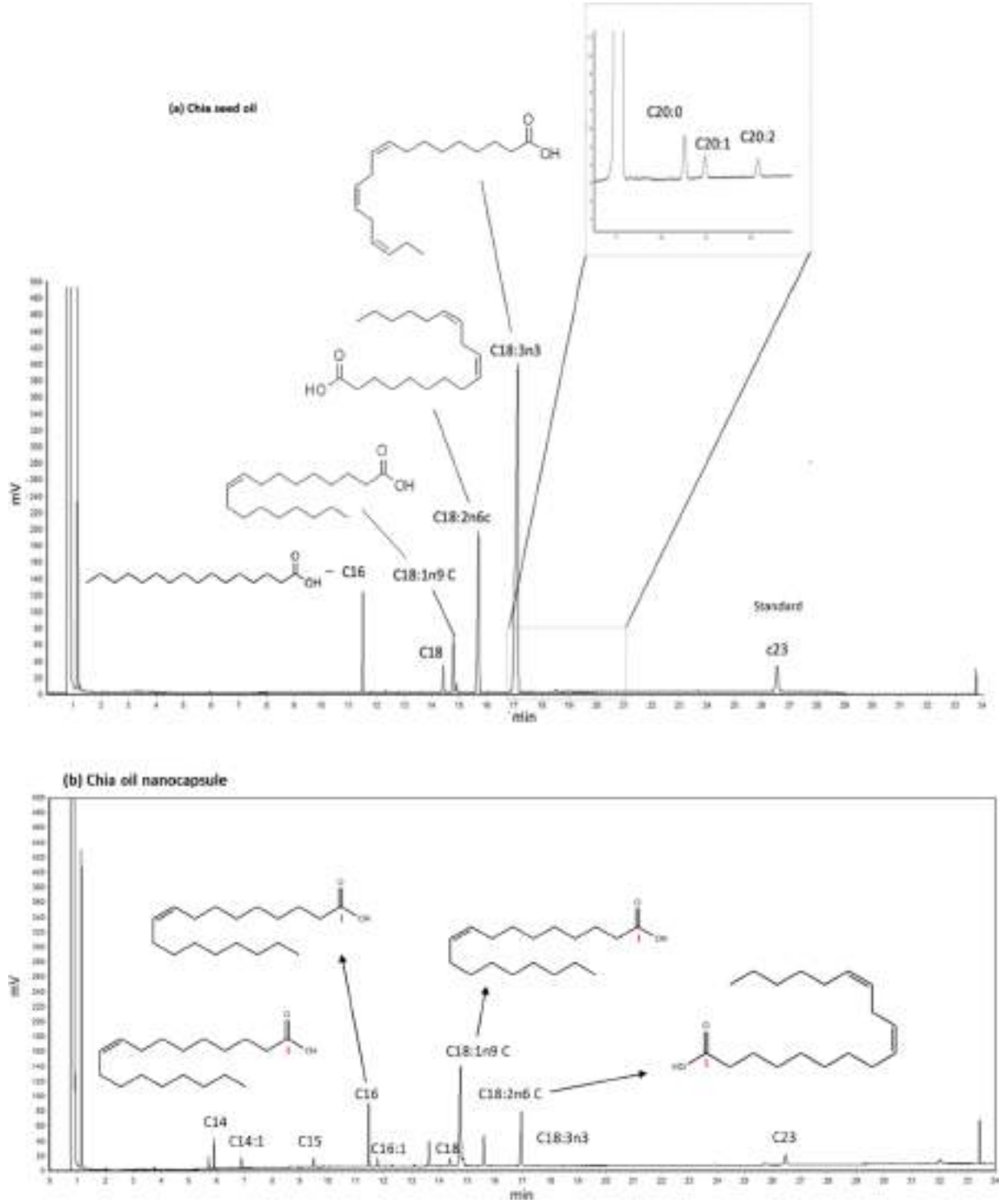



**Figure 2.** Chromatograms of (a) bulk chia seed oil and (b) the nanocapsules containing chia seed oil (P-CSO-NCs). Myristic (C14:0), palmitic (C16:0), oleic (C18:1, n-9), linoleic (C18:2, n-6), and $\alpha$-linolenic (C18:3, n-6) acids were identified.

(Figure 2a). The $^{13}$C NMR spectra (Figure 3b) confirmed the presence of other fatty acids with similar structures, corroborating the expected number of carbons and the variation in the shifts of some signals relative to linoleic acid. The greatest evidence of this is in the carbonyl region around 173 ppm, which presents two intense signals and two other less intense signals that are almost superimposed, which in the $^{13}$C NMR spectrum indicate carbons in position 1 of the structures (Figure 3a). There are also characteristic signs of sp$^2$ carbons, representing unsaturated fatty acid carbons (indicated by arrows) in the 127−132 ppm region, as well as sp$^3$ carbons in the 14−30 ppm region (Figure 3b). $^1$H and $^{13}$C NMR and the gas chromatography analyses produced similar and consistent results.

Spectroscopic analysis of the P-CSO-NCs was performed to characterize the encapsulation of CSO (Figure 4). For this endeavor, the spray-drying technique was used to dry the samples, using lactose as an adjuvant. The peaks correspond to the structure of Pluronic F-127, the polymer used in the P-CSO-NCs, in addition to lactose, which was used as an adjuvant for spray drying the samples. The characteristic fatty acid peaks of


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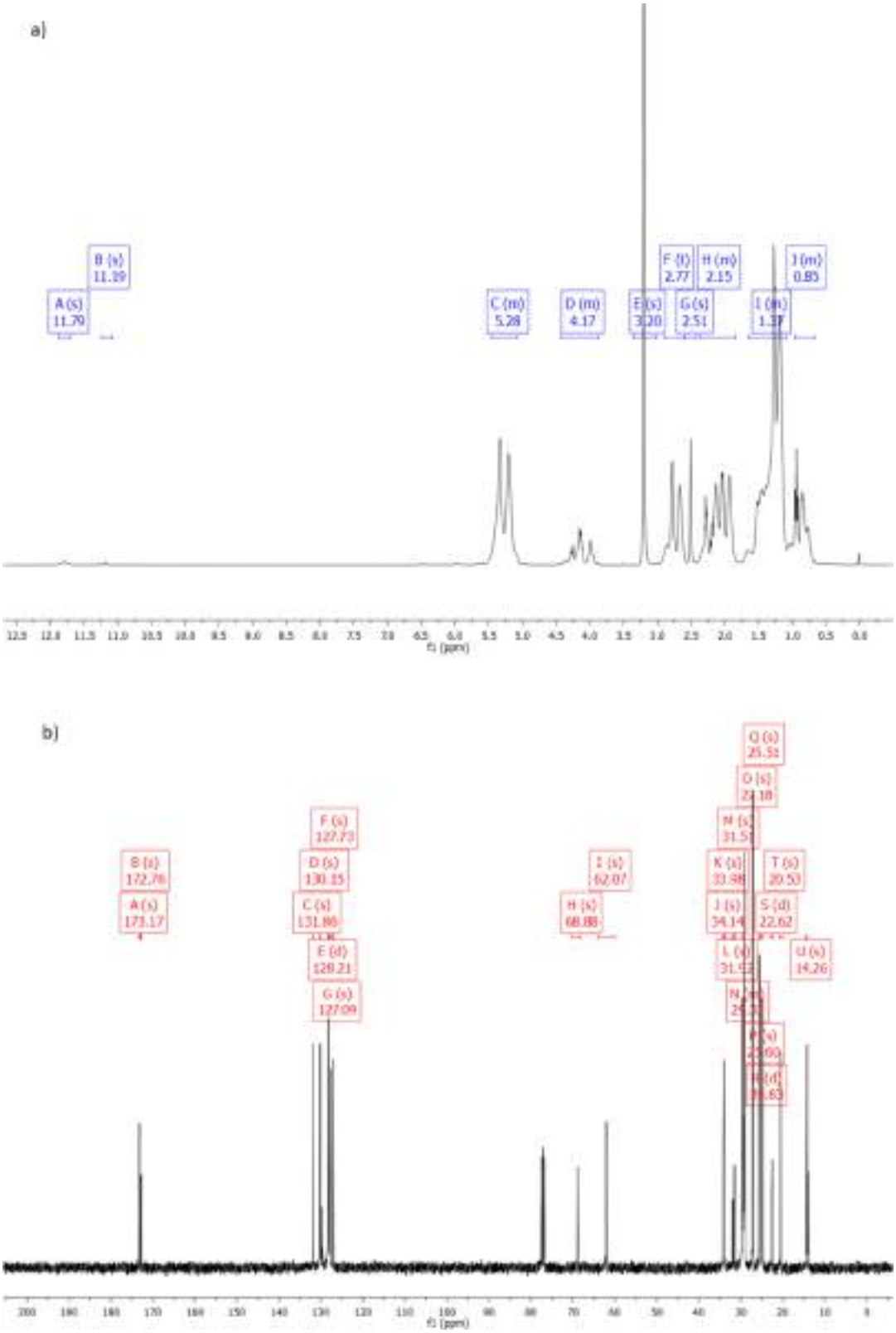



**Figure 3.** Identification of chia seed oil components by (a) proton ($^{1}$H) and (b) carbon ($^{13}$C) nuclear magnetic resonance. The spectra show the presence of the main fatty acids in the dry formulation.

CSO were not identified in the P-CSO-NCs. Considering the high sensitivity of NMR, these data indicate that CSO was encapsulated successfully.

Based on SEM, there was agglomeration of the P-NCs and P-CSO-NCs after spray drying using 10% lactose (Figure 5). The scanning electron micrographs also show an irregular surface to the nanoparticles. Considering the samples dried with lactose showed agglomerated particles, an alternative methodology was tested using 10% maltodextrin as a drying aid. In this case, there



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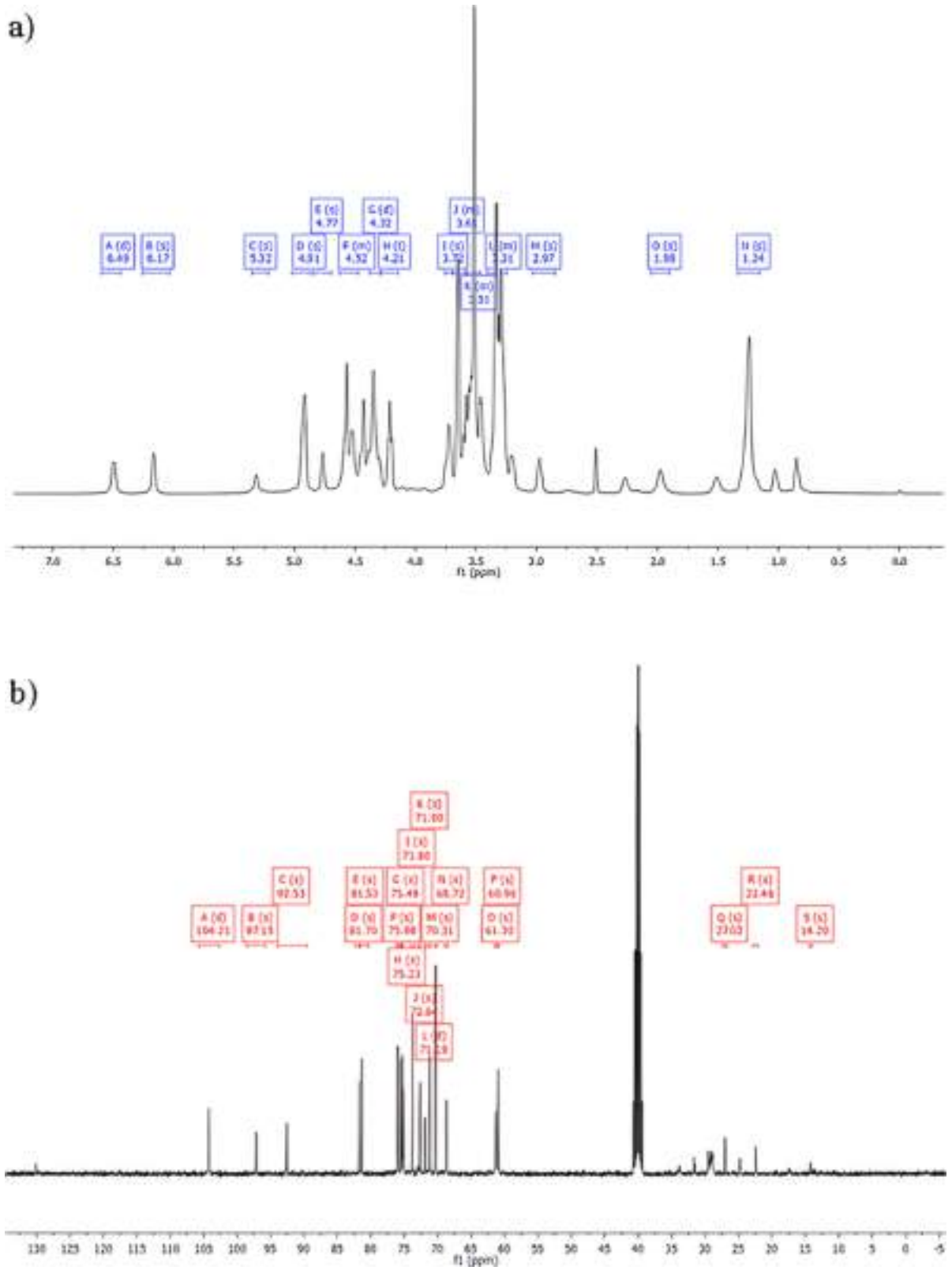



**Figure 4.** Identification of the components of the nanocapsules containing chia seed oil (P-CSO-NCs) by (a) proton ($^{1}$H) and (b) carbon ($^{13}$C) nuclear magnetic resonance. The spectra indicate the atoms present in the molecular structures of Pluronic F-127, a component of the nanocapsules, and lactose, which was used as an adjuvant for spray drying.

was still agglomeration of the P-NCs and P-CSO-NCs, but it was possible to verify their presence and a spherical shape (Figure 5).

Figure 6 shows scanning electron micrographs of the formulations dried using ambient air and vacuum aspiration (without an adjuvant). The particles had a nanometer size, similar to the laser diffraction (using a Zetasizer instrument) and light scattering (using a Microtrac instrument) results.



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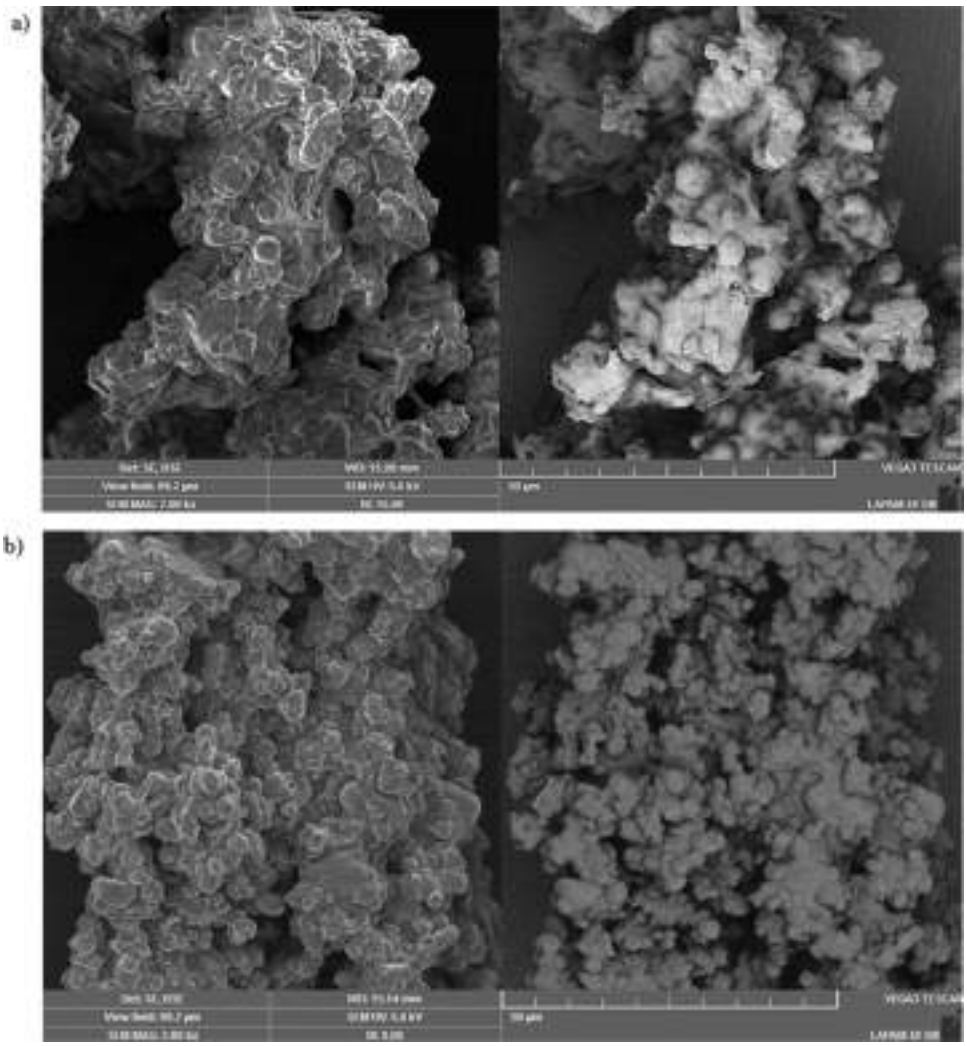



**Figure 5.** Representative scanning electron micrographs of (a) the blank nanocapsules (P-NCs) and (b) the nanocapsules containing chia seed oil (P-CSO-NCs) dried in the presence of 10% lactose (left images) and 10% maltodextrin (right images). The magnification is 2000×.

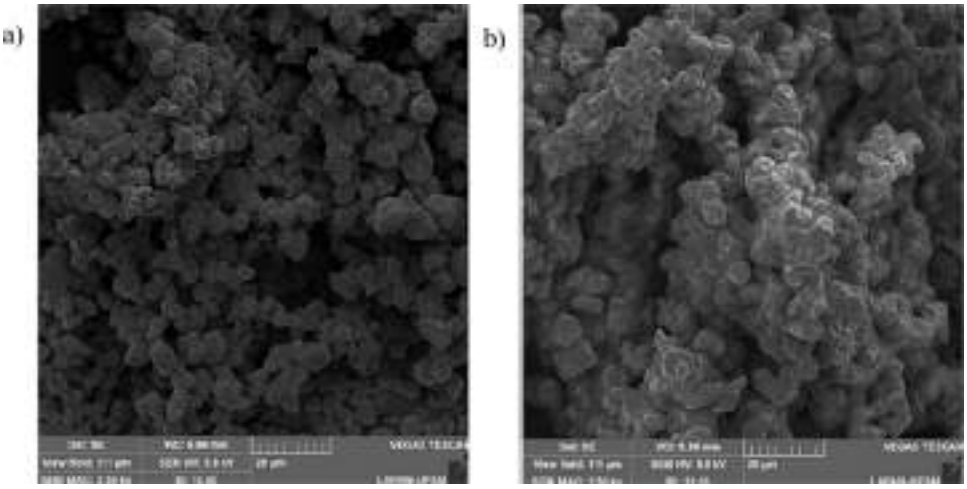



**Figure 6.** Representative scanning electron microscopes of (a) the blank nanocapsules (P-NCs) and (b) the nanocapsules containing chia seed oil (P-CSO-NCs) dried using ambient air and vacuum aspiration (without an adjuvant). The magnification is 2,500×.

As demonstrated in Figure 7, there were a small number of particles per field in the P-NCs and P-CSO-NCs. During preparation of the samples for microscopy, the particles could have adhered to the tape and thus been aspirated at the time of drying and vacuum aspiration.

Finally, the cytotoxicity of the P-NCs and P-CSO-NCs was assessed (Figure 8). For the analysis of P-NCs, the volume used



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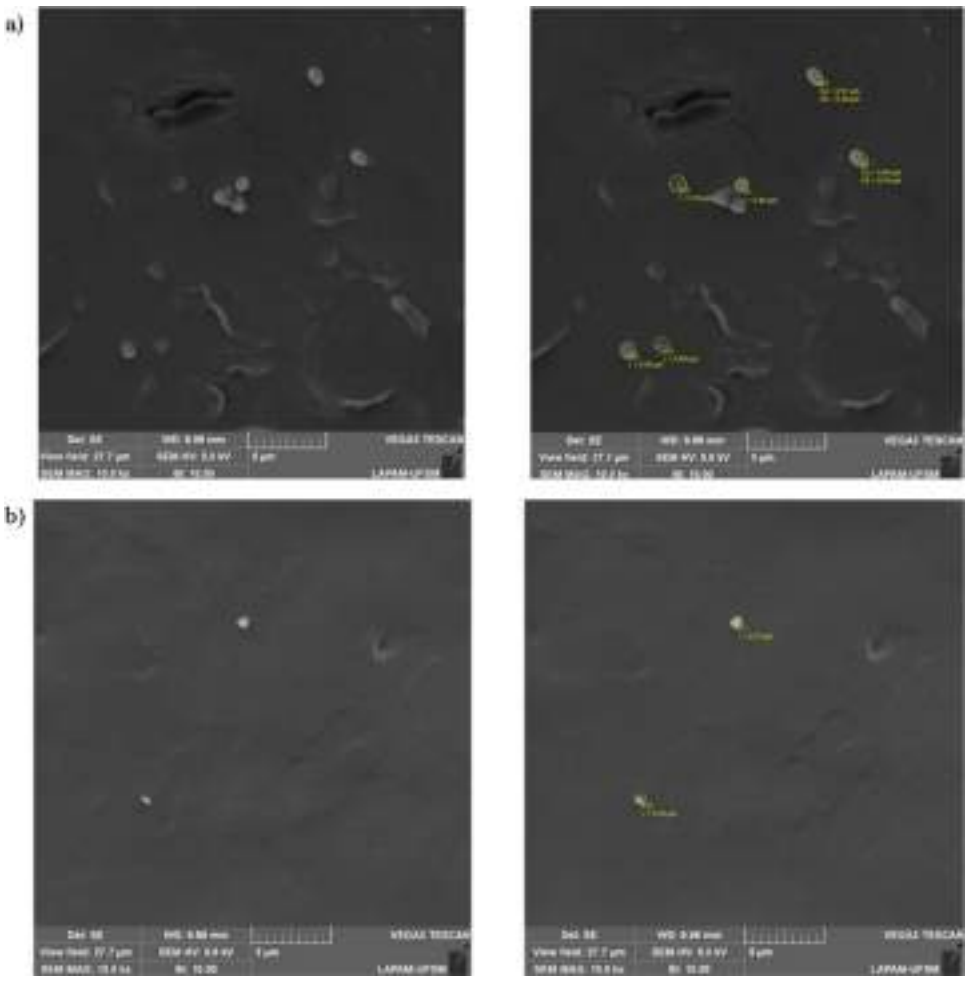



**Figure 7.** Representative scanning electron microscopes of (a) the blank nanocapsules (P-NCs) and (b) the nanocapsules containing chia seed oil (P-CSO-NCs) dried in ambient conditions without the presence of adjuvants. The magnification is 10,000×. Some particles are circled in yellow.

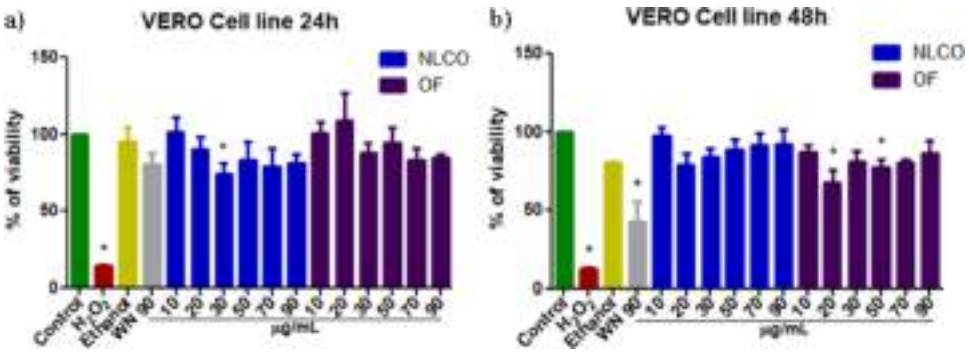



**Figure 8.** VERO cell viability (expressed as a percent relative to the control) after incubation with nanocapsules containing for chia seed oil (NLCO) or chia seed oil (OF) for (a) 24 h and (b) 48 h. Hydrogen peroxide ($H_2O_2$) used as a positive control. Ethanol used as a vehicle for the nanoformulations. WN 90 refers to blank nanocapsules at the highest volume equivalent to 90 $\mu$g/mL NLCO. The data are expressed as the mean ± standard deviation. *$p < 0.05$ compared to control (cells in culture medium without treatment).

was equivalent to the 90 $\mu$g/mL concentration of P-CSO-NCs. After incubation with VERO cells for 24 h, only 30 $\mu$g/mL of the P-CSO-NCs decreased viability compared with the control. The P-NCs, the other P-CSO-NC concentrations, and the free CSO were not toxic to the cells. After incubation for 48 h, the P-NCs decreased VERO cell viability. This effect could have been the result of a metabolic change rather than a reduction in the number of viable cells, as the VERO cells treated with the P-CSO-NCs at the same dose did not show the same response. The P-CSO-NCs were not toxic to the VERO cells at any concentration, whereas 20 and 50 $\mu$g/mL of the free CSO reduced VERO cell viability.

## DISCUSSION

Nanotechnology has become a very promising tool in the food industry as a supplementation strategy to improve nutrients. CSO contains abundant PUFAs, which are known to help prevent several diseases. Nanoencapsulation of PUFAs increases



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their bioavailability and reduces their oxidative degradation.[22] In the present work, the P-CSO-NCs presented a mean size of 231 ± 0.02 nm and a unimodal particle size distribution. This size is probably due to the presence of *in natura* oil in the formulation, which is different from the P-NCs that were produced with capric and caprylic acid triglycerides in the oily core. In the literature, differences regarding the particle diameter are often related to the nature of the nanoencapsulated oil and the viscosity, hydrophobicity, or surface tension of the materials used in the preparation of the nanocapsules.[23] Herein, a unimodal distribution and low PDI were observed, indicating the homogeneity of the nanoparticles, which is important for guaranteeing the formulation's stability. A PDI of 0.15−0.3 indicates homogeneity in the particle diameter and is a good indicator of stability,[24] corroborating the results found in this study with the two size analysis techniques (laser diffraction and dynamic light scattering). Orlistat, ibuprofen, and caffeine-loaded nanoparticles produced with Pluronic F-68 polymer resulted in nanocapsules with a mean size of 200 nm; they remained stable in an aqueous medium throughout the release process.[25]

Another relevant parameter is the zeta potential, which reflects the surface charge of a particle. The zeta potential is influenced by the particle's composition, the dispersing medium, pH, and ionic strength in the solution; a value that is greater than +30 mV or less than −30 mV indicates good colloidal stability in solution.[26] Nonionic polymers such as Pluronic F-127 tend to reduce the zeta potential, justifying the low zeta potential found in the present study. It is also known that the adsorption of steric stabilizers in the formulation decreases the zeta potential due to the change in the shear plane of the particles.[27] Moreover, in the case of nonionic surfactants, steric hindrance also increases the stability of colloidal dispersions.[28]

The pH of the suspension is another important characteristic to evaluate because its variation over time indicates the stability of the formulations.[29] The pH was similar for the P-NCs and P-CSO-NCs, demonstrating that CSO did not influence the pH. During the 120-day stability evaluation, the samples stored at 4 °C remained stable for 30 days but then deteriorated beginning at day 60. The samples stored at 25 °C changed after day 30, and those stored at 40 °C changed after day 7. The variation in pH as a function of time provides important information about the stability of nanostructures by ensuring the chemical stability of the polymer present in the nanocapsules.[30] However, a pH decrease of colloidal suspensions over a short period of time can be attributed to the ionization of carboxylic groups present in the polymer and/or its hydrolysis.[9] These results, despite indicating that the suspensions were slightly acidic, with a pH close to 7, are suitable for cutaneous administration, as the skin pH is 4−7.[31]

Gas chromatography indicated that the constituents of the bulk CSO and the P-CSO-NCs were similar to the CSO microparticles obtained by Souza et al.[32] and the bulk CSO analysis by Zanqui et al.[33] Span 80 has a high concentration of linoleic acid (C18:2, n-6), which may have influenced the quantification of this acid in the nanoformulation, making it difficult to quantify its presence in the P-CSO-NCs.

After drying the material for structural analysis, there were similar results to those reported by Lebhardt et al.,[34] who performed spray drying of polymeric nanoparticle suspensions using lactose as an adjuvant and observed that the particles agglomerated. Maltodextrin used as a drying adjuvant for passion fruit pulp showed good results compared with other adjuvants, as it reduced the moisture content of the sample and showed less deposition of the materials on the walls of the spray-drying equipment.[35] These samples showed a better size distribution and spherical morphological appearance, minimizing the effects of particle agglomeration in melatonin nanocapsules with lactose or maltodextrin.[19] The MTT assay was used to assess the cytotoxicity of the nanoformulations. Incubation with P-CSO-NCs for up to 48 h did not significantly reduce VERO cell viability compared with the control, demonstrating the safety of this nanoformulation.

Thus, the present study examined P-CSO-NCs prepared using Pluronic F-127 through nanoprecipitation. The formulation presented good characteristics in terms of particle size and PDI (<0.2). For *in natura* CSO, several fatty acids were identified by gas chromatography, mainly palmitic, oleic, linoleic, and linolenic acids. Other fatty acids were also identified from the chromatograms, corresponding to the components of the nanocapsule formulation. The $^{1}$H NMR spectrum of CSO presented the characteristic signals of its constituent fatty acids. The $^{13}$C NMR spectrum confirmed the presence of other fatty acids with similar structures, corroborating the expected number of carbons and the variation in the shifts of some signals in relation to linoleic acid, the standard and major product. SEM revealed that the particles had a nanometer dimeter, with results similar to the laser diffraction and dynamic light scattering techniques. The 120-day stability analysis indicated that 4 °C is the best temperature to store the nanocapsules. Finally, the P-NCs and P-CSO-NCs were not toxic to VERO cells. Therefore, the P-CSO-NCs are viable and stable, effectively protecting CSO from degradation. Despite the innovative nature of this study, additional investigations need to be carried out to confirm the safety and therapeutic potential of P-CSO-NCs.

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### Author Contributions

All authors contributed to the writing of the manuscript and reviewed and approved the final version.

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# Intake of Condensed Tannins (*Acacia mearnsii*) by Lambs in Confinement and Its Impact on Growth Performance, Rumen Environment, and Meat

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**Abstract:** This study determined whether adding condensed tannins to the diet of confined lamb improves performance, metabolism, health, and meat composition and quality. We used 24 Lacaune lambs with an average initial weight of 24.5 ± 0.24 kg. The animals were divided into 12 pens with two animals each. In the treatment group, a black wattle extract was added to the lamb concentrate (2.5 g/kg dry matter), while the control group received only the basal diet (without extract). Black wattle contained 98.6 g/kg of condensed tannin. There was no difference between the performance variables, which included weight gain, feed intake, and feed efficiency. However, the group fed on condensed tannins showed higher carcass yield and a higher percentage of linked intramuscular fat. In meat, a lower proportion of saturated fatty acids combined with higher concentrations of unsaturated fatty acids was observed in lambs of the condensed tannin group. The polyunsaturated fatty acids (PUFA) were no different; however, a higher PUFA/SFA ratio was observed in animals with condensed tannin intake, and a higher proportion of ω6 and ω3 was observed in the meat of these animals. The meat's protein and ash content did not differ between treatments, as did the meat's water retention capacity, color, and luminosity. Furthermore, animals that fed on condensed tannins had lower cholesterol levels and higher globulin content. The values of erythrogram variables were lower in lambs that consumed condensed tannins. More significant ruminal bacterial activity was also observed in the group receiving condensed tannins. The antioxidant effect was verified with lower thiobarbituric acid reactive species (TBARS) levels and more significant antioxidant enzyme activity. We conclude that adding condensed tannins improves carcass yield and increases the percentage of fat in the carcass, improving the fatty acid profile, which may be linked to the antioxidant effect, in addition to the health benefits of the consumer of this product.

**Keywords:** natural extracts; ruminants; antioxidant; fatty acids

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## 1. Introduction

The black wattle (*Acacia mearnsii*) is native to southeastern Australia and Tasmania and has been introduced to various regions, including the Americas. Its extract has shown

great potential as a feed additive in ruminant nutrition due to polyflavonoids, phenolic compounds, amino acids, carbohydrates, and other sugars [1].

Polyphenols are phenolic components of the natural organic compounds family [2]. Tannins (condensed and hydrolysable) are secondary plant metabolites composed of phenolic substances. When added in concentrations above 5% of condensed tannins in animal total diets, these compounds can negatively impact consumption [3,4]; however, in low concentrations, they have beneficial effects such as parasite control, reduced enteric methane production, and increased flow and absorption of protein in the intestine [4–6]. These compounds also have antioxidant action on animal fluids and tissues. Among the main compounds involved, we can mention phenolic acids, flavonoids, and tannins, which act to eliminate free radicals [7,8].

Polyphenols generally act on the rumen microbiota and can affect carbohydrate fermentation, protein degradation, and lipid metabolism [9]. Among them are tannins, which include condensed tannins with a lower risk of toxicity [10], but may be more toxic to ruminants than hydrolyzable tannins [11]. These compounds form complexes with dietary protein and are less degraded in the rumen [12], resulting in greater intestinal absorption, improved utilization, and reduced nitrogen excretion. Condensed tannins (CT) can limit the ruminal biohydrogenation of unsaturated fats in the diet during digestion, promoting changes in fermentative processes and acting on groups of bacteria, causing changes in the flow of fatty acids to tissues [13]. With all these properties and the benefits that condensed tannins can offer, they are seen as potential substitutes for commonly used additives, such as antibiotics and performance enhancers, which are being questioned due to their mode of action.

We tested the hypothesis that lower doses of CT in the diet of confined lambs promote changes in the ruminal biohydrogenation process, increasing the concentrations of unsaturated fatty acids deposited in the meat, reducing oxidative stress in tissues, and maintaining animal performance without impacting feed intake. The objective was to evaluate whether the addition of CT (black wattle extract) in the diet of confined lambs would have a positive effect on weight gain performance, feed intake, and fatty acid profile changes in ruminal fluid and meat, in addition to enhancing the antioxidant system and reducing oxidative stress.

## 2. Materials and Methods

### *2.1. Additive*

We used the commercial product (735 g/kg minimum guarantee of CT; NutreSet PNT, SETA S.A., Estancia Velha, RS, Brazil), a natural antioxidant based on CT, as well as other polyflavonoids, together with other phenolic components and complex mixtures of carbohydrate gums, sugars and amino acids extracted and purified from the bark of black wattle extract (*Acacia mearnsii*). The product is obtained from the secondary metabolism of plants and added to the lambs' diet at a dose of 2.5 g/kg dry matter, with an active ingredient of 111.13 g/kg DM of gallic acid equivalent. Following the manufacturer's recommendation, the concentration of condensed tannin in the diet is similar to that of another recently published study with tannin for lambs [14].

### *2.2. Animals, Experiment Site, and Diets*

The study was conducted in the ruminants sector of the UDESC Experimental Farm (FECEO), located in Guatambu/SC. Twenty-four Lacaune lambs were used with an average age of 70 ± 2.5 days and an average initial weight of 24.5 ± 0.24 kg. They were accommodated in stalls (1.2 cm × 2.5 m) equipped with feeders and drinkers with a float for controlling water flow.

### *2.3. Experimental Diets and Feed Intake*

The experiment lasted 89 days (82 days experimental period + seven adaptation days), during which the animals were divided into two groups, with six repetitions per group

and a total of two animals per repetition/pen, being considered an adaptation period of for environment and feed [15] due to the animals already receiving a similar diet, adapting only to the additive. Animals in the control group received a basal diet. In contrast, animals in the treated group and the basal diet received the polyphenol-based additive at 2.5 g/kg of dry matter. Powder concentrate was used, and additives were added during its production, carried out at 15-day intervals.

The diet was formulated according to the nutritional requirements of the animals [16], considering the feeds of concentrate and corn silage, provided at different times in the feeders; that is, first, the concentrate was provided, and after consumption was finished (30 min), silage was supplied. It is important to note that the animals consumed all the concentrate provided throughout the experiment, meaning that the additive was consumed in full. Feed was divided into two daily meals, at 8:00 a.m. and 4:00 p.m., while water was provided ad libitum.

The amount of feed (dry matter) provided was 4.5% of live weight, with a diet in the concentrate:silage ratio of 55:45, respectively. The volume of feed provided was adjusted at 15-day intervals based on the body weight of the animals; however, the feeder was monitored in order to have a 2–3% surplus of silage. The chemical composition of the feedstuffs used in the diet was measured according to the technique described below, and the results are shown in Table 1.

**Table 1.** Chemical composition of corn silage and basal concentrate used.

| Items [1] | Corn Silage | Control Concentrate [2] | Treatment Concentrate [2] |
|---|---|---|---|
| Dry matter (DM), % | 28.8 | 89.4 | 89.5 |
| Crude protein (CP), % DM | 9.59 | 18.0 | 19.2 |
| EE, % DM | 3.06 | 3.90 | 3.88 |
| NDF, % DM | 34.8 | 18.3 | 19.5 |
| ADF, % DM | 16.7 | 6.18 | 6.33 |
| Ash, % DM | 4.42 | 10.6 | 9.75 |
| Fatty acid (%) | | | |
| C12:0 (Lauric) | 0.25 | 0.008 | 0.008 |
| C14:0 (Myristic) | 0.24 | 0.05 | 0.05 |
| C14:1 (Myristoleic) | 0.01 | - | - |
| C15:0 (Pentadecanoic) | 0.05 | 0.02 | 0.02 |
| C16:0 (Palmitic) | 11.1 | 8.79 | 8.81 |
| C16:1 (Palmitoleic) | 0.38 | 0.10 | 0.10 |
| C17:0 (Heptadecanoic) | 0.28 | 0.05 | 0.06 |
| C18:0 (Stearic) | 3.47 | 2.16 | 2.15 |
| C18:1n9c (Oleic) | 13.3 | 14.5 | 14.6 |
| C18:2n6c (Linoleic) | 17.1 | 22.8 | 22.2 |
| C20:0 (Arachidic) | 0.43 | 0.24 | 0.23 |
| C20:1n9 (cis-11-Eicosenoic) | - | 0.18 | 0.17 |
| C18:3n3 (a-Linolenic) | 5.00 | 1.03 | 1.01 |
| C20:2 (cis-11.14-Eicosadienoic) | - | 0.02 | 0.02 |
| C22:0 (Behenic) | 0.50 | 0.15 | 0.16 |
| C22:1n9 (Erucic) | 0.04 | 0.03 | 0.04 |
| C20:4n6 (Arachidonic) | 0.07 | 0.06 | 0.07 |
| C24:0 (Lignoceric) | 0.63 | 0.16 | 0.15 |
| Σ SFA | 17.0 | 11.6 | 11.6 |
| Σ MUFA | 13.8 | 14.8 | 14.7 |
| Σ PUFA | 22.2 | 23.6 | 23.7 |

[1] DM (dry matter), CP (crude protein), EE (ether extract), NDF (neutral detergent fiber), ADF (acid detergent fiber), and Ash. [2] Ingredients present in 100 kg of concentrate: ground corn (550 g/kg), soybean bran (255 g/kg), wheat bran (155 g/kg), calcite limestone (10 g/kg) and mineral and vitamin premix (30 g/kg). Concentration of mineral and vitamin premix (concentration/kg): Ca min. 175 max. 245 g; P min. 40 g; S min. 25 g; Mg min. 12 g; At max. 100 g; Ash max. 980 g; with min. 40 mg; Fe min. 1800 mg; I min. 80 mg; Mn me. 1400 mg; If min. 12 mg; Zn min. 4000 mg; Vit A min. 300,000 IU; Vit D3 min 60,000 IU. Vit E min. 600 IU; F max. 600 mg; Monensin sodium 600 mg.

### *2.4. Body Weight and Sample Collection*

Lambs were weighed individually every 15 days. All weighings were carried out in the morning, with the lambs fasting for 12 h, using a digital electronic bar scale (DIGITRON®, UL-300, Nunawading, Australia). Fasting was only carried out 12 h prior to the day of data and sample collection (nighttime); on the other days, the animals remained with the silage. Daily mean intake (DMI) was measured daily in the morning, and leftovers were removed and weighed per stall. The average daily gain (ADG) was calculated as the regression coefficient of individual live weight on time [17]. Feed efficiency (ADG/DMI) was calculated.

Blood collections were carried out on days 0, 30, 65, and 81 (before feeding). The tubes containing an anticoagulant (EDTA 10%) were used for the complete blood count, and tubes without an anticoagulant were used for serum preparation. After collection, all these samples were transported to the laboratory (styrofoam box with ice), and tubes without an anticoagulant were centrifuged at 7000 rpm for ten minutes to separate the serum. Then, a serum fraction was stored in 1.8-mL microtubes (Eppendorf, Hamburg, Germany) at −15 °C.

On days 30, 65, and 81 of the experiment, 100 mL of ruminal fluid per animal was collected two hours after morning feeding (the first 50 mL were discarded, and the rest of the collected material was used for analysis) with an esophageal probe coupled to a suction motor. The collected content was placed in tubes to measure pH and the time of functional activity of the rumen microbiota was measured using the Methylene blue reduction test (MBRT), described by [18]. A subsample of ruminal fluid was reserved for the determination of SCFAs. These samples after collected were immediately filtered and submitted to freezing to avoid SCFAs degradation.

All animals were slaughtered one day after the trial period ended (day 82) at a slaughterhouse specific for small ruminants (Guatapará in Guatambu—SC), following current regulations and supervised by a veterinarian. Muscle pH was measured after the stunning, bleeding, skin removal, and evisceration procedures. The hot carcasses were weighed, and after 24 h, the weighing was carried out again to obtain the cold carcass weight and a new pH measurement. The carcasses were cooled in the slaughterhouse's cold chambers (between −2 and 0 °C). After 24 h of slaughter, the quadriceps femoris muscle (left limb) was removed and used for meat analyses.

### *2.5. Laboratory Analysis*

#### 2.5.1. Determination of Total Phenolic Compounds (TPC), Antioxidant Activity and Tannins in the Additive

For extraction, 0.5 g of the sample was used, and 50 mL of distilled water was added. The mixture was placed in an ultrasound bath (70 W) for three hours and left to rest in the dark for another three hours. The supernatant was filtered with quantitative filter paper (Whatman n° 40) and stored in a 100 mL volumetric flask wrapped in aluminum foil. Immediately after extraction, the extracts were stored in Eppendorf tubes and kept frozen at −83 °C, if necessary, and not for more than 24 h, until use [19].

Total phenolic compound quantification was performed using the Folin–Ciocalteu colorimetric method [20]. An aliquot of each diluted sample was mixed with 0.5 mL of Folin–Ciocalteu and stirred for one minute; 2 mL of sodium carbonate (20%) was added to the mixture and stirred for 30 s. After incubation for two hours in the dark, the absorbance at 750 nm was read relative to the prepared blank. The standard curve was prepared by solutions of gallic acid in methanol. The concentration of TPC in the extracts was determined in gallic acid equivalents using an equation obtained from the standard gallic acid graph and expressed in mg of gallic acid equivalent per 100 g of dry sample (mg EGA·g$^{-1}$). Data were presented as the mean of analyses ± SD of triplicates [21].

The antioxidant activity was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, by the scavenging of the 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical (ABTS) with measurements in triplicate. The assay of DPPH radical scavenging

was performed according to the literature [22], with modifications. In methanol, 0.1 mL of the sample was mixed with 3.9 mL of 0.06 mM DPPH solution. In the control solution, methanol was used in place of the sample. The mixtures were incubated at 25 °C for one hour in the dark. The absorbance reading was performed at 515 nm, with methanol to calibrate the equipment. Quantitation was performed based on a standard curve of DPPH in methanol (at concentrations of 0 µM, 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, and 60 µM) using the linear equation y = 0.0104x + 0.0248 ($R^2$ = 0.9919), where the *y*-axis is absorbance and *x*-axis is DPPH concentration.

The ABTS radical scavenging assay was performed according to the literature [23]. A stock solution of 7 mM ABTS was prepared. The ABTS working solution was prepared by mixing the ABTS stock solution with the 2.45 mM $K_2S_2O_8$ solution in a ratio of 1:1 (*v*/*v*). 20 µL of the sample was mixed with 980 µL of ABTS working solution. The absorbance of the mixture was measured in a spectrophotometer at 734 nm after six min of incubation in the absence of light. Quantitation was performed using a standard curve of Trolox in ethanol (at concentrations of 100 µM, 500 µM, 750 µM, 1000 µM, 1500 µM, and 2000 µM), using the linear equation y = −0.000289x + 0.53447 ($R^2$ = 0.9176), where the *y*-axis is absorbance and *x*-axis is Trolox concentration.

Total tannins were quantified by the Folin–Ciocalteau method. The tannin extract was carried out with aqueous acetone (70%, *v*/*v*). Five replicates of tannin extract were analyzed. For total tannins, standard analysis was used, whereas CT concentration was calculated as a leucocyanidin equivalent through the equation: [Abs 550 nm × 78.26 × dilution factor]/% DM [24].

#### 2.5.2. Chemical Composition Analysis

In the laboratory, the feed samples were pre-dried in a forced ventilation oven at 55 °C for 72 h, then removed from the oven and weighed again to determine the partial dry matter content, followed by grinding in a Wiley mill (Marconi, model: MA340), using a 1 mm mesh sieve. The pre-dried and ground samples were heated at 105 °C for 24 h to determine dry matter (DM) and ashed in a muffle furnace at 600 °C [25]. The micro-Kjeldahl method determined the N content (Method 984.13 [26]), which allowed predicting the crude protein (CP, method 976.05) content through mathematical calculation (N × 6.25 = CP). To determine the neutral detergent fiber (NDF) content, the samples were placed in polyester bags [27] and treated with a neutral detergent solution in an autoclave at 110 °C for 40 min [28]. For concentrate samples, we included α-amylase [29]. Acid detergent fiber (ADF) concentrations were determined according to AOAC [26], method 973.18). The values of starch, carbohydrates, and ether extract were obtained using a Near Infrared Reflectance Spectrometer, model Spectra Star 2600 XT series of Near Infrared Analyzers (Unity Scientific®, Brookfield, WI, USA). The results are presented in Table 1.

Fatty acids from feed samples (silage and concentrate) were extracted by the method of [30] using a quantity of 1.5 g of sample, which had been pre-dried (oven 60 °C, 72 h), for stabilization and the possibility of grinding. The amount of water in the extraction method for food samples was 1.5 mL. Other procedures for feed analysis were similar to those performed on meat, as will be described below (Section 2.6).

#### 2.5.3. Hemogram and Serum Biochemistry

Erythrocyte and total leukocyte counts, hemoglobin concentration, hematocrit percentage, and leukocyte differentials were performed immediately upon sample arrival at the laboratory using an automatic hematologic analyzer (three parts EQUIP VET 3000®, Pleasanton, CA, USA).

Serum levels of total protein, albumin, cholesterol, triglyceride, glucose, and urea were evaluated in serum using commercial kits (Analisa, Belo Horizonte, MG, Brazil) and semi-automatic equipment (BIO PLUS 2000®, Barueri, SP, Brazil), following manufacturers' recommendations. Globulin levels were obtained using globulin = total proteins − albumin [31,32].

#### 2.5.4. Oxidant Status

The meat (0.5 g) was weighed and allocated into test tubes. The tissue was homogenized separately in 10 mmol Tris–HCl buffer, pH 7.2 to verify the oxidative status variables. The samples were stored in tubes and frozen at −20 °C until analyzed. All the procedures described above were performed with refrigerated temperature (4 °C).

Lipid peroxidation was measured as the amount of TBARS, according to literature for serum [33] and for meat [34]. The reaction was read in a spectrophotometer Kasuaki IL-593-S at 535 nm (serum) and 532 nm (meat). The results were expressed as nmol of malondialdehyde (MDA)/mL of serum or homogenate [35].

Serum and meat glutathione S-transferase (GST) activity was measured based on researchers [36] and expressed as μmol 1-Chloro-2,4-dinitrobenzene (CDNB)/min/mg protein. According to the literature [37], protein thiols were measured, and the results were expressed as nmol thiols/mg of protein.

Superoxide dismutase activity measurement in meat homogenate was based on the inhibition of $O_2$ reaction with adrenalin as described by researchers [38]. A unit of SOD is defined as the amount of enzyme that inhibits by 50% the speed of adrenalin oxidation. It leads to the formation of the red-colored product, adrenochrome, which is detected by a spectrophotometer. SOD activity is determined by measuring the speed of adrenochrome formation, observed at 480 nm, in a reaction medium containing 50 mM glicine–NaOH, pH 10, and 1 mM adrenalin. The results were expressed as UI SOD per milligram protein.

#### 2.5.5. Ruminal Volatile Fatty Acids

Immediately after collecting the rumen fluid, the pH was measured using a digital pH meter (KASVI®, K39-1420, Pinhais, Brazil) while still on site. Immediately after collection, the functional activity of the rumen microbiota was analyzed using the methylene blue reduction test (MBRT), described by researchers [18]. For the determination of short-chain fatty acids, the rumen fluid samples were thawed to 5 °C and manually agitated to homogenize them. One milliliter of aliquots of the supernatant from rumen fluid samples were collected in polypropylene microtubes (2 mL) and centrifuged for five min (12,300× *g*). After that, 250 μL of the supernatant was removed and transferred to a new microtube containing 250 μL of formic acid. The mixture was manually shaken and centrifuged again for three minutes. Then, 250 μL of the supernatant of the mixture was collected into an injection vial, and 500 μL of 3-octanol solution (665 μg $mL^{-1}$ in methanol) was used as an internal standard and homogenized. The samples were analyzed in a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3600, Palo Alto, CA, USA) and an autosampler (Varian 8200CX, Palo Alto, CA, USA); 1 μL of the extract was injected in split mode at 1:10. The carrier gas used was hydrogen at a constant pressure of 20 psi. The analytes (acetic, propionic, butyric, valeric, and isovaleric acids) were separated by a CP-WAX 52CB capillary column (60 m × 0.25 mm; 0.25 μm stationary phase thickness). The initial column temperature was set at 80 °C for one minute and increased to 120 °C at 8 °C $min^{-1}$, then up to 230 °C by 15 °C $min^{-1}$, where it remained for one min. Injector and detector temperatures were set at 250 °C. The validation of the method included the following parameters: selectivity, linearity, linear range, repeatability, precision, limit of detection (LOD), and limit of quantification (LOQ) for acetic, propionic, butyric, valeric, and isovaleric acids. Analytical parameters are shown in Table S1. Linearity was evaluated by calculating a regression equation using the least squares method. LOD and LOQ values were achieved by sequential dilutions up to signal-to-noise ratios of 3:1 and 6:1, respectively. Precision was assessed by analyzing the repeatability of six replicate samples. Accuracy was determined by recovering known amounts of standard substances added to a diluted sample. The results were expressed as mmol $L^{-1}$ of each SCFA in rumen fluid.

### *2.6. Meat*

The meat was cooled at 6 °C for 24 h, after which a cut in half (separating into two 2 cm steaks each) was made to measure pH using a portable penetration electrode (HANNA, HI

99163, Smithfield, RI, USA). In this cut of meat, the color of the meat on the surface was also evaluated in four different locations (the average was determined for data analysis), using a CR-400 chromometer (Minolka, Osaka, Japan); CIE1976 L* a* b*, with a D65 standard illuminant, compared to a CIE 1931 standard observer (x2λ, y λ, z λ), to a calibration plate (number 1849-701). They are defined as lightness (L*), redness (a*) and yellowness (b*), measured at three random sample points. In another portion of meat, we evaluated the water holding capacity (WHC) using compression according to the Hamm methodology, adapted by the researchers [39]. A total of 5 g of meat was used, a sample without ribs, connective tissue and fat. This meat was placed between two sheets of filter paper, then compressed for 5 min with a weight of 2.5 kg. This meat sample was then weighed to assess how much water was lost. The WHC was then calculated, and the results were presented as a percentage.

The extraction of fatty methyl esters was performed using Bligh and Dyer [30] method with some modifications: 1.5 g of meat samples, 0.5 mL of water, 5 mL of methanol, and 2.5 mL of chloroform were added to a 15-mL polypropylene tube, and mechanical shaking was performed for 60 min. After that, 2.5 mL of chloroform and $Na_2SO_4$ 1.5% solution were added to promote a biphasic system. This mixture was shaken for two minutes and then centrifuged for 15 min at 2000 rpm. Lipids obtained from the chloroform phase were subjected to fatty acid analysis.

Fatty acid methylation was performed using a transesterification method proposed by [40]. The extracted lipids were added to one milliliter of 0.4 M KOH methanolic solution in a test tube and shaken in a vortex for one minute. Samples were kept in a water bath for ten minutes at boiling point. Subsequently, they were cooled at room temperature, and 3 mL of 1 M $H_2SO_4$ methanolic solution was added, shaken in a vortex, and maintained in a water bath for ten minutes. After cooling, 2 mL of hexane was added and centrifuged at 2000 rpm for ten min. Lastly, hexane with the fatty acid methyl esters (FAME) was subjected to chromatography analysis.

For the FAME determination, a gas chromatograph model TRACE 1310 was equipped with a flame ionization detector (Thermo Scientific, Waltham, MA, USA). One microliter of samples was injected in a splitless injector, operated in 1:10 ratio split mode at 250 °C. Hydrogen was used as carrier gas at a constant flow rate of 1.5 mL/min. FAME separation was performed using an RT 2560 (100 m × 0.25 mm × 0.20 µm of thickness film, Restek, Bellefonte, PA, USA) chromatography column. The initial oven temperature was programmed at 100 °C for five min and increased to 180 °C at an 8 °C/min rate. Then, it increases to 210 °C at 4 °C/min, and finally, until 240 °C, it increases to 20 °C/min, and it is maintained for 20 min in isothermal. The detector temperature was kept constant at 250 °C. The FAME compounds were identified by comparison of experimental retention time with those from authentic standards (FAME Mix-37, Sigma Aldrich, St. Louis, MO, USA). The results were presented as a percentage of each fatty acid identified in the lipid fraction, considering the chain size equivalent factor of FAME for FID and conversion factor of ester to the respective acid, according to the literature [41].

### *2.7. Statistical Analysis*

The experimental data were first analyzed descriptively, and measures of central tendency (median) and data dispersion (range that stands for the interval between the minimum and maximum values in the data) were computed. Further, all variables were subjected to the Shapiro–Wilk W-test, which revealed a normal data distribution. Skewness, kurtosis, and homogeneity were evaluated using the Levene test, and linearity was used using linear regression. All data were analyzed immediately using the PROC MIXED procedure (SAS Inst. Inc., Cary, NC, USA; version 9.4), with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed effects test. Weight gain, ADG, feed efficiency, and feed conversion were tested for treatment fixed effects using a pen (treatment) and animal (pen) as random variables (i.e., pen within treatment and animal within pen). Body weight and intake feed were tested for fixed effects of treatment,

day, and treatment × day; and pen (treatment) as random variables. All other variables (blood and ruminal liquid) were analyzed as repeated measurements and tested for fixed effects of treatment, day, and treatment × day, and pen (treatment) and animal (pen) as random variables. Post-mortem variables analysis (meat, carcass, and fatty acid profile) was tested using animal (treatment) as random variables for treatment fixed effects. All results obtained on the first day for each variable were also included as covariates; however, the covariate was removed from the model when $p > 0.05$. Means were separated using PDIFF (student test), and all results were reported as LSMEANS followed by standard error. Significance was defined when $p \leq 0.05$ and tendency when $p > 0.05$ and $p \leq 0.10$.

We calculated the post hoc power for the groups, considering body weight as output since it is one of the main variables of importance in adding performance-enhancing additives. We use the mean of each group and its SD with an alpha of 0.05, and we finish with a reasonable power of 86.4%.

## 3. Results

### *3.1. Additive*

This methodology verified that the TPC concentration is 2534.7 ± 111.13 mg GAE/g DM. The activity was 0.27 ± 0.02 EC50, µg/mL, i.e., the result was expressed as EC50, which is the sample concentration required for DPPH radical scavenging by 50%. The concentration was 10514.02 ± 1099.53 µM Trolox/g of the sample, with 87.8 ± 10.5% inhibition. We detected 986 ± 2.4 g tannin per kg additive (fresh material) used in the experiment.

### *3.2. Growth Performance and Feed Intake*

The growth performance results are presented in Table 2 and Figure S1. We observed no treatment effect on ADG, average weight gain, average daily weight gain, total feed intake, and feed efficiency. There was also no treatment effect on concentrate and silage intake, when evaluated separately.

**Table 2.** Growth performance of lambs fed or not with condensed tannins added to the concentrate.

| Variables | Control [1] | Condensed Tannins [1] | SEM | *p*-Value: Treat |
|---|---|---|---|---|
| Initial body weight, kg | 24.8 | 24.3 | 0.247 | 0.89 |
| Final body weight, kg | 46.3 | 44.9 | 0.240 | 0.65 |
| Average daily gain, g/d (d 1 to 81) | 265 | 256 | 1.68 | 0.53 |
| Silage intake (kg DM/day) | 0.50 | 0.50 | 0.08 | 0.90 |
| Concentrate intake (kg DM/day) | 0.61 | 0.62 | 0.00 | 0.98 |
| [2] Daily mean intake (DMI) (kg DM) | 1.12 | 1.13 | 0.12 | 0.96 |
| Feed efficiency | 0.23 | 0.22 | 0.03 | 0.90 |

[1] The treatments: Lambs that received a diet with added condensed tannins in the concentrate (treatment); Lambs that did not receive the addition of condensed tannins in the concentrate (control). [2] Concentrate + silage per day (DM).

As there was 100% consumption of the concentrate (with CT) and knowing the daily feed intake of the lambs per pen, the concentration of the additive per animal/daily was calculated, i.e., it was 2.82 g.

### *3.3. Hemogram, Serum Biochemistry and Metabolites*

Results of serum biochemistry and metabolism are presented in Tables 3 and S2. The treatment affected cholesterol levels, which were lower in the serum of the animals in the treated group. Globulin levels were higher than the control in the entire experimental period, which was linked to a trend toward higher levels of total proteins. The variables albumin, triglycerides, glucose, and urea did not affect treatment ($p > 0.05$). Effect of treatment for TBARS and thiols, treatment × day interaction was verified with lower concentrations of TBARS and higher concentrations of thiols in the treated group (d 29, 65, and 81) ($p < 0.05$). There was no treatment effect for GST ($p > 0.05$).

**Table 3.** Serum biochemistry and oxidation biomarkers in the serum of lambs fed or not with condensed tannins added to the concentrate.

| Variable | Treatments [1] | | SEM | *p*-Value [4] | | |
|---|---|---|---|---|---|---|
| | Control | Condensed Tannins | | Treat | Treat × Day [5] | Day [6] |
| Albumin (g/dL) | 3.01 | 3.10 | 0.08 | 0.85 | 0.59 | 0.64 |
| Globulin (g/dL) | 3.56 [b] | 3.84 [a] | 0.09 | 0.05 | 0.01 | 0.01 |
| Total protein (g/dL) | 6.45 [b] | 6.95 [a] | 0.25 | 0.07 | 0.16 | 0.22 |
| Urea (mg/dL) | 42.0 | 42.8 | 2.06 | 0.92 | 0.90 | 0.91 |
| Cholesterol (mg/dL) | 72.9 [a] | 65.9 [b] | 2.32 | 0.05 | 0.02 | 0.01 |
| Tryglicerídes (mg/dL) | 33.6 | 31.7 | 4.57 | 0.85 | 0.89 | 0.78 |
| Glucose (mg/dL) | 79.8 | 83.9 | 3.90 | 0.51 | 0.65 | 0.45 |
| TBARS (nmol MDA/mL) | 25.3 [a] | 21.4 [b] | 1.62 | 0.04 | 0.03 | 0.05 |
| Thiols (nmol SH/mg protein) | 115 [b] | 140 [a] | 8.05 | 0.01 | 0.01 | 0.01 |
| GST [2] (µmol CDNB [3]/min/mg protein) | 367 | 365 | 18.2 | 0.94 | 0.86 | 0.82 |

[1] The treatments: Lambs that received a diet with added condensed tannins in the concentrate (treatment); Lambs that did not receive the addition of condensed tannins in the concentrate (control). [2] Glutathione S-Transferase (GST). [3] 1-cloro-2,4-dinitrobenzeno (CDNB). [4] Means differ between groups ($p \leq 0.05$), illustrated by different letters (a, b) on the same line to demonstrate the treatment effect. [5] when there was interaction between treatment and day, results are shown in Table S2. [6] when there was day effect, results are shown in Table S2.

Blood count results are presented in Tables 4 and S2. There was a treatment effect for hematocrit percentage, hemoglobin concentration, erythrocytes, and granulocytes, which was lower in the treated group ($p < 0.05$). Treatment × day interaction on total leukocyte concentration, being highest on day 29 in the treated group and lowest on day 65. The trend of treatment effect and treatment × day interaction for lymphocyte count is highest in the treated group and at certain moments (d 29 and 81). There was a tendency for treatment effect and treatment × day interaction for the number of monocytes lower in the treated group, especially on day 81.

**Table 4.** Blood parameters of lambs fed on with the control and condensed tannin diets.

| Variable | Treatment [1] | | SEM | *p*-Value [2] | | |
|---|---|---|---|---|---|---|
| | Control | Condensed Tannins | | Treat | Treat × Day [3] | Day [4] |
| Hematocrit (%) | 33.5 [a] | 31.1 [b] | 0.27 | 0.02 | 0.05 | 0.38 |
| Hemoglobin (g/dL) | 13.0 [a] | 11.8 [b] | 0.14 | 0.04 | 0.05 | 0.21 |
| Erythrocytes ($\times 10^6$µL) | 11.1 [a] | 10.4 [b] | 0.15 | 0.05 | 0.11 | 0.44 |
| Leukocytes ($\times 10^3$ µL) | 11.3 | 11.4 | 1.08 | 0.83 | 0.03 | 0.01 |
| Granulocytes ($\times 10^3$ µL) | 2.13 [a] | 1.76 [b] | 0.06 | 0.01 | 0.01 | 0.45 |
| Lymphocytes ($\times 10^3$/µL) | 8.20 [b] | 8.97 [a] | 0.31 | 0.07 | 0.01 | 0.01 |
| Monocytes ($\times 10^3$/µL) | 0.93 [a] | 0.63 [b] | 0.05 | 0.06 | 0.01 | 0.02 |

[1] Treatments: Lambs that received a diet with added condensed tannins in the concentrate (treatment); Lambs that did not receive the addition of condensed tannins in the concentrate (control). [2] Means differ between groups ($p \leq 0.05$), illustrated by different letters (a, b) on the same line to demonstrate treatment effect. [3] when there was interaction between treatment and day, results were shown in Table S2. [4] When there was a day effect, results were shown in Table S2. Note: In addition to the variables described in the table, we measured the number of platelets and MCV, MCH, MCHC, and RDW-CV, which did not differ between treatments.

Results of the day effect were presented in Table S2. The day effect for globulin levels was observed only in the lambs of the control group, which decreased over time. On the other hand, TBARS levels decreased, and thiols increased over time in the lambs of the condensed tannin group. The day effect for cholesterol was also observed, with the levels of this biomarker increasing compared to the beginning of the experiment in both groups. Likewise, the total leukocyte and lymphocyte counts increased in the blood of both groups of animals. The monocyte count fluctuated, with a higher count at the end of the experiment in the control group lambs; on the other hand, in the lambs fed condensed

tannins, the count was lower at the end of the research. No day effect was observed for hemoglobin and hematocrit.

### *3.4. Ruminal Fermentation Activity*

Rumen fluid results are presented in Table 5 and Figure 1. There was a tendency toward a shorter time of bacterial activity in the treated group and treatment × day interaction at the end of the experiment (d 81). The pH variable of the rumen fluid showed no effect of treatments ($p > 0.05$). There was a tendency for interaction between treatment × day at time 81 for the total short-chain fatty acids concentration, which was lower in the rumen fluid of the animals in the treatment group. This result occurred due to the lower concentration of acetic and propionic acids on day 81 in lambs in the treatment group. In rumen fluid, treatment effect and treatment × day interaction were observed for butyric, isovaleric, and valeric acids. These three volatile acids were in higher concentrations in lambs from the treatment group compared to the control. There was no treatment effect or treatment × day interaction for the acetic/propionic ratio.

**Table 5.** pH and bacterial activity (MBRT), total volatile fatty acids (VFA), in the rumen fluid of lambs with or without added non-concentrated condensed tannins.

| Variable | Treatment [1] | | SEM | *p*-Value [3] | | |
|---|---|---|---|---|---|---|
| | Control | Condensed Tannins | | Treat | Day [5] | Treat × Day [4] |
| pH | 6.42 | 6.38 | 0.12 | 0.77 | 0.25 | 0.32 |
| MBRT [2] (seconds) | 86.8 [a] | 73.2 [b] | 4.21 | 0.08 | 0.01 | 0.01 |
| VFA (mmol/L) | 101 | 99.3 | 2.58 | 0.40 | 0.03 | 0.10 |
| Acetic (mmol/L) | 70.1 | 66.6 | 2.40 | 0.24 | 0.01 | 0.05 |
| Propionic (mmol/L) | 18.3 | 17.1 | 0.88 | 0.56 | 0.04 | 0.03 |
| Butyric (mmol/L) | 9.65 [b] | 11.7 [a] | 0.66 | 0.05 | 0.01 | 0.01 |
| Isovaleric (mmol/L) | 2.05 [b] | 2.73 [a] | 0.16 | 0.01 | 0.01 | 0.01 |
| Valeric (mmol/L) | 1.02 [b] | 1.28 [a] | 0.11 | 0.10 | 0.19 | 0.01 |
| Acetic/propionic | 3.83 | 3.88 | 0.15 | 0.86 | 0.66 | 0.74 |

[1] The treatments: Lambs that received a diet with added condensed tannins in the concentrate (treatment); Lambs that did not receive the addition of condensed tannins in the concentrate (control). [2] Methylene blue reduction test (MBRT)—microbial activity indicator); the shorter the time, the greater the action of bacteria. That is, the less time is needed to consume methylene blue. [3] Means differ between groups ($p \leq 0.05$), illustrated by different letters (a, b) on the same line to demonstrate the treatment effect. [4] When there is interaction between the treatment and day, results are shown in Figure 1. [5] When there is a day effect, the results are shown in Figure 1.

Results of ruminal fluid biomarkers were presented in Figure 1. Bacterial activity was affected by the day, being lower in both groups in the middle of the experiment; we also highlighted the higher bacterial activity at the end of the experiment when lambs consumed condensed tannin. VFA concentration was affected by the day, being significantly lower in the ruminal fluid of lambs that consumed condensed tannin compared to d 81 to d 29. In both groups, an effect of the day was observed on the percentage of acetic, propionic, butyric and isovaleric acid, but no effect on valeric acid. In general, these VFA reduced when the animals consumed condensed tannin as detailed in Figure 1. The other biomarkers had no day effect in this sample.

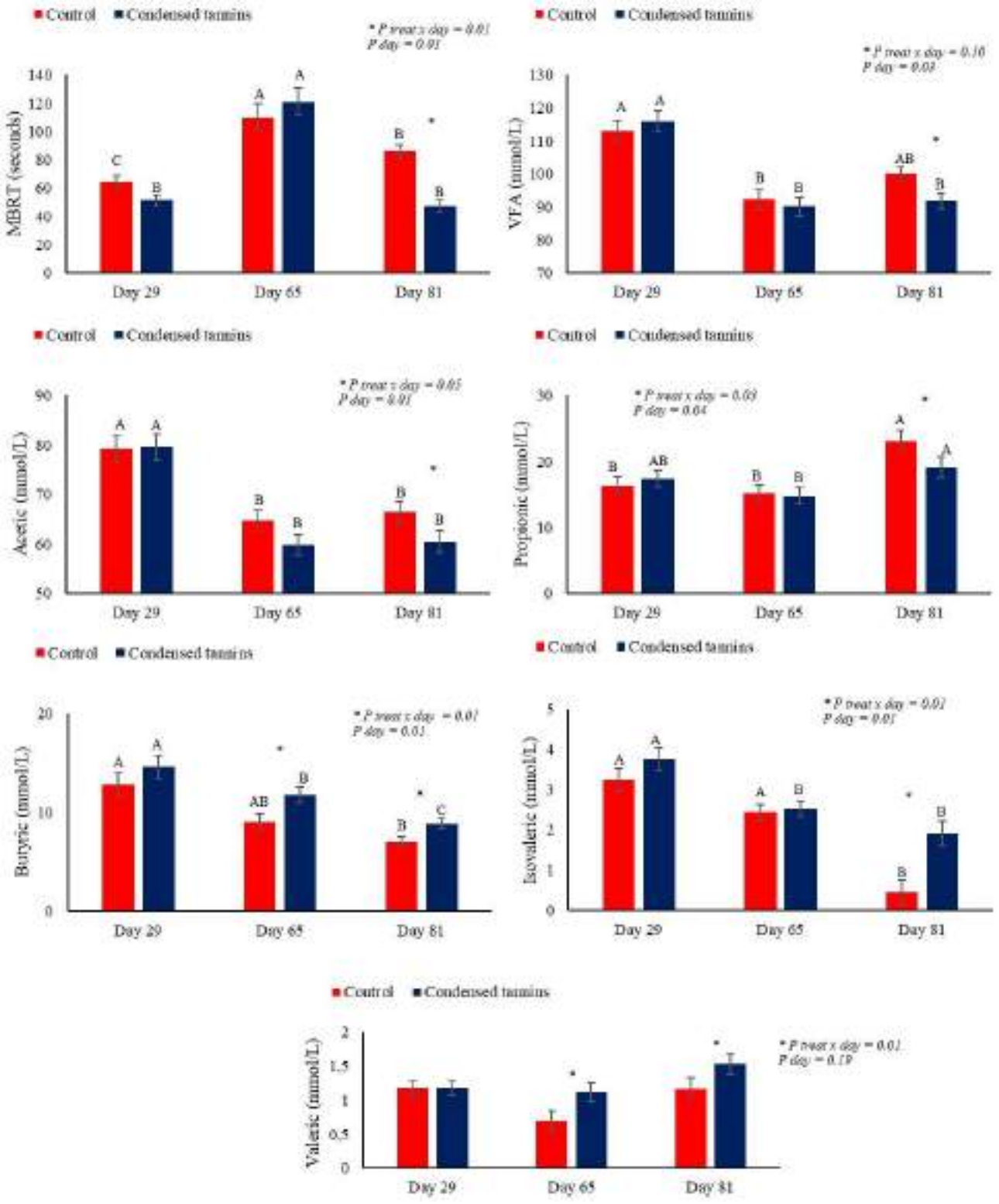



**Figure 1.** Treatment vs. day interaction and day effect for bacterial activity (MBRT), total volatile fatty acids (VFA), in the rumen fluid of lambs with or without added non-concentrated condensed tannins. Note: 2 Methylene blue reduction test (MBRT)—microbial activity indicator); the shorter the time, the greater the action of bacteria; that is, the less time is needed to consume methylene blue. Means differ between groups ($p \leq 0.05$), illustrated by an asterisk (*) on the bars to demonstrate the interaction between treatment and day. The day effect ($p \leq 0.05$) was illustrated by different capital letters (A, B, C) on the bars.

### 3.5. Carcass Characteristics

Table 6 presents the meat quality results. We verified a treatment effect for cold carcass yield and percentage of fat in the meat, which was more significant in the treated group ($p < 0.05$). The other variables, such as hot carcass weight, cold carcass weight, hot carcass pH, cold carcass pH, luminosity color, and water retention capacity, had no treatment effect ($p > 0.05$).

**Table 6.** Analysis of carcass and meat of lambs fed or not with condensed tannins added to the concentrate.

| Variable | Control [1] | Condensed Tannins [1] | SEM | *p*-Value: Treat [2] |
|---|---|---|---|---|
| Hot carcass weight (kg) | 21.4 | 21.3 | 1.21 | 0.96 |
| Cold carcass weight (kg) | 20.4 | 20.3 | 1.20 | 0.95 |
| Cold carcass yield (%) | 44.1 [b] | 45.2 [a] | 0.07 | 0.05 |
| Hot carcass pH | 6.45 | 6.35 | 0.09 | 0.70 |
| Cold carcass pH | 5.81 | 5.81 | 0.03 | 0.97 |
| Lightness | 45.3 | 45.3 | 1.06 | 0.98 |
| Redness | 12.2 | 11.8 | 0.14 | 0.90 |
| Yellowness | 6.54 | 6.38 | 0.29 | 0.82 |
| Water holding capacity (%) | 82.2 | 80.9 | 1.51 | 0.86 |
| **Chemical composition of meat** | | | | |
| Dry matter (g/kg) | 262 | 273 | 2.61 | 0.24 |
| Protein (g/kg) | 217 | 213 | 2.03 | 0.69 |
| Intramuscular fat (g/kg) | 20.7 [b] | 34.7 [a] | 1.12 | 0.01 |
| Ashes (g/kg) | 24.9 | 25.8 | 0.87 | 0.16 |

[1] Treatments: Lambs that received a diet with added condensed tannins in the concentrate (treatment); Lambs that did not receive the addition of condensed tannins in the concentrate (control). [2] Means differ between groups ($p \leq 0.05$), illustrated by different letters (a, b) on the same line to demonstrate the treatment effect.

There was a treatment effect for lower TBARS concentrations in the treated group ($p < 0.05$—Figure 2). Higher concentrations of thiols ($p < 0.05$) and a tendency toward higher concentrations of SOD (Figure 2). Fatty acid profiles are displayed in Table 7 and Figure 3. There was a treatment effect in the concentrations of C17:1(cis-10-Heptadecenoic), C18:0 (Stearic), and C20:4n6 (arachidonic) with lower concentrations in the treated group ($p < 0.05$). Higher amounts of C18:1n9c (oleic), C18:2n6c (linoleic), and C20:3n3 (cis-11,14,17-eicosatrienoic) in the treatment ($p < 0.05$). A significant effect for saturated fatty acids (SFAs) in lower amounts in the treatment and higher concentrations of monosaturated fatty acids (MUFAs) ($p < 0.05$). The polyunsaturated fatty acids (PUFA) were no different, but a higher PUFA/SFA ratio was observed in animals with CT intake, and a higher proportion of ω6 and ω3 was observed in the meat of these animals (Figure 3).

**Table 7.** Profile of fatty acids in lamb meat fed or not with condensed tannins added to the concentrate.

| Fatty Acids (%) | Control [1] | Condensed Tannins [1] | SEM | *p*-Value: Treat [2] |
|---|---|---|---|---|
| C12:0 (Lauric) | 0.056 | 0.063 | 0.009 | 0.12 |
| C14:0 (Myristic) | 1.300 | 1.385 | 0.135 | 0.54 |
| C14:1 (Myristoleic) | 0.081 | 0.078 | 0.004 | 0.82 |
| C15:0 (Pentadecanoic) | 0.215 | 0.226 | 0.028 | 0.70 |
| C16:0 (Palmitic) | 24.24 | 22.80 | 0.975 | 0.16 |
| C16:1 (Palmitoleic) | 1.457 | 1.539 | 0.198 | 0.72 |
| C17:0 (Heptadecanoic) | 0.897 | 0.913 | 0.117 | 0.85 |
| C17:1 (cis-10-Heptadecenoic) | 0.581 [a] | 0.000 [b] | 0.060 | 0.01 |
| C18:0 (Stearic) | 16.69 [a] | 13.70 [b] | 0.999 | 0.01 |
| C18:1n9c (Oleic) | 40.70 [b] | 43.99 [a] | 0.526 | 0.01 |
| C18:2n6c (Linoleic) | 9.292 [b] | 12.11 [a] | 0.178 | 0.01 |

**Table 7.** *Cont.*

| Fatty Acids (%) | Control [1] | Condensed Tannins [1] | SEM | *p*-Value: Treat [2] |
|---|---|---|---|---|
| C20:0 (Arachidic) | 0.120 | 0.154 | 0.010 | 0.23 |
| C18:3n6 (Linolenic) | 0.126 | 0.107 | 0.011 | 0.18 |
| C20:1n9 (cis-11-Eicosenoic) | 0.081 | 0.103 | 0.002 | 0.21 |
| C18:3n3 (a-Linolenic) | 0.370 | 0.517 | 0.125 | 0.32 |
| C20:2 (cis-11.14-Eicosadienoic) | 0.071 | 0.087 | 0.001 | 0.42 |
| C22:0 (Behenic) | 0.064 | 0.083 | 0.011 | 0.11 |
| C20:3n6 (cis-8.11.14-Eicosatrienoic) | 0.193 | 0.183 | 0.013 | 0.95 |
| C22:1n9 (Erucic) | 0.040 | 0.037 | 0.005 | 0.63 |
| C20:3n3 (cis-11.14.17-Eicosatrienoic) | 0.000 [b] | 0.026 [a] | 0.002 | 0.01 |
| C20:4n6 (Arachidonic) | 3.117 [a] | 1.611 [b] | 0.202 | 0.01 |
| C24:0 (Lignoceric) | 0.050 | 0.073 | 0.019 | 0.28 |
| C20:5n3 (cis-5.8.11.14.17-Eicosapentaenoic) | 0.091 | 0.075 | 0.009 | 0.15 |
| C24:1n9 (Nervonic) | 0.032 | 0.032 | 0.001 | 0.97 |
| C22:6n3 (cis-4.7.10.13.16.19-Docosahexaenoic) | 0.107 | 0.104 | 0.056 | 0.98 |

[1] Treatments: Lambs that received a diet with added condensed tannins in the concentrate (treatment); Lambs that did not receive the addition of condensed tannins in the concentrate (control). [2] Means differ between groups ($p \leq 0.05$), illustrated by different letters (a, b) on the same line to demonstrate the treatment effect.

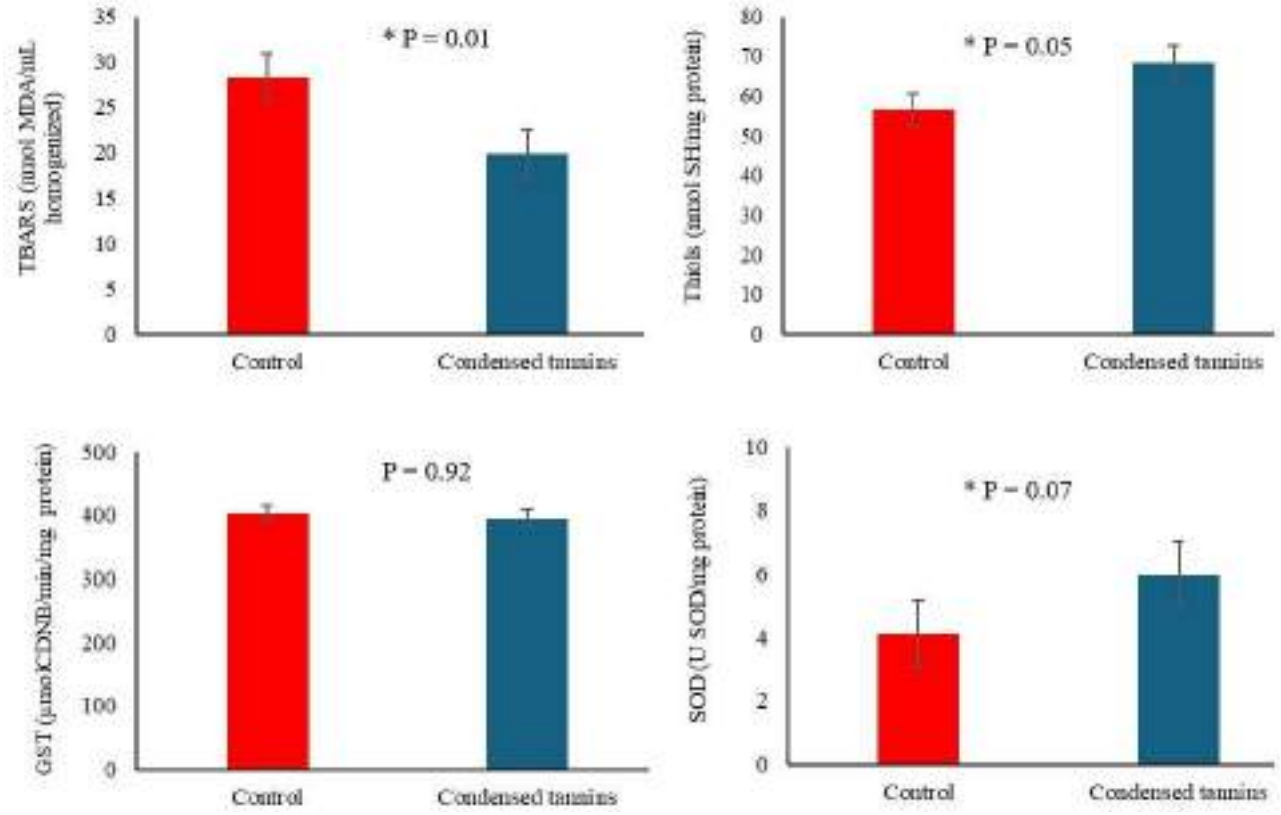



**Figure 2.** Oxidative in meat of lambs fed or not with condensed tannins added to the concentrate (* treatment effect when $p \leq 0.05$ and tendency when $p \leq 0.05$ and $p > 0.10$).

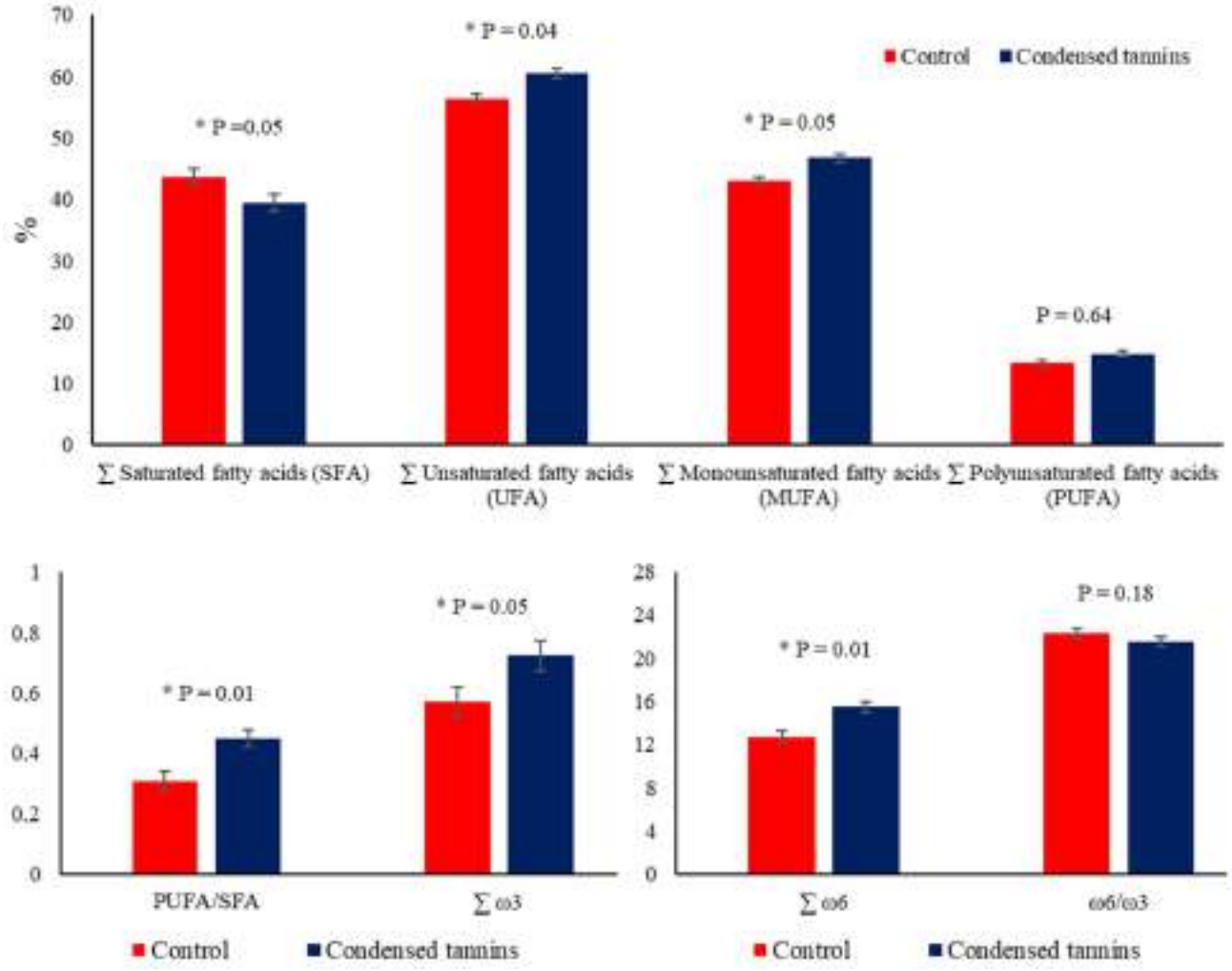



**Figure 3.** Effect of treatment on the sum of saturated and unsaturated fatty acids in the meat of lamb fed condensed tannins, as well as omega 3 and 6. * Difference between groups was demonstrated when $p \leq 0.05$.

## 4. Discussion

Condensed tannins derived from black acacia (*A. mearnsii*) extract has the characteristic of inducing a reduction in dry matter intake (DMI). When consumed in doses higher than 5% of the total diet DM, due to anti-nutritional factors that confer an astringent taste, they can impact intake and performance [4]. Tannins can complex with dietary proteins and carbohydrates, reducing their degradation at the ruminal level. After passing through the more acidic environment of the abomasum, they are decomplexed, allowing for a greater supply of nutrients at the intestinal level, including essential amino acids [42], thus promoting weight gain. In our study, when using 2.5 g/kg of DM, no changes in feed intake or animal performance were observed, indicating that this lower dose does not affect DMI or weight gain in animals. The researchers [10], when adding increasing doses of CT to lamb diets, found that amounts above 40 g/kg DM reduced intake and weight gain. This is because they negatively affect the activity of the microbiota and ruminal digestive efficiency [43], inhibiting the activity of enzymes that digest fibers and carbohydrates, altering permeability and the capacity for nutrient absorption.

Tannins act primarily on groups of bacteria that digest fibers and carbohydrates, which can lead to changes in these populations within the ruminal environment, causing shifts in the profile of short-chain fatty acids produced by these bacteria through the fermentation of substrates provided by the diet. The reduction in acetate and propionate concentrations and the increase in butyrate concentration observed here can be caused by tannin ingestion.

There was a treatment × day interaction for the short-chain fatty acid profile in the rumen, with the difference between groups for all these acids being observed on day 81, and only for butyric and valeric acid on day 65. This clearly demonstrates that a long adaptation period to tannin in the diet was necessary. The ruminal fermentation profile of dairy cows supplemented with increasing doses of CT showed a higher proportion of butyrate and a lower proportion of propionate and acetate in the ruminal fluid [44]. Changes in the ruminal microbiome may have mediated this change in the ruminal fermentation profile. Tannins can alter the ruminal environment due to the formation of ammonia ions, which promote changes in the pH of the environment. This pH alteration favors the multiplication of certain bacteria, especially gram-negative bacteria, leading to reduced fermentation of fibers and carbohydrates, as most bacteria that degrade these substrates are gram-positive. Consequently, there is a reduction in the production of acetate and propionate, resulting from the lower fermentation of these compounds. The addition of tannins to the diet of ruminants can interfere with the production of branched-chain fatty acids, which are produced from the breakdown of amino acid carbon skeletons [45], as seen in the increase in isovaleric acid in our study.

There were effects of tannin consumption on lamb meat, and it is important to emphasize that the ingestion of this additive does not affect food safety, since tannins are commonly consumed by ruminants. The alterations in the fatty acid profile were also observed in the meat composition, where a higher percentage of fat was found in animals from the group that consumed condensed tannins. Upon analysis, it was found that the fatty acid profile was modified, reducing SFA due to the lower biohydrogenation of unsaturated fatty acids at the ruminal level. This is caused by the reduced activity of gram-positive bacteria, resulting in greater metabolization of unsaturated fatty acids, which are then directed to the tissues, promoting changes in the fatty acid profile found in the meat [46]. It is worth highlighting the increase in concentrations of omega C18:1n9c (oleic) and C18:2n6c (linoleic) acids [47,48], which are directly linked to cardiovascular health benefits both in the animals and in consumers of the products derived from them, in this case, the meat [49]. The enzyme Δ9-desaturase is involved in the metabolism of SFA. It is responsible for introducing a double bond between the ninth and tenth carbon of the saturated fatty acid chain, converting SFA, such as stearic acid (C18:0), into monounsaturated fatty acids, such as oleic acid [50]. The activity of this enzyme is crucial for the regulation of membrane fluidity. In our study, we observed high activity of this enzyme, which reduced the concentrations of C18:0 and increased the concentration of monounsaturated fatty acids. With this improvement in the lipid profile, we suggest that the reduction in serum cholesterol levels can be attributed to this alteration, facilitating the flow of lipids in the bloodstream, preventing adhesion to artery walls, and reducing the risk of cardiovascular diseases typically associated with animal-derived products.

The values found for the polyunsaturated:saturated fatty acid ratio (PUFA:SFA) in animals that intake CT (0.45) achieved the values recommended for a healthy diet, as reported by the Ministry of Health of the United Kingdom, that should be greater than 0.4 [51], which is also recommended by the literature [52], which is 0.45 in foods. These data are significant because typically, according to the literature, this ratio in meat is generally low, around 0.1 [53], characterizing the products derived from ruminant animals, which have a ratio of PUFA:SFA fatty acids lower due to the biohydrogenation of dietary unsaturated fatty acids by rumen bacteria [54]. However, these results demonstrate that although lamb meat has higher levels of SFA and lower ratio of polyunsaturated:SFA, supplementation with CT improved this health attribute. The n6:n3 ratio did not change as a function of the supplementation with CT. On average, it was 22.7. Higher amounts of unsaturated fatty acids and this relationship is positive, since this the consumption of SFA can be considered harmful to consumers because this presentation of fatty acid is associated with coronary disease, while many UFAs are linked to reductions in inflammation, stroke, oxidative stress and liver disorders.

Condensed tannins have a positive effect on stimulating the immune, anti-inflammatory, and antioxidant response [55]. In addition, the intake of flavonols and procyanidins favors the production of anti-inflammatory molecules [56], which can interfere with the proliferation of B and T lymphocytes, cells involved in immunological function with antibody production [57]. We observed an increase in the number of lymphocytes in the treatment group, which can be attributed to the action of the additive with flavonoids and gallic acid in its composition. They can interfere with the number of cells in the blood count [58]. The increase in B lymphocytes can stimulate the increase in globulins, which include immunoglobulins, which are responsible for the immune response.

Numerous factors can contribute to oxidative stress and increased levels of oxidizing compounds in the body. This imbalance in the oxidant/antioxidant relationship increases levels of oxidizing compounds that can potentiate some pathologies [59]. Oxidation processes typically occur due to the action of oxygen on the cell membrane, which contains lipids in its composition and is more prone to oxidation and the action of free radicals. We found that the provision of black wattle tannin extract in the lambs' diet improved the antioxidant response, increasing the activity of enzymes responsible for neutralizing free radicals and helping to minimize the impacts of lipid peroxidation on the cell membrane and its integrity. The CT in the extract helps reduce the production of reactive oxygen species, which reduces the production of free radicals and preserves cellular integrity, enhancing animal performance. This can be verified by the decrease in lipid peroxidation levels both in serum levels and in the meat of lambs from the group that received the extract, indicating that the oxidation of lipid compounds is occurring at a lower intensity. This is because polyphenols such as gallic acid and catechins, which belong to the flavonoid family and are typically found in the extract of black wattle, have an antioxidant function [60], allowing the antioxidant system to be stimulated by providing these compounds through the diet. The increase in antioxidant enzymes was seen (GSH at the serum level and in meat) measured indirectly through total thiols and Superoxide Dismutase (SOD) in meat. Other studies verified the antioxidant action of these compounds [61–63], reporting the ability of polyphenols to neutralize free radicals, increasing the activity of antioxidant enzymes and promoting relief from oxidative stress. Similar results were found by researchers [64], who reported an increase in the levels of antioxidant enzymes when providing grape residue containing flavonoids, including catechins, anthocyanins, and procyanidins.

## 5. Conclusions

The inclusion of black acacia CT in the lambs' diet showed benefits in modulating the antioxidant response and in the fatty acid profile of the meat, as well as improving carcass yield and meat fat percentage. However, there was no significant impact on growth performance and feed intake. These results highlight the effectiveness of CT in improving meat quality and overall animal health without compromising productive performance.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10120630/s1, Table S1: Standardization of the methodology used to quantify the levels of short-chain fatty acids in rumen liquid; Table S2: Treatment vs. day interaction and day effect in serum biochemistry, oxidation biomarkers, and hematological variables of lambs fed or not with condensed tannins added to the concentrate; Figure S1: Growth curve (mean and standard error of body weight) during the experimental period: control vs. condensed tannins. Day effect was observed ($p < 0.001$).

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Research Article

# Inclusion of exogenous enzymes in feedlot cattle diets: Impacts on physiology, rumen fermentation, digestibility and fatty acid profile in rumen and meat

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ABSTRACT

The objective of this study was to evaluate if the inclusion of a blend composed of exogenous enzymes (amylase, protease, cellulase, xylanase and beta glucanase) in the individual and combined form in the feedlot steers diet has benefits on the physiology, rumen fermentation, digestibility and fatty acid profile in rumen and meat. The experiment used 24 animals, divided into 4 treatments, described as: T1-CON, T2-BLEND (0.5 g mixture of enzyme), T3-AMIL (0.5 g alpha-amylase), T4-BLEND+AMIL (0.5 g enzyme blend+ 0.5 g amylase). The concentration of mineral matter was higher in the meat of cattle of T4-BLEND+AMIL. A higher proportion of monounsaturated fatty acids was observed in the T3-AMIL group when compared to the others. The percentage of polyunsaturated fatty acids was higher in the T2-BLEND and T4-BLEND+AMIL compared to the T1-CON. The combination of exogenous enzymes in the diet positively modulate nutritional biomarkers, in addition to benefits in the lipid and oxidative profile meat.

## 1. Introduction

The demand for beef increases every year, which is why the need to intensify beef production systems has arisen. However, there is a huge challenge to this intensification since the 90 s, which is to produce in a profitable and sustainable way [1]. Still in the 1980s, there was the perception that a reduction in the production cycle, an increase in carcass yield, greater production per area, freeing up pasture areas for other categories and a faster return on investment are some of the advantages of the confined system [2]. However, the high cost of the diet provided to the animals was a limitation of this production system, due to the high added value of the ingredients, mainly cereals; nonetheless, the animal confinement system has grown worldwide.

Aiming to maximize the diet, the use of enzymatic additives is a good alternative to increase the performance of the animals through the mutual action of the enzymes provided and produced by the ruminal microbiota [3], which aims to increase digestion and seeks greater use of feed, and consequently greater weight gain. In order to maximize the use of the diet, the process of protein degradation in the rumen undergoes the action of the protease enzyme, involved in the breakdown of molecules to generate free amino acids through hydrolysis caused by the action of water and the action of enzymes [4]. In this context, in addition to the new formation of protein chains, the remaining amino acids serve as a substrate for microorganisms that will be sources of microbial protein, which is characterized as the main metabolizable source in ruminants, which leads to the understanding that the multiplication of microorganisms promotes improvements both in performance and in the health of these animals. The use of fibrolytic enzymes such as cellulase and xylanase has shown good results in the digestibility of dry matter and fiber, since the forage digestion process is often considered incomplete [5], which justifies the use of exogenous fibrolytic enzymes to act in conjunction with enzymes produced by microorganisms in the ruminal environment, thus enhancing fiber degradation [6–8].

Starchy grains are the main source of energy in confined ruminant

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diets, mainly corn [9]. With the processing it is possible to change the physical and chemical characteristics of the starch [10]. Knowing this, research with the inclusion of alpha amylase enzymes aims to optimize the digestion of starch, which can pass intact through the rumen. Due to the consistency of the endosperm, hard or farinaceous and the protein matrix that covers the starch, because it works as a physical and chemical barrier that prevents the hydrolysis of the starch molecule [11, 12]. However, this is extremely necessary in the digestion process to generate a maltose molecule that is equivalent to two glucose molecules, necessary to increase feed efficiency.

There are studies on the addition of exogenous enzymes in the diet of production animals, but it is considered insufficient when the experimental condition is in enzymatic association in order to evaluate digestibility, rumen environment and their effects on performance [13, 14]. We also agree that further studies are needed in order to seek to improve production in confinement, where a detail can reflect on profitability and consequently sustainability. Therefore, our hypothesis is that the combination of protease and amylase as an additive in the bovine diet will improve the digestibility. For this reason, this study was to evaluate if the inclusion of a blend composed of exogenous enzymes (amylase, protease, cellulase, xylanase and beta glucanase) in the individual and combined form in the feedlot steers diet has benefits on the physiology, rumen fermentation, digestibility and fatty acid profile in rumen and meat.

## 2. Material and methods

### 2.1. Facilities and animals

The study was carried out in the ruminant sector at the experimental farm of the Universidade do Estado de Santa Catarina (FECEO/UDESC) in the municipality of Guatambú (Latitude: 27° 8′ 5″ South, Longitude: 52° 47′ 15″ West), located in the western region of Santa Catarina. The animals were housed in appropriate confinement, allocated in individual pens measuring 1.5 × 7.0 m with a concrete floor, equipped with automatic drinkers and a freely accessible feeder. The animals' feeding area had cover, which allowed the animals to shelter from the weather conditions. The shed had north-south solar orientation, which allowed contact of the animals with sunlight. Twenty-four castrated male Holstein steers with an average weight of 336±4.68 kg and an average age of 12 months were used as an experimental model.

### 2.2. Test products

Tecmax Pro-Ruminantes® (Toledo – PR, Brazil) is a fermented product of *Bacillus subtilis*, algaroba bran, inactivated and dehydrated sugar cane yeast, fermentation product of *Aspergillus Niger*; which presents the following guarantee levels in its technical sheet: Protease (min.) 7500 U/g, cellulase (min.) 2700 U/g, xylanase (min.) 1200 U/g, beta glucanase (min.) 300 U/g. The alpha-amylase (corn starch and amylase) have guaranteed levels of amylase (min.) 1000 U/g. Therefore, the exogenous enzymes used were amylase and an enzyme mixture containing mainly protease in addition to cellulase, xylanase and beta glucanase; as well as they combined.

### 2.3. Experimental design, diets and feeding

The experiment was a randomized controlled design to standardize the initial body weight, with the animals divided into 4 treatments with 6 repetitions each: T1-CON (traditional confinement diet, T2-BLEND (0.5 g mixture of protease enzymes, cellulase, xylanase and beta glucanase per kg of DM diet), T3-AMIL (0.5 g alpha-amylase per kg of DM diet), T4-BLEND+AMIL (0.5 g enzyme blend + 0.5 g amylase per kg of DM).

Previously, the animals went through a period of adaptation to the diet (15 days), where the protocol of gradual inclusion of concentrate was used (40:60, 60:40, 70:30–concentrate ratio: roughage) in the periods 1–5, 5–10, 10–15 days of experiment, respectively. This gradual adaptation of the animals occurred because previously the animals were on winter pasture (oats and ryegrass), receiving protein in the feeder once a day. The diets were calculated according to the nutritional requirements of the animal category for an estimated average daily gain (ADG) of 1.5 kg of body weight (BR-CORTE, 2016). The daily diet was divided into two similar meals (08 AM and 16 PM), being supplied as a Total Mixed Ration (TMR: concentrate + silage). Enzymes were added to the TMR in the animal feeder, a top-dressed mixture.

### 2.4. Sample and data collection

#### 2.4.1. Growth performance

Zootechnical performance was evaluated as complementary information for this research. Steers were weighed individually at 9 times (days 1, 15, 30, 45, 60, 75, 90, 105 and 120 of the experiment). All weighings were performed in the morning, with the animals fasting, with the aid of a digital electronic scale. With the body weight data, it was possible to calculate weight gain (WG) (WG = initial BW – final BW) and average daily gain (ADG) [(final BW–initial BW) / number of days)]. This body weight information was used to individually calculate the animals' diet for the subsequent 15 days; thus, each animal received the volume of feed proportional to its body weight. Feed leftovers were weighed and recorded daily during the morning; important information to calculate feed efficiency.

#### 2.4.2. Sample collection

Blood collections were performed on days 1, 15, 60 and 120, through the caudal vein, with the aid of needles and vacuolated tubes, without anticoagulants to obtain serum for biochemical analysis and levels of oxidants and antioxidants. Vacuolated tubes with anticoagulants (EDTA) were also used for hematological analyses. The tubes were kept refrigerated at 10 °C, in an isothermal box until arrival at the laboratory. For serum separation, tubes were centrifuged without anticoagulant (7500 RPM for 10 min). The serum was transferred to microtubes, identified and stored at -20 °C until laboratory analysis. Using an esophageal probe with a vacuum system, the collection of ruminal fluid from bovines was carried out. This material had pH analyzed instantly and the rest of the material was frozen for analysis of volatile fatty acids.

On day 121 of the experiment, the animals were slaughtered in a specialized slaughterhouse for cattle. The slaughters followed current legislation, and were under veterinary inspection. A fragment of liver and *longissimus dorsi* muscle were collected for analysis of fatty acid profiles, stored at −20 °C until analysis.

### 2.5. Laboratory analysis

#### 2.5.1. Chemical composition of diet and feces

Feed and feces samples were dried in a forced-air oven at 56 °C for at least 72 h and ground in a 1 mm sieve for analysis. Total DM contents in feed and feces samples were determined by oven drying at 110 °C for 24 h. Ash was determined by combustion at 600 °C for 3 h, and organic matter was determined by mass difference. Total nitrogen was assayed using the Kjeldahl method (Method 984.13; AOAC 1997). The neutral detergent fiber (NDF) analyses included ash and were based on the procedures described by Mertens (2002). The chemical composition of feeds is shown in Table 1.

#### 2.5.2. Apparent digestibility coeficiente

Indigestible neutral detergent fiber (iNDF) was used as an internal marker to calculate apparent feed digestibility (Cochran et al., 1986; Huhtanen et al., 1994). The feed and feces samples were weighed in bags with 16-μm porosity and incubated in the rumen of cattle for 288 h. Then they were washed with tap water, treated with neutral detergent in an autoclave (SENGER et al., 2008), and dried in a forced-air ventilation

**Table 1**
Ingredients and chemical composition of the diet.

| Feed | Dry matter (kg/animal/day) | |
|---|---|---|
| Corn silage | 2.29 | |
| Concentrate[1] | 7.04 | |
| Total | 9.33 | |
| **Chemical composition%** | **Corn silage** | **Concentrate** |
| Dry matter | 35.33 | 89.07 |
| Crude protein | 5.97 | 17.70 |
| NDF | 43.02 | 31.24 |
| ADF | 21.76 | 5.93 |
| Ethereal extract | 1.16 | 4.10 |
| Ashes | 4.56 | 6.59 |
| Starch | 22.6 | 37.35 |

oven at 55 °C. Digestibility was calculated as 1 – (iNDF in feed (% of DM)/iNDF in feces (% of DM).

### 2.5.3. Hematology

Hemoglobin concentration, total erythrocyte and leukocyte count and percentage of hematocrit were performed immediately upon arrival at the laboratory, with the aid of the Sysmex electronic device (model KX-21 N). The leukocyte differential followed the technique described by [15], using blood smear and light microscope identification.

### 2.5.4. Serum biochemistry

Levels of total proteins, glucose, albumin, cholesterol and urea were made using the Bio-2000 (BioPlus®) semi-automatic analyzer and commercial kits Analisa®, and their respective methodologies. The levels of globulins were obtained with mathematical calculation (total proteins - albumin).

### 2.5.5. Tissue oxidative status

Liver and meat fragments were homogenized (1v/9v) in saline solution, centrifuged for 10 min at 5600 g [16]. Then, the supernatant was collected, stored in microtubes under freezing (-20 °C) until analysis.

The levels of non-enzymatic antioxidants in meat and liver (protein thiols) were evaluated, following the methodology described by [17] and the results were expressed in nmol SH/mg protein. Glutathione S-transferase (GST) activity in the homogenate was measured based on the method described by researchers [18,19] and the result was expressed as μmol CDNB/min/mg protein. Superoxide dismutase (SOD) activity was performed according to the methodology described by [20], with the results expressed U SOD/mg protein. The catalase activity (CAT) in the homogenized meat and liver followed the technique described by [21], and the data were expressed U CAT/mg protein.

Serum lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS) according to researchers [22,23]. The reaction was read in a spectrophotometer at 535 nm. The result will be expressed in nmoles of malondialdehyde/ml of homogenate. The determination of reactive oxygen species (ROS) was based on the technique described by researchers [24–26] and the results were expressed in U DCFH/mg protein.

### 2.5.6. Determination of short chain fatty acids in ruminal liquid

The rumen fluid samples were thawed to 5 °C and agitated manually in order to homogenize them. 1 mL aliquots of the supernatant from rumen fluid samples were collected in polypropylene microtubes (2 mL) and then centrifuged for 5 min (12,300 × *g*). Then 250 μL of the supernatant was removed and transferred to a new microtube containing 250 μL of formic acid. The mixture was manually shaken and centrifuged for 3 min. After centrifugation, 250 μL of the supernatant of the mixture was collected into an injection vial. 500 μL of 3-octanol solution (665 μg $mL^{-1}$ in methanol) was added, used as an internal standard, and homogenized. The samples were injected into a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3400, Palo Alto, USA) and an autosampler (Varian 8200CX, Palo Alto, USA). 1 μL of the extract was injected in split mode at 1:10. The carrier gas used was hydrogen at a constant pressure of 20 psi. The analytes (acetic, propionic, butyric, valeric, and isovaleric acids) were separated by a CP WAX 52CB capillary column (60 m x 0.25 mm; 0.25 μm stationary phase thickness). The initial column temperature was set at 80 °C for 1 min and increasing to 120 °C at a rate of 8 °C $min^{-1}$, than up to 230 °C by 20 °C $min^{-1}$, where it remained for 1 min. Injector and detector temperatures were set at 250 °C. The validation of the method comprised the following parameters: selectivity, linearity, linear range, repeatability, precision, limit of detection (LOD) and limit of quantification (LOQ) for acetic, propionic, butyric, valeric and isovaleric acids. Analytical parameters are shown in Table Supplementary 1. Linearity was evaluated by calculating a regression equation using the least squares method. LOD and LOQ values were achieved by sequential dilutions up to signal-to-noise ratios of 3:1 and 6:1, respectively. Precision was assessed by analyzing the repeatability of six replicate samples. Accuracy was determined by recovering known amounts of standard substances added to a diluted sample. The results were expressed in mmol $L^{-1}$ of each SCFA in rumen fluid.

### 2.5.7. Profile of fatty acids in meat and feed

The extraction was performed by the Bligh and Dyer method [27] with some modifications. 1.5 g of bovine muscle samples, 0.5 mL of water, 5 mL of methanol and 2.5 mL of chloroform were added to a 15 mL polypropylene tube and mechanical stirring was performed for 60 min. Subsequently, 2.5 mL of chloroform and 1.5 % Na2SO4 solution were added to promote a biphasic system [28]. This mixture was shaken for 2 min, and then centrifuged for 15 min at 2000 rpm. Lipids obtained from the chloroform phase were subjected to fatty acid analysis.

Methylation was performed by a transesterification method proposed by [29]. We added to the extracted lipids 1 mL of 0.4 M koh methanolic solution in a test tube and vortexed for 1 min. The samples were kept in a water bath for 10 min at the boiling point. Subsequently, they were cooled to room temperature and 3 mL of 1 M $H_2SO_4$ solution was added and vortexed and kept in a water bath for 10 min. After cooling, 2 mL of hexane was added and centrifuged at 2000 rpm for 10 min. Finally, hexane with fatty acid methyl esters (FAME) was subjected to chromatography analysis.

For FAME determination, a TRACE 1310 gas chromatograph model equipped with a flame ionization detector (Thermo Scientific) was used. One microliter of samples was injected into a split/splitless injector operated in the 1:20 split ratio mode at 250 °C. Hydrogen was used as carrier gas at a constant flow rate of 1.5 mL/min. Separation of FAMEs was performed using an RT 2560 chromatography column (100 *m* × 0.25 mm × 0.20 μm thick film, Restek, USA). The initial oven temperature was programmed at 130 °C for 5 min and increased to 180 °C at a rate of 8 °C/min. Then, increasing to 210 °C, at a rate of 4 °C/min, and finally to 250 °C, increasing 20 °C/min, and maintained for 7 min in isotherm. The detector temperature was held constant at 250 °C. FAME compounds were identified by comparing the experimental retention time with the authentic standard (FAME Mix-37, Sigma Aldrich, St. Louis, MO). The results were presented as a percentage of each FA identified in the lipid fraction, considering the equivalent factor of PPI chain size for FID and ester conversion factor for the respective acid, according to Visentainer [30,31]. Results of the fatty acid profile of feeds were presented in Supplementary Material 1.

### 2.5.8. Statistical analysis

All data were analyzed using the SAS MIXED procedure (SAS Inst. Inc., Cary, NC, USA; version 9.4), with Satterthwaite approximation to determine denominator degrees of freedom for the fixed effects test. Growth performance data (exception for body weight -BW), carcass traits were tested for fixed treatment effect using animal (treatment) as random effect. BW, blood count, serum biochemistry, and ruminal

variables were analyzed as repeated measures and tested for fixed effects of treatment, day, and treatment × day, using animal (treatment) as the random effect. The d 1 results were included as an independent covariate. Also, for these variables, to generate the average per treatment, the d 1 results were removed from the dataset, but were kept as a covariate. The first-order autoregressive covariance structure was selected according to the lowest Akaike information criterion. Means were separated using the PDIFF method (Tukey test) and all results were reported as LSMEANS followed by SEM. Significance was defined when $P \leq 0.05$ and trend when $P > 0.05$ and $\leq 0.10$.

## 3. Results

### 3.1. Performance of steers supplemented with exogenous enzymes

The zootechnical results are shown in Fig. 1 and Supplementary material 3. The body weight gain tended to be greater with the treatment in the T4-BLEND+AMIL in the period between days 15 and 120, ($P = 0.08$–Fig. 1), when compared to the T3-AMIL treatment, as well as verified, T4-BLEND+AMIL had greater body weight gain throughout the entire experimental period, from days 1 to 120 ($P = 0.05$–Fig. 1) when compared to T3-AMIL. Mean daily gain tended to be higher on treatment at T4, on days 16 to 120 ($P = 0.09$), when compared to T3-AMIL. Total dry matter intake, feed conversion and feed efficiency did not differ between treatments.

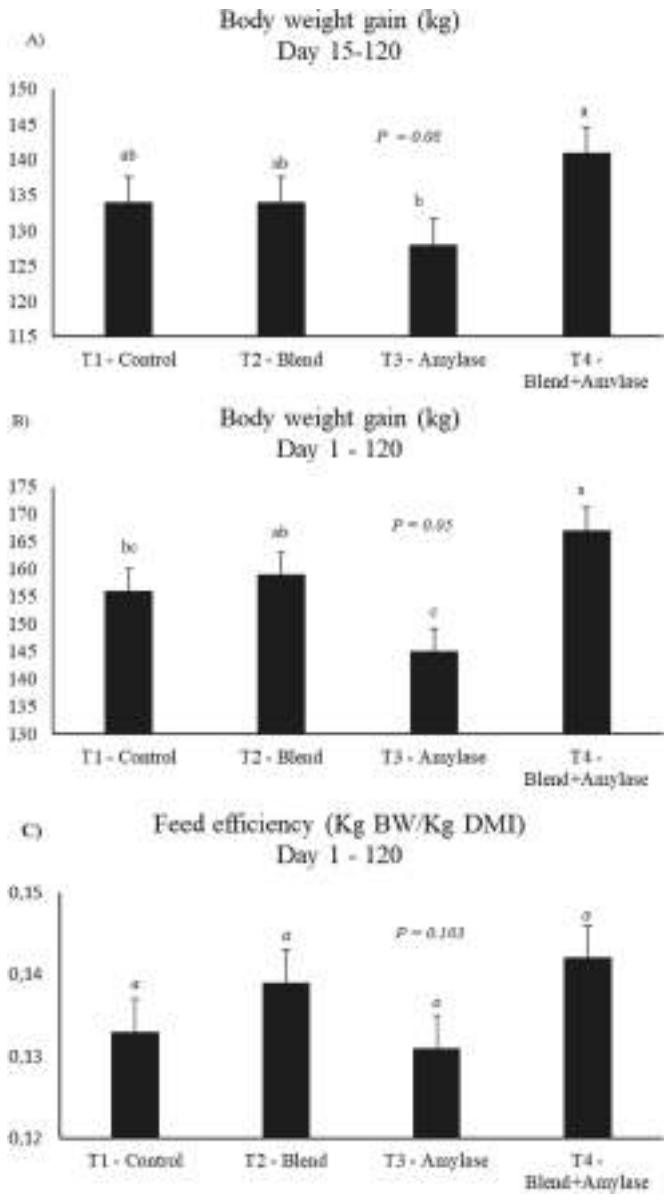



**Fig. 1.** Mean and standard error (SEM) of body weight gain and feed efficiency of steers ($n = 6$ per group) fed with exogenous enzymes during the finishing period in a confinement system. $P \leq 0.05$ (different) and $P \geq 0.05$ to $\leq 0.1$ (trend) were illustrated by different letters on the bar (a, b, c), which reports each group.

#### 3.1.1. Blood count of steers

The results of the hemograms are shown in Table 2. Higher erythrocyte counts showed a trend of interaction between treatment x day, being higher ($P = 0.08$) in the T3-AMIL animals compared to the other treatments on day 15, on day 120 the treatment T4-BLEND+AMIL presented higher counts when compared to T2-BLEND. The percentage of hematocrit showed a trend towards treatment effect, that is, lower ($P = 0.10$) in T3-AMIL at 15 days. There was also an interaction of days versus treatment for hematocrit, with T2-BLEND being lower in the blood of cattle ($P = 0.05$) in the 120 days of the experiment. Hemoglobin levels showed a trend of interaction between days and treatment ($P = 0.08$) on days 15 and 120 of the experiment, following a similar behavior for erythrocyte count and hematocrit. The numbers of leukocytes, lymphocytes, monocytes, granulocytes, distribution of red cells and number of platelets did not differ between treatments.

#### 3.1.2. Serum biochemistry

The biochemistry results are shown in Table 3. There was a tendency for treatment to influence glucose levels, the T1-CON group was superior to T2-BLEND and T3-AMIL, while T4-BLEND+AMIL was superior to T2-BLEND and equal to T3-AMIL ($P = 0.005$). Albumin, globulin, total protein, urea and cholesterol levels did not differ between treatments.

#### 3.1.3. Apparent digestibility coeficiente

Apparent digestibility coefficients are presented in Table 4. There was a treatment effect for digestibility of ether extract (EE) and starch. The T3-AMIL and T4-BLEND+AMIL treatments showed higher EE digestibility compared to the others ($P = 0.01$), which did not differ from each other. Starch digestibility was higher in groups T3-AMIL and T4-BLEND+AMIL compared to T1-CON; T2-BLEND did not differ ($P = 0.05$). The crude protein digestibility coefficient tended to be higher in groups T2-BLEND and T3-AMIL compared to T1-CON ($P = 0.07$). The coefficients of dry matter, organic matter, insoluble fiber in neutral detergent, insoluble fiber in acid detergent did not differ between treatments.

#### 3.1.4. Profile of short-chain fatty acids in ruminal fluid

The results of the profile of short-chain fatty acids in the rumen are shown in Table 5. The T4-BLEND+AMIL group had a higher concentration of acetic acid than the other groups that were equal to each other ($P = 0.05$). T3-AMIL and T4-BLEND+AMIL had a higher concentration of propionic acid ($P = 0.01$) than T1-CON, with T2-BLEND being similar for all groups. Butyric acid concentration had a strong tendency to be higher in T4-BLEND+AMIL ($P = 0.06$) compared to T1-CON. There was no treatment effect for isovaleric and valeric acid concentration between groups.

Total short-chain fatty acids (SCFA) in the ruminal fluid was higher in T4-BLEND+AMIL animals, referring to the interaction between treatment and day (day 60) compared to the other groups. Interaction treatment x day was also verified on day 120, with higher SCFA in all groups that consumed the enzymes compared to T1-CON. Treatment effect was verified in the SCFA concentration, higher in all treatments (T1, T2 and T3) compared to control (T1).

#### 3.1.5. Meat composition and carcass weight/yield

The results of the centesimal composition of meat and carcass are

**Table 2**
Mean and standard error (SEM) of blood count of steers supplemented with amylase and blend enzymes.

| Items | Treatments[1] | | | | SEM | P – values | |
|---|---|---|---|---|---|---|---|
| | T1 ($n = 6$) Control | T2 ($n = 6$) Blend | T3 ($n = 6$) Amylase | T4 ($n = 6$) Blend+Amylase | | Treat | Treat × Day |
| Erythrocytes (x10$^6$ µL) | | | | | | 0.31 | **0.08** |
| d 1 | 7.92 | 7.03 | 6.73 | 7.69 | 0.44 | | |
| d 15 | 8.21$^a$ | 7.58$^a$ | 6.47$^b$ | 8.03$^a$ | 0.42 | | |
| d 60 | 6.82 | 6.69 | 6.64 | 7.17 | 0.44 | | |
| d 120 | 7.09$^{ab}$ | 6.52$^b$ | 7.29$^{ab}$ | 7.89$^a$ | 0.42 | | |
| Average[2] | 7.40 | 6.93 | 6.80 | 7.70 | 0.34 | | |
| Hematocrit (%) | | | | | | **0.10** | **0.05** |
| d 1 | 32.3 | 30.1 | 30.1 | 31.9 | 1.44 | | |
| d 15 | 32.9$^a$ | 32.0$^{ab}$ | 29.3$^b$ | 32.6$^a$ | 1.41 | | |
| d 60 | 29.9 | 29.8 | 31.5 | 30.6 | 1.62 | | |
| d 120 | 32.0$^{bc}$ | 28.8$^c$ | 34.1$^{ab}$ | 36.2$^a$ | 1.41 | | |
| Average[2] | 31.6$^{ab}$ | 30.2$^b$ | 31.7$^{ab}$ | 33.1$^a$ | 0.66 | | |
| Hemoglobin (g/dL) | | | | | | 0.26 | **0.08** |
| d 1 | 11.0 | 10.4 | 10.2 | 11.0 | 0.55 | | |
| d 15 | 11.3$^a$ | 10.9$^{ab}$ | 9.75$^b$ | 11.7$^a$ | 0.55 | | |
| d 60 | 10.7 | 10.8 | 11.0 | 11.3 | 0.63 | | |
| d 120 | 11.5$^{ab}$ | 10.4$^b$ | 12.3$^a$ | 12.9$^a$ | 0.55 | | |
| Average[2] | 11.2 | 10.6 | 11.0 | 12.0 | 0.47 | | |
| Leukocytes (x10$^3$ µL) | | | | | | 0.93 | 0.88 |
| d 1 | 3.84 | 3.79 | 3.32 | 2.87 | 0.71 | | |
| d 15 | 4.79 | 4.57 | 4.71 | 4.39 | 0.71 | | |
| d 60 | 5.51 | 6.30 | 6.50 | 6.22 | 0.81 | | |
| d 120 | 4.56 | 5.41 | 5.74 | 6.21 | 0.71 | | |
| Average[2] | 5.09 | 5.55 | 5.61 | 5.39 | 0.58 | | |
| Lymphocytes (x10$^3$ µL) | | | | | | 0.49 | 0.88 |
| d 1 | 2.63 | 2.69 | 2.43 | 2.39 | 0.49 | | |
| d 15 | 3.50 | 3.14 | 3.53 | 2.61 | 0.49 | | |
| d 60 | 3.65 | 3.55 | 4.38 | 3.98 | 0.56 | | |
| d 120 | 3.25 | 3.25 | 4.11 | 3.74 | 0.49 | | |
| Average[2] | 3.50 | 3.36 | 3.98 | 3.39 | 0.30 | | |
| Monocytes (x10$^3$ µL) | | | | | | | |
| d 1 | 0.28 | 0.27 | 0.28 | 0.24 | 0.09 | 0.19 | 0.29 |
| d 15 | 0.31 | 0.27 | 0.32 | 0.28 | 0.10 | | |
| d 60 | 0.85 | 1.21 | 0.95 | 0.81 | 0.12 | | |
| d 120 | 0.63 | 0.88 | 0.74 | 0.77 | 0.10 | | |
| Average[2] | 0.60 | 0.79 | 0.67 | 0.62 | 0.07 | | |
| Granulocytes (x10$^3$ µL) | | | | | | 0.12 | 0.59 |
| d 1 | 1.15 | 0.95 | 0.69 | 0.89 | 0.20 | | |
| d 15 | 1.30 | 1.30 | 0.87 | 1.09 | 0.21 | | |
| d 60 | 1.33 | 1.69 | 1.16 | 0.99 | 0.24 | | |
| d 120 | 0.99 | 1.42 | 0.87 | 1.29 | 0.21 | | |
| Average[2] | 1.20 | 1.47 | 0.97 | 1.13 | 0.18 | | |
| RDW-CV | | | | | | 0.88 | 0.50 |
| d 1 | 21.2 | 22.3 | 21.8 | 21.3 | 1.30 | | |
| d 15 | 21.2 | 23.6 | 22.4 | 21.6 | 1.39 | | |
| d 60 | 26.2 | 24.7 | 25.9 | 25.3 | 1.67 | | |
| d 120 | 22.9 | 21.0 | 23.2 | 22.2 | 1.39 | | |
| Average[2] | 23.4 | 23.1 | 23.8 | 23.0 | 1.00 | | |
| Platelets (x10$^3$ µL) | | | | | | 0.89 | 0.16 |
| d 1 | 395 | 345 | 329 | 372 | 55.7 | | |
| d 15 | 380 | 289 | 305 | 423 | 62.0 | | |
| d 60 | 235 | 119 | 145 | 161 | 70.7 | | |
| d 120 | 130 | 283 | 223 | 229 | 62.0 | | |
| Average[2] | 248 | 230 | 224 | 271 | 45.9 | | |

[1] Treatments were: T1 Control- Control treatment, T2 blend– Treatment with 0.5 g of blend per kg of DM in the diet, T3 Amylase- Treatment with 0.5 g of amylase per kg of DM in the diet, T4 blend + Amylase- Treatment with 0.5 g enzyme blend+ 0 0.5 g amylase per kg of DM in the diet.
[2] The d 1 results were removed from the data set to generate the average per treatment in the statistical analysis.
[a,b,c] Note: $P \leq 0.05$ (different) and $P \geq 0.05$ to $\leq 0.1$ (trend) were illustrated by different letters (a,b,c) on the same line.

shown in Table 6. There was no effect of treatment on weight and carcass yield. There was influence of the treatment on the percentage of fat in the meat ($P = 0.007$) in animals from T3-AMIL, higher levels when compared to T2-BLEND and T4-BLEND+AMIL. The concentration of mineral matter was higher in the meat of cattle that consumed the enzymatic combination T4-BLEND+AMIL when compared to T1-CON and T2-BLEND. As for the content of dry matter and protein there was no difference between treatments.

### 3.1.6. Meat fatty acid profile

The results of the fatty acid profile in meat are shown in Table 7. We verified the influence of treatments for the presence of saturated, monounsaturated and polyunsaturated fatty acids in different treatments. We highlight the lower amount of saturated fatty acids in the meat of cattle that consumed the mixture of enzymes T4-BLEND+AMIL compared to T1-CON and T3-AMIL. A higher proportion of monounsaturated fatty acids was observed in the T3-AMIL group when compared to the others. The percentage of polyunsaturated fatty acids was higher in the T2-BLEND and T4-BLEND+AMIL groups when compared to the T1-CON control, and lower in the meat of the T3-AMIL cattle compared to the other groups.

These results are related to individual changes in fatty acids, which when added together changed the fatty acid profile of the meat. We found that C8:0 levels were higher ($P = 0.010$) in T4-BLEND+AMIL

**Table 3**
Mean and standard error (SEM) of serum biochemistry of steers supplemented with amylase and blend enzymes.

| Items | Treatments[1] | | | | SEM | P – values | |
|---|---|---|---|---|---|---|---|
| | T1 ($n = 6$) Control | T2 ($n = 6$) Blend | T3 ($n = 6$) Amylase | T4 ($n = 6$) Blend+Amylase | | Treat | Treat × Day |
| Albumin (g/dL) | | | | | | 0.88 | 0.45 |
| d 1 | 6.36 | 5.86 | 6.07 | 5.58 | 0.29 | | |
| d 15 | 2.50 | 2.63 | 2.64 | 2.78 | 0.29 | | |
| d 60 | 2.76 | 2.83 | 2.52 | 3.40 | 0.29 | | |
| d 120 | 3.05 | 3.01 | 2.89 | 2.90 | 0.29 | | |
| Average[2] | 2.89 | 2.79 | 2.72 | 2.91 | 0.14 | | |
| Globulin (g/dL) | | | | | | 0.48 | 0.20 |
| d 1 | 3.30 | 2.91 | 3.51 | 3.21 | 0.79 | | |
| d 15 | 6.02 | 5.81 | 5.99 | 5.71 | 0.79 | | |
| d 60 | 9.12 | 11.8 | 12.3 | 9.99 | 0.79 | | |
| d 120 | 4.15 | 4.99 | 3.9 | 5.14 | 0.79 | | |
| Average[2] | 6.45 | 7.43 | 7.48 | 6.94 | 0.53 | | |
| Total protein (g/dL) | | | | | | 0.73 | 0.36 |
| d 1 | 9.68 | 8.74 | 9.61 | 8.78 | 0.83 | | |
| d 15 | 8.53 | 8.41 | 8.65 | 8.48 | 0.83 | | |
| d 60 | 11.9 | 14.6 | 14.8 | 13.4 | 0.83 | | |
| d 120 | 7.21 | 7.98 | 6.8 | 8.03 | 0.83 | | |
| Average[2] | 9.38 | 10.2 | 10.3 | 9.81 | 0.57 | | |
| Glucose (mg/dL) | | | | | | **0.06** | 0.86 |
| d 1 | 97.7 | 98.6 | 97.0 | 93.9 | 6.37 | | |
| d 15 | 84.8 | 77.1 | 82.4 | 87.7 | 6.37 | | |
| d 60 | 105 | 86.6 | 89.4 | 106 | 6.37 | | |
| d 120 | 96.4 | 72.9 | 85.4 | 84.9 | 6.37 | | |
| Average[2] | 95.5$^{a}$ | 78.9$^{c}$ | 85.7$^{bc}$ | 92.6$^{ab}$ | 3.24 | | |
| Urea (mg/dL) | | | | | | 0.98 | 0.18 |
| d 1 | 23.6 | 20.8 | 22.6 | 21.6 | 1.87 | | |
| d 15 | 26.2 | 23.3 | 22.9 | 23.3 | 1.87 | | |
| d 60 | 26.6 | 25.3 | 25.6 | 28.1 | 1.87 | | |
| d 120 | 27.1 | 31.7 | 32.1 | 28.4 | 1.87 | | |
| Average[2] | 27.1 | 26.3 | 27.1 | 26.4 | 1.51 | | |
| Cholesterol (mg/dL) | | | | | | 0.75 | 0.51 |
| d 1 | 108 | 97.6 | 86.8 | 97.2 | 11.7 | | |
| d 15 | 103 | 87.1 | 90.4 | 117 | 11.7 | | |
| d 60 | 98.6 | 106 | 97.1 | 103 | 11.7 | | |
| d 120 | 116 | 113 | 138 | 125 | 11.7 | | |
| Average[2] | 109 | 102 | 105 | 115 | 9.40 | | |

[1] Treatments were: T Control- Control treatment, T2 blend– Treatment with 0.5 g of blend per kg of DM in the diet, T3 Amylase- Treatment with 0.5 g of amylase per kg of DM in the diet, T4 blend + Amylase- Treatment with 0.5 g enzyme blend + 0 0.5 g amylase per kg of DM in the diet.
[2] The d 1 results were removed from the data set to generate the average per treatment in the statistical analysis.
[a,b,c] Note: $P \leq 0.05$ (different) and $P \geq 0.05$ to $\leq 0.1$ (trend) were illustrated by different letters (a,b,c) on the same line.

**Table 4**
Mean and standard error (SEM) of apparent digestibility coeficiente (ADC) of cattle fed exogenous enzymes.

| Variables (%) | T1 ($n = 6$) Control | T2 ($n = 6$) Blend | T3 ($n = 6$) Amylase | T4 ($n = 6$) Blend+Amylase | SEM | P- trat |
|---|---|---|---|---|---|---|
| Dry Matter | 61.7 | 65.4 | 67.6 | 66.4 | 2.41 | 0.39 |
| Organic Matter | 65.4 | 68.8 | 71.2 | 70.3 | 2.93 | 0.26 |
| Crude protein | 54.2$^{b}$ | 58.1$^{a}$ | 61.3$^{a}$ | 57.1$^{ab}$ | 1.08 | **0.07** |
| NDF | 59.6 | 62.9 | 61.5 | 64.5 | 2.58 | 0.46 |
| ADF | 46.1 | 46.6 | 51.0 | 49.5 | 2.36 | 0.37 |
| Ether extract | 36.6$^{b}$ | 42.8$^{b}$ | 53.0$^{a}$ | 55.2$^{a}$ | 2.79 | **0.01** |
| Starch | 73.0$^{b}$ | 76.9$^{ab}$ | 81.3$^{a}$ | 81.8$^{a}$ | 2.01 | **0.05** |

[1]Treatments were: T1 Control- Control treatment, T2 blend– Treatment with 0.5 g of blend per kg of DM in the diet, T3 Amylase- Treatment with 0.5 g of amylase per kg of DM in the diet, T4 blend + Amylase- Treatment with 0.5 g enzyme blend+ 0 0.5 g amylase per kg of DM in the diet.
[a,b,c] Note: $P \leq 0.05$ (different) and $P \geq 0.05$ to $\leq 0.1$ (trend) were illustrated by different letters (a,b,c) on the same line.

when compared to T3-AMIL, the same was repeated for C14:0 ($P = 0.010$), but T4-BLEND+AMIL showed lower concentration than T3-AMIL, for C16:0 ($P = 0.071$) the T4-BLEND+AMIL treatment and T2-BLEND showed a lower concentration than the control treatment, for C18:0 ($P = 0.100$) the T2-BLEND and T3-AMIL were superior when compared to T4-BLEND+AMIL, for C:20 ($P = 0.023$) all treatments had a higher concentration than the control, for C22:0 ($P = 0.050$) T4-BLEND+AMIL was superior when compared to all other treatments, for C24:0 ($P = 0.050$) T2-BLEND was superior when compared to T3-AMIL.

The concentration of C18:1n9c ($P = 0.001$) had a treatment effect, with higher concentrations in T2-BREND when compared to control, T1-CON and T4-BLEND+AMIL; for C20:1n9 ($P = 0.060$) all treatments showed lower concentration compared to control (T1-CON).

The concentration of C18:3n3 ($P = 0.001$) in meat was higher in T4-BLEND+AMIL when compared to T3-AMIL, T2-BLEND, and T1-CON; for the levels of C20:3n6 ($P = 0.043$) we found that T4-BLEND+AMIL had a higher concentration when compared to T3-AMIL, but did not differ from the other treatments; the levels of C20:4n6 (0.001) in T4-BLEND+AMIL were higher when compared to T3-AMIL, T2-BLEND, and T1-CON.

### *3.1.7. Oxidative status in liver and meat*

The results of oxidative status in liver and meat are shown in Table 8. In the liver, in general, we observed a lower concentration of TBARS in

**Table 5**
Mean and standard error (SEM) of total short-chain fatty acids (SCFA) in ruminal fluid and profile of volatile fatty acids in the rumen of cattle that consumed exogenous enzymes.

| Variables | T1 ($n = 6$) Control | T2 ($n = 6$) Blend | T3 ($n = 6$) Amylase | T4 ($n = 6$) Blend+Amylase | SEM | P- trat | P- trat x day |
|---|---|---|---|---|---|---|---|
| Acetic acid (mmol/L | | | | | | **0.05** | **0.05** |
| d60 | 64.7$^{ab}$ | 51.5$^{b}$ | 58.2$^{ab}$ | 69.0$^{a}$ | 1.98 | | |
| d120 | 68.4$^{b}$ | 77.2$^{a}$ | 75.1$^{ab}$ | 77.9$^{a}$ | 1.57 | | |
| Average | 66.5$^{b}$ | 64.3$^{b}$ | 66.6$^{b}$ | 73.4$^{a}$ | 1.45 | | |
| Propionic acid (mmol/L) | | | | | | **0.01** | **0.01** |
| d60 | 15.6$^{bc}$ | 13.7$^{c}$ | 17.0$^{ab}$ | 19.7$^{a}$ | 0.74 | | |
| d120 | 18.0$^{b}$ | 26.9$^{a}$ | 29.4$^{a}$ | 28.8$^{a}$ | 0.81 | | |
| Average | 16.8$^{b}$ | 20.3$^{ab}$ | 23.2$^{a}$ | 24.2$^{a}$ | 0.75 | | |
| Butiric acid (mmol/L) | | | | | | **0.06** | **0.04** |
| d60 | 10.9 | 10.81 | 10.6 | 12.6 | 0.65 | | |
| d120 | 13.3$^{b}$ | 15.5$^{ab}$ | 15.6$^{ab}$ | 17.1$^{a}$ | 0.66 | | |
| Average | 12.1$^{b}$ | 13.1$^{ab}$ | 13.1$^{ab}$ | 14.9$^{a}$ | 0.62 | | |
| Isovaleric acid (mmol/L) | | | | | | 0.59 | 0.23 |
| d60 | 1.82 | 1.60 | 1.49 | 1.70 | 0.11 | | |
| d120 | 1.72 | 2.23 | 1.74 | 2.05 | 0.09 | | |
| Average | 1.77 | 1.91 | 1.61 | 1.87 | 0.1 | | |
| Valeric acid (mmol/L) | | | | | | 0.44 | 0.62 |
| d60 | 1.77 | 1.51 | 1.59 | 1.85 | 0.21 | | |
| d120 | 2.01 | 2.50 | 2.35 | 2.61 | 0.36 | | |
| Average | 1.89 | 2.00 | 1.97 | 2.23 | 0.25 | | |
| SCFA (mmol/L) | | | | | | **0.01** | **0.01** |
| d60 | 94.7$^{b}$ | 79.1$^{c}$ | 88.8$^{b}$ | 104.8$^{a}$ | 2.04 | | |
| d120 | 103.4$^{b}$ | 124.3$^{a}$ | 124.2$^{a}$ | 128.4$^{a}$ | 1.96 | | |
| Average | 99.0$^{b}$ | 101.7$^{b}$ | 106.5$^{b}$ | 116.6$^{a}$ | 2.01 | | |

[1]Treatments were: T1 Control- Control treatment, T2 blend– Treatment with 0.5 g of blend per kg of DM in the diet, T3 Amylase- Treatment with 0.5 g of amylase per kg of DM in the diet, T4 blend + Amylase- Treatment with 0.5 g enzyme blend+ 0 0.5 g amylase per kg of DM in the diet.
[2]The d 1 results were removed from the data set to generate the average per treatment in the statistical analysis. [a,b,c] Note: $P \leq 0.05$ (different) and $P \geq 0.05$ to $\leq 0.1$ (trend) were illustrated by different letters (a,b,c) on the same line.

**Table 6**
Mean and standard error (SEM) of meat and carcass composition of steers supplemented with amylase and protease enzymes.

| Items | Treatments[1] | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | T1 ($n = 6$) Control | T2 ($n = 6$) Blend | T3 ($n = 6$) Amylase | T4 ($n = 6$) Blend+Amylase | | Trat |
| Carcass variables | | | | | | |
| Carcass weight (kg) | 251 | 244 | 239 | 246 | 9.79 | 0.86 |
| Carcass yield (%) | 50.5 | 49.2 | 49.5 | 48.9 | 0.71 | 0.43 |
| Meat variables | | | | | | |
| Dry matter (%) | 31.8 | 30.6 | 32.3 | 33.1 | 1.13 | 0.48 |
| Protein (%) | 25.1 | 25.7 | 23.8 | 25.7 | 0.78 | 0.30 |
| Fat (%) | 2.79$^{b}$ | 2.05$^{b}$ | 3.84$^{a}$ | 1.97$^{b}$ | 0.38 | **0.007** |
| Ash (%) | 3.91$^{bc}$ | 2.85$^{c}$ | 4.66$^{ab}$ | 5.43$^{a}$ | 0.40 | **0.001** |

[1] Treatments were: T1 Control- Control treatment, T2 blend– Treatment with 0.5 g of blend per kg of DM in the diet, T3 Amylase- Treatment with 0.5 g of amylase per kg of DM in the diet, T4 blend + Amylase- Treatment with 0.5 g enzyme blend+ 0 0.5 g amylase per kg of DM in the diet.
[a,b,c]Note: $P \leq 0.05$ (different) and $P \geq 0.05$ to $\leq 0.1$ (trend) were illustrated by different letters (a,b,c) on the same line.

T2-BLEND animals ($P = 0.050$), when compared to T1-CON. The lowest production of ROS ($P = 0.001$) was verified in T3-CON, when compared to T2-BLEND and control, on the other hand SOD activity ($P = 0.015$) did not differ between T2-BLEND, T3-AMIL and T4-BLEND+AMIL, but it was higher in the T1-CON control. We also verified the highest CAT activity ($P = 0.052$) in the liver of the T4-BLEND+AMIL animals when compared to the other treatments. As for meat, we found that animals from T4-BLEND+AMIL ($P = 0.094$) tended towards a higher proportion of ROS than T3-AMIL and T1-CON, in contrast to meat from steers from T4-BLEND+AMIL ($P = 0.001$) showed higher GST activity when compared to T3-AMIL and control. PSH levels ($P = 0.050$) were higher in T3-AMIL when compared to T4-BLEND+AMIL and T2-BLEND; while the highest SOD activity ($P = 0.045$) was higher in T4-BLEND+AMIL and T2-BLEND when compared to control T1-CON.

## 4. Discussion

Addition of exogenous enzymes amylase, protease, cellulase, xylanase and beta glucanase positively affected weight gain, but without affecting the final weight. Similar results were verified by Rodríguez-Carías et al. [32] who used a commercial product based on exogenous enzymes beta-glucanase, xylanase, pectinase, mannanase, xyloglucanase, laminarase, β-glucosidase, β-xylosidase, α-L-arabinofuranosidase, amylase and protease in lambs, and found an effect on animal performance. These authors explain that this greater weight gain occurs due to the improvement in nutrient digestibility, since the product did not affect DM intake [32]. It is believed that when using proteolytic enzymes they act by removing protein structures from the cell wall of the forage, allowing faster access for ruminal microorganisms [33], which leads us to justify the gains in performance when using the enzymes in combination.

Researchers found that the inclusion of amylase promotes rapid release of starch oligosaccharides that are used by both amylolytic and non-amylolytic bacteria [34], for this reason starch is fermented quickly. This was observed in our study, in both groups of steers that consumed amylase in the diet, the starch digestibility coefficient was higher; as well as increasing the concentration of total volatile fatty acids in the rumen; but this was not enough when the exogenous enzyme was added to the diet to enhance weight gain. The reasons for this are not known and complementary results do not help explain the mechanisms

**Table 7**
Mean and standard error (SEM) of profile of fatty acids in the meat of steers supplemented with amylase and blend enzymes.

| Fatty acid (g/kg) | Treatments | | | | SEM | P-value |
|---|---|---|---|---|---|---|
| | **T1 ($n = 6$) Control** | **T2 ($n = 6$) Blend** | **T3 ($n = 6$) Amylase** | **T4 ($n = 6$) Blend+Amylase** | | |
| C8:0 (Caprylic) | 0.908$^{ab}$ | 1.605$^{a}$ | 0.579$^{b}$ | 1.744$^{a}$ | 0.121 | **0.010** |
| C12:0 (Lauric) | 0.472 | 0.650 | 0.532 | 0.521 | 0.048 | 0.654 |
| C14:0 (Myristic) | 20.12$^{ab}$ | 19.18$^{ab}$ | 23.598$^{a}$ | 17.861$^{b}$ | 0.823 | **0.010** |
| C14:1 (Myristoleic) | 5.232 | 4.776 | 5.616 | 4.497 | 0.337 | 0.102 |
| C15:0 (Pentadecanoic) | 2.279 | 2.401 | 2.498 | 2.204 | 0.083 | 0.856 |
| C16:0 (Palmitic) | 314.8$^{a}$ | 300.0$^{b}$ | 310.0$^{ab}$ | 300.9$^{b}$ | 2.397 | **0.071** |
| C16:1 (Palmitoleic) | 33.59 | 34.29 | 36.56 | 33.02 | 0.969 | 0.258 |
| C17:0 (Heptadecanoic) | 6.437 | 6.309 | 7.475 | 6.261 | 0.240 | 0.182 |
| C18:0 (Stearic) | 148.1$^{a}$ | 150.9$^{a}$ | 148.6$^{a}$ | 144.1$^{b}$ | 1.966 | **0.100** |
| C18:1n9c (Oleic) | 384.0$^{b}$ | 380.0$^{b}$ | 406.0$^{a}$ | 378.9$^{b}$ | 4.689 | **0.001** |
| C18:2n6c (Linoleic) | 53.33$^{b}$ | 67.96$^{a}$ | 37.33$^{c}$ | 73.74$^{a}$ | 4.202 | **0.001** |
| C20:0 (Arachidic) | 0.645$^{b}$ | 1.173$^{a}$ | 0.923$^{a}$ | 0.982$^{a}$ | 0.061 | **0.023** |
| C18:3n6 (?-Linolenic) | 0.558 | 0.626 | 0.420 | 0.589 | 0.047 | 0.362 |
| C20:1n9 (cis-11-Eicosenoic) | 2.382$^{a}$ | 1.000$^{b}$ | 1.304$^{b}$ | 1.347$^{b}$ | 0.054 | **0.060** |
| C18:3n3 (a-Linolenic) | 1.772$^{b}$ | 1.548$^{b}$ | 1.337$^{b}$ | 3.201$^{a}$ | 0.245 | **0.001** |
| C21:0 (Henicosanoic) | 2.014 | 1.864 | 2.534 | 1.867 | 0.105 | 0.201 |
| C20:2 (cis-11,14-Eicosadienoic) | 0.799 | 0.832 | 0.592 | 0.632 | 0.027 | 0.120 |
| C22:0 (Behenic) | 0.362$^{b}$ | 0.369$^{b}$ | 0.195$^{c}$ | 0.457$^{a}$ | 0.048 | **0.050** |
| C20:3n6 (cis-8,11,14-Eicosatrienoic) | 4.371$^{a}$ | 4.567$^{a}$ | 2.770$^{b}$ | 4.531$^{a}$ | 0.258 | **0.043** |
| C22:1n9 (Erucic) | 0.283 | 0.310 | 0.204 | 0.384 | 0.038 | 0.258 |
| C20:4n6 (Arachidonic) | 14.92$^{cd}$ | 16.21$^{bc}$ | 9.575$^{d}$ | 19.28$^{a}$ | 1.217 | **0.001** |
| C22:2 (cis-13,16-Docosadienoic) | 0.187 | 0.072 | 0.109 | 0.185 | 0.011 | 0.350 |
| C24:0 (Lignoceric) | 0.399$^{bc}$ | 0.551$^{a}$ | 0.266$^{c}$ | 0.461$^{ab}$ | 0.032 | **0.050** |
| C20:5n3 (cis-5,8,11,14,17-Eicosapentaenoic) | 0.692 | 0.680 | 0.374 | 0.792 | 0.051 | 0.135 |
| C24:1n9 (Nervonic) | 0.220 | 0.148 | 0.116 | 0.162 | 0.016 | 0.186 |
| C22:6n3 (cis-4,7,10,13,16,19-Docosahexaenoic) | 1.035 | 0.792 | 0.361 | 1.187 | 0.163 | 0.632 |
| AGS | 496.5$^{a}$ | 485.0$^{ab}$ | 497.2$^{a}$ | 477.4$^{b}$ | 2.053 | **0.038** |
| MUFA | 425.7$^{b}$ | 420.5$^{b}$ | 449.8$^{a}$ | 418.3$^{b}$ | 1.970 | **0.001** |
| PUFA | 77.67$^{b}$ | 93.29$^{a}$ | 52.87$^{c}$ | 104.14$^{a}$ | 2.381 | **0.001** |

[1]Treatments were: T1 Control- Control treatment, T2 blend– Treatment with 0.5 g of blend per kg of DM in the diet, T3 Amylase- Treatment with 0.5 g of amylase per kg of DM in the diet, T4 blend + Amylase- Treatment with 0.5 g enzyme blend+ 0 0.5 g amylase per kg of DM in the diet.
[a,b,c] Note: $P \leq 0.05$ (different) and $P \geq 0.05$ to $\leq 0.1$ (trend) were illustrated by different letters (a,b,c) on the same line.

**Table 8**
Mean and standard error (SEM) of oxidative status in the liver and meat of steers supplemented with amylase and blend enzymes.

| Tissue | Treatments | **TBARS** | **ROS** | **GST** | **PSH** | **SOD** | **CAT** |
|---|---|---|---|---|---|---|---|
| Liver($n = 6$ per group) | T1 Control | 1.436$^{a}$ | 4.623$^{a}$ | 1082 | 579.7 | 3.976$^{a}$ | 2.044$^{ab}$ |
| | T2 Blend | 1.183$^{b}$ | 3.235$^{b}$ | 1188 | 464.0 | 1.281$^{b}$ | 2.454$^{ab}$ |
| | T3 Amylase | 1.273$^{ab}$ | 2.206$^{c}$ | 1119 | 566.0 | 2.061$^{b}$ | 2.375$^{ab}$ |
| | T4 Blend + Amylase | 1.270$^{ab}$ | 2.434$^{c}$ | 1087 | 488.1 | 1.729$^{b}$ | 2.880$^{a}$ |
| | SEM | 0.025 | 0.067 | 4.142 | 8.369 | 0.042 | 0.013 |
| | p-value | **0.050** | **0.001** | 0.657 | 0.241 | **0.015** | **0.052** |
| Meat($n = 6$ per group) | T1 Control | 2.704 | 1.119$^{ab}$ | 2832$^{b}$ | 757.2$^{c}$ | 9.080$^{b}$ | 1.505 |
| | T2 Blend | 2.702 | 1.498$^{a}$ | 3202$^{ab}$ | 803.6$^{b}$ | 13.04$^{a}$ | 1.662 |
| | T3 Amylase | 2.442 | 0.699$^{b}$ | 2858$^{b}$ | 853.6$^{a}$ | 12.60$^{ab}$ | 1.185 |
| | T4 Blend + Amylase | 3.110 | 1.350$^{a}$ | 3478$^{a}$ | 808.0$^{b}$ | 14.38$^{a}$ | 1.876 |
| | SEM | 0.169 | 0.056 | 6.521 | 5.978 | 1.006 | 0.240 |
| | p-value | 0.147 | **0.094** | **0.001** | **0.050** | **0.045** | 0.109 |

Note: ROS (U DCFH/mg protein); TBARS (nmol MDA/mL), GST (μmolCDNB/min/mg protein), PSH (nmol SH/mg protein), SOD (U SOD/mg protein), CAT (U CAT/mg of protein).
[1]Treatments were: T1 Control- Control treatment, T2 blend– Treatment with 0.5 g of blend per kg of DM in the diet, T3 Amylase- Treatment with 0.5 g of amylase per kg of DM in the diet, T4 blend + Amylase- Treatment with 0.5 g enzyme blend+ 0 0.5 g amylase per kg of DM in the diet.
[a,b,c] Note: $P \leq 0.05$ (different) and $P \geq 0.05$ to $\leq 0.1$ (trend) were illustrated by different letters (a,b,c) on the same column.

involved.

Feed efficiency, feed conversion and dry matter intake showed no treatment effect; which is a consequence of the greater digestibility of the EE and starch nutrients, which may be related to the greater weight gain of T4-BLEND+AMIL cattle. Rose et al. (2010) found similar results when testing a product based on protease and amylase in the diet of Guzerat cattle in confinement, in which there was also no influence on the same productive parameters mentioned above. Andreazzi et al. [35] when testing amylase in dairy cows, found that there was no change in dry matter intake, therefore, in milk production, it generated an increase with diets of at least 30 % starch. In the 90 s, researchers already said that the use of exogenous enzymes could be more efficient in diets with low moisture content, less than 30 %, than in diets with high moisture content [36], as when corn silage is present, which presents approximately 70 %, which explains the results obtained in our study, since the silage had a high moisture content.

With the blood count results, we noticed a high percentage of hematocrit, with interaction of treatment × day for T4-BLEND+AMIL, which was really not expected and we have no explanation of the mechanisms involved. According to Santos [37], normal blood hematocrit levels are 30 to 35 %, in this work we noticed that there was a positive effect when using the enzymes in a combined form T4-BLEND+AMIL and a negative effect at times when using only amylase (T3-AMIL), as the percentage was less than 30, indicating mild anemia, which may have contributed to the body weight not differing.

There is a tendency for treatment to affect glucose levels, with

animals in the T2-BLEND and T3-AMIL groups having lower serum concentrations; however, we expected that the animals that consumed the enzyme diet would have a higher concentration of glucose in the blood. This increase in glucose concentration in amylase treatments may have resulted from increased ruminal starch fermentation and propionate uptake for gluconeogenesis [38]. According to Denardin Silva [39], starch is composed of amylose and amylopectin, formed by glucose joined by a-1,4 and a-1,6 bonds, part of which is broken down into glucose by hydrolysis and another part by the enzymatic action of amylase. As mentioned by Vigne et al. [14], when using amylase-based enzyme complexes in feedlot steers, there was an improvement in starch digestion. We also observed that the addition of α-amylase improved the digestibility of starch and ether extract, which demonstrates the action of this enzyme in the degradation of these compounds. As a result of the greater utilization of starch, a higher concentration of short-chain fatty acids was observed, especially propionic acid, the main precursor of glucose in ruminants. However, the concentration of glucose in the blood tended to be lower when the animals consumed the enzymes individually; which may be related to the difficulty of measuring this enzyme in ruminants, since the animal is rarely fasting; as well as the rate of passage of feed through the gastrointestinal tract differ between animals.

Rocha et al. [40] concluded that the optimal level of starch in cattle supplemented with essential oils and amylase is 35 %, thanks to the possibility that the amylase remains active until the intestine. Furthermore Toseti et al. [41] observed potential for increased microbial protein with combined supplementation of amylase and essential oils. Upregulation of several enzymes that promote carbohydrate degradation in glycolic pathways, gluconeogenesis and oxidative phosphorylation was observed in cattle supplemented with amylase and essential oils combined [40]. This justifies the increase in starch digestibility and increase in body weight gain when amylase was given in combination. Salem et al. [42] observed a 16 % increase in weight gain when providing exogenous enzymes. Cattle supplemented with an amylase-based enzymatic complex [43] concluded that starch from corn caused improvements in subcutaneous fat thickness in feedlot super-early steers. Another point that we can observe was the concentration of mineral matter, which was higher in the meat of cattle that consumed the enzymatic combination T4-BLEND+AMIL, but it was not evaluated which minerals were deposited in the muscle tissue.

Another point that we can observe is the concentration of mineral matter, which was higher in the meat of cattle that consumed the enzymatic combination T4-BLEND+AMIL, but it was not evaluated which minerals were deposited in the muscle tissue. Vigne et al. [44] used a similar enzyme complex in feedlot steers consuming diets with high starch content, and also observed greater fat deposition in the carcass, a consequence of better feed conversion and increased starch digestibility. As can be seen, the addition of exogenous enzymes can alter lipid metabolism in meat from cattle confined with high percentage of concentrate. In addition to greater fat deposition, treatments influenced the fatty acid profile in the meat. We emphasize that the lower amount of saturated fatty acids in the meat of cattle that consumed the mixture of T4-BLEND+AMIL enzymes is an excellent result for the consumer, since saturated fats must be avoided, since in the lipid metabolism it can increase the levels of LDL (known as bad cholesterol), which is a low-density lipoprotein, capable of carrying cholesterol particles from the liver and other places to the arteries, that is, when in excess in the circulation, it causes accumulation in the vessels that can, over time, clog or form thrombi [45]. The benefits of reducing LDL cholesterol in humans are observed on a large scale in the literature [46].

In addition, when isolated or combined enzymes were used in the bovine diet, we verified a higher percentage of unsaturated fatty acids, the fat considered ideal for human consumption, since we have the omegas that increased when consumed in a diet containing only the analysis, such as acid oleic. Observed effect on the concentration of polyunsaturated fatty acids (PUFA) in the T4-BLEND+AMIL group; which is extremely beneficial for human health with potential effect on cancer protection (Chikwabha et al. 2018). We believe that the mechanisms that led to changes in the fatty acid profile are indirect, altering the digestive process, and consequently the lipid metabolism; the mechanisms involved are unclear and need further research to avoid speculation.

With changes in the profile of fatty acids in meat, an effect on the oxidative status of liver and meat was already expected. This is because it is known that lipids are among the main tissues responsible for producing oxidative instability and raising levels of free radicals. Consumption of combined exogenous enzymes and isolated amylase had a positive effect on redox balance, mainly in meat, but also in liver. We verified less lipid peroxidation and reactive oxygen species, as well as greater activity of antioxidant enzymes. In the literature there is no explanation of how exogenous enzymes modulate the oxidative status, but it is known that proteases are able to improve the oxidative stability of feeds with fermented processed items [47].

## 5. Conclusion

The results allow us to conclude that the combination of digestive enzymes (amylase, protease, cellulase, xylanase and beta glucanase) in the bovine diet improves growth performance, in addition to increasing the amount of unsaturated fatty acids and reducing the amount of saturated fatty acids in the meat. Consumption of exogenous enzymes also has a positive effect on oxidative stability in meat.

## Ethics committee

Project approved at CEUA/UDESC, protocol number 8717230921.

## CRediT authorship contribution statement

**Alexandre L. Simon:** Writing – original draft, Methodology, Data curation, Conceptualization. **Priscila M. Copetti:** Validation, Methodology, Formal analysis. **Rafael V.P. Lago:** Writing – original draft, Methodology, Investigation, Data curation. **Maksuel G. Vitt:** Writing – original draft, Methodology, Investigation, Formal analysis. **Aline L. Nascimento:** Writing – original draft, Methodology, Investigation. **Luiz Eduardo Lobo e Silva:** Writing – original draft, Methodology. **Roger Wagner:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Bruna Klein:** Writing – review & editing, Validation, Supervision, Methodology. **Camila Soares Martins:** Writing – original draft, Methodology. **Gilberto V. Kozloski:** Writing – review & editing, Validation, Supervision, Resources, Methodology, Investigation, Data curation. **Aleksandro S. Da Silva:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of Competing Interest

The authors declare no competing or financial interests.

## Data availability

No data was used for the research described in the article.

## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2023.e00824.

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REGULAR ARTICLES

# Grape seed oil in the diet of primiparous Jersey cows before and after parturition: effects on performance, health, rumen environment, and milk quality

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## Abstract

The objective of the study was to determine whether adding grape seed oil (GSO) to the diet of primiparous Jersey breeds during the transition period would improve animal health by measuring effects on the rumen environment, serum biochemistry, oxidative response, and the composition and quality of milk. We used 14 Jersey heifers, weighing an average of 430 kg and 240 days of gestation. The animals were divided into two groups and offered a basal diet, including GSO in the concentrate for the GSO group (dose of 25 mL per animal day) and the same dose of soybean oil (SO) for the control group. The animals were allocated and maintained in a compost barn system, receiving an anionic diet (pre-partum) and a diet for postpartum lactating animals. Dry matter intake (DMI), milk production, serum biochemistry, serum and milk oxidative stability, ruminal fluid and milk fatty acid profile, milk qualitative aspects, and ruminal parameters such as pH, bacterial activity, and protozoan count were evaluated. The addition of GSO had a positive effect on the health of the cows, especially on the oxidative stability of the cows, by increasing total thiols ($P=0.03$), higher plasma ferric reducing capacity (FRAP) ($P=0.01$), and total antioxidant capacity (TAC) ($P=0.01$). In the oxidative stability of the milk produced by the treated animals, there was also an increase in TAC ($P=0.05$) and FRAP ($P=0.03$). Discreet changes were observed in the ruminal environment with a decreasing trend in pH ($P=0.04$) but an increase in bacterial activity ($P=0.05$) and protozoa counts ($P=0.07$) in cows that consumed the additive. GSO consumption affected the fatty acid profile in milk, increasing saturated fatty acids (SFA) ($P=0.05$) and reducing unsaturated fatty acids (UFA) ($P=0.03$). The oil did not affect milk production or efficiency in the postpartum period. Based on this information, it is concluded that the addition of GSO positively affects the cow's antioxidant system.

**Keywords** Antioxidant · Biochemistry · Dairy Cows · Grape Seed Oil · Transition

## Introduction

The dairy cow's transition period occurs from three weeks before to three weeks after calving and the beginning of lactation (Mota et al., 2006); it is a critical period from an immunological point of view for these animals, who have difficulty maintaining homeostasis, with changes in metabolic and hormonal processes associated with parturition, the beginning of milk synthesis, and adaptation to new nutritional management (Ortolani 2009). The challenges of transition in cows make researchers and companies in animal nutrition look for ways to reduce the occurrence of disorders and abnormalities. Among the ways are the introduction of food alternatives, supplements, and additives

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that have a positive effect on the cows' organism and that promote better health, reduction of disorders and diseases, which consequently favor a smaller number of medicinal interventions, and reduction of productive and reproductive losses throughout lactation.

The use of vegetable oils in cow feed is already known (Kra et al. 2021; Pi et al. 2019; Vargas-Bello-Pérez et al. 2016, 2020). Grape seed oil (GSO) is a co-product produced from residue from the wine industry. It is not yet used in pure cow feed but in grape residue fed to cows in silage or flour (Gessner et al. 2015; Santos et al., 2014).

Molosse et al. (2023) found that replacing wheat bran and soybean hulls with grape pomace silage stimulates beef cattle's immune and antioxidant system, with health benefits and possible reduction in animal feed costs. The immune and antioxidant stimulus was also ascertained in suckling lambs when they received a grape meal (Molosse et al. 2021). The demand is due to the sustainable bias of the reuse of waste, which has high biological value, phenols, flavonoids, and anthocyanins with high antioxidant potential, which can be helpful to nutraceuticals (Sirohi et al. 2020). A review by Silva et al. (2022) also highlights the antioxidant benefits in meat products from animals that received the inclusion of grape residue-based products. Recent literature affirms the potential of this co-product and its different forms of use in different categories of production animals, mainly to improve animal health, which, consequently, can increase their productive performance.

Grape seeds represent 15% of the solid residue and contain 14 to 17% oil, depending on the variety used (Göktürk and Akkurt 2001). The seed and oil are composed of linoleic and oleic acid. The omega 3 (ω3), omega 6 (ω6) and omega (ω9) fatty acid families, including oleic acid (ω 9), linoleic acid (ω6) and α-linolenic acid (ω3), are described as essential fatty acids (Rockenbach, 2010; Palmquist and Mattos 2006). The oil also contains considerable vitamin E (Freitas 2007), a vitamin that promotes reproductive rates and milk production in cows when offered during the transition period (Moghimi-Kandelousi et al. 2020). Studies in human medicine found that GSO has several pharmacological activities against the oxidation of low-density lipoproteins, preventing cardiovascular diseases and reducing cholesterol (Jabeen et al., 2021).

Omega-3 fatty acids, such as linoleic acid, have been linked to cardiovascular health benefits. Studies show that adequate consumption can help reduce the incidence of cardiovascular disorders in cattle (Glasser et al. 2008). There is involvement in hormonal regulation that contributes to reproductive success. They have anti-inflammatory and immune system-modulating properties, helping to reduce inflammation and promoting a healthy immune response (Bobe et al. 2009; Benjamin et al. 2016). Increasing the intake of unsaturated fats may result in an increase in unsaturated fats and a reduction in saturated fats in milk, which may benefit human health. Traditionally, milk and dairy products are among the main contributors to human diets, and in these products, linoleic acid and α-linolenic acid compose the milk fat. The ω3 can reduce excess fat in the liver and improve liver function (Glasser et al. 2008; Benjamin et al. 2016).

Primiparous cows in the transition phase are more susceptible to oxidative stress than multiparous cows because of their lower total antioxidant capacity (TAC) (Moretti et al. 2017). According to Santos et al. (2014), the use of grape residues in the diet of dairy cows improves the antioxidant response. The development of dietary strategies using grape by-products was effective in the production of milk and dairy products characterized by implemented nutritional properties and better oxidative stability, with health benefits for consumers due to the presence of compounds credited with high biological value. Furthermore, it has environmental and sustainable benefits with reusing waste (Ianni, 2020). In other animal categories, GSO, when used to control dyslipidemia and hyperglycemia in diabetic rats, effectively controls metabolic disorders (Shiri-Shahsavar et al., 2023). Dietary inclusion of GSO improved the productivity of growing rabbits compared to control and antibiotic-treated groups (Nasr et al. 2022).

The chemical composition of the oil leads us to believe that it has potential for use in dairy production, and it would also be a possibility of adding value to grape residue as a feed in dairy cattle farming (Olegário, 2015). Therefore, the objective of the study was to verify whether the addition of GSO to the diet of primiparous Jersey breeds during the transition period had a positive effect on animal health and investigate the effects on the rumen environment, serum metabolism, oxidative response, and the composition and quality of milk.

## Materials and methods

### Location and facilities

The experiment was carried out in the dairy cattle sector of the Experimental Farm of the Center for Higher Education of the West – FECEO of the State University of Santa Catarina – UDESC, located in Guatambu – SC / Brazil. The animals were housed for the experimental period in a compost barn system, with bedding made up of sawmill waste (sawdust) turned over daily and with an area of 20 $m^2$ per animal. The shed consists of a rest area (bed), waiting room, milking room, feeding lane equipped with individual feeders and drinkers separated by metal canziles, feed room, cooler room, and office.

The rest area had a tilting collective drinking fountain MX300 (MX do Brasil®) with 300 L of water capacity and a retractable system to facilitate daily cleaning, ensuring animal ad libitum consumption. Furthermore, the area had five Eco Fan MX 160 fans (MX do Brasil®) to control the warehouse's internal microclimate and dry the litter. The milking parlor was in a herringbone format with a capacity for three animals simultaneously and a single milking side. The system had three AMI 5550 automatic extractors with total production, milk flow, and temperature indicators, coupled to three Flo-Star MAX anterior–posterior collectors and Guardian JW automatic washing station, all BOUMATIC® equipment. Combined with a plumbing, filtration, milk transfer, and containment system from ORDEMILK®.

The feeding lane had two fans, 20 individual feeders with automatic closing, and plastic drinkers distributed along the feeder line (one for every two animals) with the possibility of depriving/releasing access to water according to demand; the Installation of these drinking fountains was due to the experimental need to control access to food for a restricted time and in a guided manner, requiring the availability of water during the period in which the animals remain deprived of access to the resting area.

The floor of the facilities (except for the rest area) is concrete, with friezes to increase the animals' grip and ensure safety. Animal waste and cleaning residues from these areas flow through channels to waste lagoons, where they remain for a stabilization period before distribution to agricultural areas as organic fertilizer.

## Characterization of the animals

We used 14 nulliparous Jersey breed animals with an average body weight of 430 kg, 26 months old, and pregnant for 240 days, coming from a Fixed Time Artificial Insemination protocol with Angus breed semen. These animals were arranged until the experimental period in a field pasture area composed of annual winter grasses, oats, and ryegrass, overseeded in Jiggs. They received commercial mineral supplementation for pregnant heifers until transfer to the compost barn and the beginning of the breeding period.

## Experimental design

The design used was a randomized block design; the animals ($n = 14$) were divided into two groups with seven replications each; the SO group received 25 mL/day of SO in the diet, while the GSO group received 25 mL/day of GSO. Both diets provided were isoproteic and isoenergetic. The experimental period lasted 60 days, given the necessary variations, as it began with 240 days of pregnancy (approximately 30 days before the expected date of birth) and continued until the first 30 days of lactation.

## GSO

GSO was added to the diet of animals in the treatment group throughout the experimental period at a 25 mL/cow/day dose. In the absence of similar studies in the literature, the dose used is based on levels that suggest economic viability of production and use, as it is a product with high added value. The levels do not impact the diet's nutritional value and, therefore, do not pose a risk of poisoning and productive losses through treatment. The quantity of oil was measured individually and mixed with the concentrate when providing the total diet in the animals' morning treatment. The animals in the control group received the same dose of commercial SO to make the diets isoenergetic. The Vitis vinifera vegetable oil is a commercial product from the HARMONIE® Aromaterapia brand, purchased in 1-L bottles. The oil is extracted through cold pressing and subsequent deodorization in Chile. The product is mainly composed of linoleic acid (68.57%), oleic acid (15.86%), palmitic acid (8.7%), stearic acid (5.48%), as well as other minority or unidentified compounds. The report obtained in chromatography agrees with the results obtained by Rockenbach et al. (2010). The chromatography results for GSO and SO are displayed in Table 1.

## Diet

The animals received a diet formulated following the nutritional requirements for Jersey heifers of the NRC 2001. The diet was formulated based on Tifton 85 hay, corn silage, and concentrate. The prepartum concentrate had soybean bran, cornmeal (ground through a 2-mm sieve), wheat bran, and soybean hulls in its composition. Also, in this concentrate, the BOVIGOLD® Pre-Partum OVN nucleus (DSM Tortuga®) was included to reduce the diet's dietary cation–anion difference (DCAD). During this phase, the concentrate was previously supplied to the other ingredients in

**Table 1** Chromatographic composition of grape seed oil (Treatment) and soybean oil (Control)

| Identification | % in grape oil | % in soybean oil |
|---|---|---|
| C14:0 – myristic acid | 0.18 | 0.14 |
| C16:1 – palmitoleic acid | 0.10 | 0.09 |
| C16:0 – palmitic acid | 8.70 | 12.6 |
| C18:2 – linoleic acid | 68.5 | 50.4 |
| C18:1 – oleic acid | 15.8 | 25.4 |
| C18:0 – stearic acid | 5.48 | 4.20 |

Individual fatty acids are calculated as % of total fatty acids

the diet to maximize the consumption of anionic salts, which have less palatability but are essential for good performance in the transition period. In the postpartum diet, animals aged 26 months were considered empty, with 20 days in milk (DEL = 20) and an expected 18 kg milk/day production. This includes hay, corn silage and cornmeal-based concentrate (2-mm sieve), soybean bran, wheat bran, soybean hulls, livestock urea (YARA®), and BOVIGOLD® Plus core (DSM Tortuga). All ingredients were supplied by COOPERALFA (Chapecó – SC). Table 2 shows the ingredients and basic chemical composition of the total pre-partum and postpartum diets analyzed in the laboratory after sampling when the diet was fed.

## Milking management

The herd was milked twice daily during the experimental period, at 7:00 AM and 4:30 PM. Upon entering the milking parlor, the teats were disinfected (pre-dipping) with a 0.5% Chlorhexidine-based product. After disinfection, the first three jets of milk were removed, and pre-dipping was applied again. Then, the milk was dried using paper towels. The teat cups were attached manually and extracted automatically. After milking, post-dipping was carried out before the animal was released. Due to delays in installing the milking system, one animal in the experimental group had to be milked manually during the initial week of lactation. During the period, pre- and post-dipping were done in the same way.

## Data and sample collection

Different data and samples were collected to achieve the proposed objectives: weighing the animals and collecting blood, ruminal fluid, and milk. DMI was quantified by weighing the diet provided and leftovers. Figure 1 describes the timeline of the experimental period, pointing out the period in which the respective collections were made. Milk production was also measured daily.

The animals were weighed using a bar scale (DIGITRON®) before the animals entered the experiment. Blood collections were performed by puncturing the jugular vein of the animals, collecting blood in vacuolated tubes without anticoagulant (VACUOPLAST®) to obtain blood serum and vacuolated tubes (VACUOPLAST®) with anticoagulant (EDTA) to perform a blood count. After collection, the samples were stored below five °C for transport to the laboratory. The samples collected in tubes without anticoagulant were centrifuged to obtain blood serum for ten minutes at 8000 revolutions per minute. Subsequently, the serum was stored in microtubes (EPPENDORF®) at –20 °C until analysis.

Ruminal fluid was collected four hours after the animals' first day of treatment, using an esophageal probe to

**Table 2** Ingredients and elemental chemical composition of total pre-partum and post-partum diets analyzed in the laboratory after sampling at the time of diet feeding

| Ingredients (Kg DM/day) | | |
|---|---|---|
| | Pre-delivery Diet | Postpartum Diet |
| Corn silage | 4.69 | 5.21 |
| Tifton 85 hay | 1.23 | 1.3 |
| Ground corn (2 mm) | 0.24 | 1.98 |
| Soybean meal (45% CP) | 1.61 | 2.67 |
| Soy Hull | 0.12 | 0.63 |
| Wheat bran | 0.12 | 0.63 |
| Pre-delivery nucleus [1] | 0.28 | - |
| Postpartum nucleus [2] | - | 0.21 |
| Urea | - | 0.04 |
| Total | 8.3 | 12.7 |
| Inclusion of Grape oil/Soybean oil | | |
| mL/day | 0.025 | 0.025 |
| Chemical composition of diets | | |
| | Diet SO | Diet GSO |
| Pre-delivery | | |
| Dry matter (%) | 67.2 | 67.9 |
| Crude protein (%/DM) | 14.8 | 14.7 |
| Ethereal extract (%/DM) | 2.23 | 2.13 |
| Ash (%/DM) | 7.66 | 7.50 |
| NDF (%/DM) | 48.6 | 46.8 |
| DCAD[3] | -115 | -115 |
| Postpartum | | |
| Dry matter (%) | 47.1 | 47.0 |
| Crude protein (%/DM) | 18.1 | 18.0 |
| Ethereal extract (%/DM) | 2.72 | 2.72 |
| Ash (%/DM) | 6.93 | 7.39 |
| NDF (%/DM) | 34.4 | 34.9 |
| ADF (%/DM) | 17.9 | 17.3 |
| NEL[3] (Mcal/Kg DM) | 1.58 | 1.58 |
| RDP[3] (g/day) | 1355 | 1355 |
| RUP[3] (g/day) | 714 | 714 |
| DCAD[3] | 197 | 197 |
| Fatty acid (%) | | |
| C10:0 (Capric) | 0.05 | 0.06 |
| C12:0 (Lauric) | 0.13 | 0.16 |
| C14:0 (Myristic) | 0.37 | 0.50 |
| C16:0 (Palmitic) | 20.1 | 19.0 |
| C16:1 (Palmitoleic) | 0.20 | 0.20 |
| C17:0 (Heptadecanoic) | 0.20 | 0.21 |
| C18:0 (Stearic) | 4.25 | 4.67 |
| C18:1n9c (Oleic) | 24.5 | 22.6 |
| C18:2n6c (Linoleic) | 42.5 | 46.2 |
| C20:0 (Arachidic) | 0.66 | 0.60 |
| C20:1n9 (cis-11-Eicosenoic) | 0.26 | 0.24 |
| C18:3n3 (a-Linolenic) | 4.99 | 3.88 |
| C21:0 (Henicosanoic) | 0.06 | 0.08 |

**Table 2** (continued)

| Ingredients (Kg DM/day) | | |
|---|---|---|
| | Pre-delivery Diet | Postpartum Diet |
| C20:2 (cis-11,14-Eicosadienoic) | 0.09 | 0.10 |
| C22:0 (Behenic) | 0.63 | 0.57 |
| C20:4n6 (Arachidonic) | 0.16 | 0.14 |
| C24:0 (Lignoceric) | 0.66 | 0.61 |
| ∑ Saturated fatty acids (SFA) | 27.1 | 26.5 |
| ∑ Unsaturated fatty acids (UFA) | 72.8 | 73.4 |

Note 1. Pre-calving core guarantee levels: Calcium (min): 106 g/Kg – (max): 130 g/Kg; Phosphorus (min): 30 g/Kg; Sulfur (min): 90 g/Kg; Magnesium (min): 20 g/Kg; Sodium (min): 31 g/Kg; Chlorine (min): 130 g/kg; Cobalt (min): 12 mg/Kg; Copper (min): 600 mg/Kg; Chromium (min): 30 mg/Kg; Iron (min): 600 mg/Kg; Iodine (min): 60 g/Kg; Manganese (min): 1600 mg/Kg; Selenium (min): 16 mg/Kg; Zinc (min): 2400 mg/Kg; Biotin (min): 80 mg/Kg; Vitamin A (min): 480000 IU/Kg; Vitamin D3 (min): 200000 IU/Kg; Vitamin E (min): 12000 IU/Kg; Saccharomyces cerevisae:$1.5 \times 10^9$ CFU/Kg; Monensin: 500 mg/kg; Fluoride (max): 300 mg/Kg

Note 2. Postpartum core guarantee levels: Calcium (min): 230 g/Kg – (max): 249 g/Kg; Phosphorus (min):40 g/Kg; Sulfur (min):20 g/Kg; Magnesium (min):25 g/Kg; Sodium (min):70 g/Kg; Cobalt (min):15 mg/Kg; Copper (min): 750 mg/Kg; Chromium (min): 20 mg/Kg; Iron (min): 600 mg/Kg; Iodine (min): 40 g/Kg; Manganese (min): 2000 mg/Kg; Selenium (min): 22 mg/Kg; Zinc (min): 2850 mg/Kg; Biotin (min): 80 mg/Kg; Vitamin A (min): 350000 IU/Kg; Vitamin D3 (min):100000 IU/Kg; Vitamin E (min): 2000 IU/Kg; Monensin: 1000 mg/kg

Note 3. NRC 2001 estimated the net lactation energy (NEL), dietary cation–anion difference (DCAD), rumen degradable protein (RDP), and rumen non-degradable protein (RUP) of the two diets

access and suction the rumen. After obtaining the fluid, its pH was measured. A portion was filtered to reduce methylene blue and stored to determine the rumen fatty acid profile. At the same time, the remainder was stored below 5 °C until arrival at the laboratory for storage and further analysis.

Milk was collected with individual samplers from the milking system, and a homogeneous sample of the entire milking was collected. The milk was inserted into tubes sterilized with Azidiol for standard plate counts (SPC) and with Bronopol for somatic cell counts (SCC) and chemical composition, then sent to the laboratory for analysis.

## Feed composition analysis

To analyze the bromatological composition of the feeds used, homogeneous samples were collected from both experimental groups' total pre- and postpartum diet. These samples were pre-dried in a forced air oven at 55 ºC for 72 h, removed from the oven, and weighed to determine the partial dry matter content (DM), followed by grinding in a Wiley mill (Marconi, model: MA340), using a 1 mm mesh sieve. The pre-dried and ground samples were heated at 105 ºC to obtain the DM and the mineral material in a muffle furnace at 600 ºC (Silva and Queiroz 2002). Crude protein was determined by the micro-Kjeldahl method (Method 984.13; AOAC, 1977). To determine the neutral detergent fiber (NDF) content, the samples were placed in polyester bags (Komarek, 1993) and treated with a neutral detergent solution in an ANKOM 200® fiber analyzer at 100° C for 1 h. The concentrations of acid detergent fiber (ADF) were determined using the same equipment using the ANKOM methodology, following the methods proposed by Van Soest et al. (1994). The portion of ether extract in the samples was quantified using an automatic fat extractor SER158 (VELP® Scientifica) according to the AOAC (1975).

## Hemogram

Hematologic variables were obtained using EDTA blood tubes collected using the VET3000 automatic hematologic analyzer (EQUIP®), which determines the following variables: leukocytes, lymphocytes, granulocytes, monocytes, erythrocytes and platelet count in cells $\times 10^3$/µl. It also determines hemoglobin concentration (g/dL), hematocrit (%), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and red blood cell distribution width (RDW-SD).

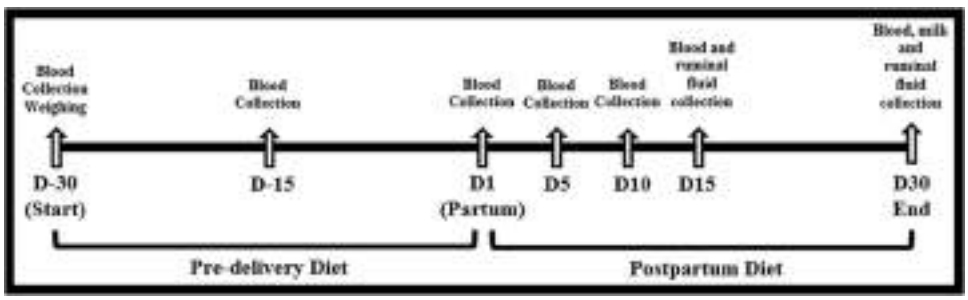



**Fig. 1** Flowchart of sample collections carried out throughout the experimental period

## Serum biochemistries

The samples collected in tubes without anticoagulant were centrifuged in a QUIMIS® tube centrifuge to obtain blood serum for ten minutes at 8000 revolutions per minute. Subsequently, the serum was stored in microtubes at –20 °C until analysis. The levels of total protein, albumin, glucose, urea, triglycerides, and total cholesterol were evaluated in blood serum. The ANALISA® kits were used in semi-automatic equipment (Bio Plus 2000®). Globulin concentration was calculated using the PT-AL equation.

## Serum oxidative status

Lipid peroxidation was measured through the amount of thiobarbituric acid reactive substances (TBARS), according to Jentzsch et al. (1996). The reaction is read in a spectrophotometer at 535 nm, and the result is expressed in nmoles of malondialdehyde/ml of serum. Total thiols were quantified in nmol SH/mg of protein using the methodology of Ellman (1959). Serum glutathione S-transferase (GST) activity was measured based on the method described by Habig et al. (1974) and expressed as U GST/mg protein. According to Erel (2004), the colorimetric method measured the serum antioxidant levels as TAC. Plasma ferric reducing capacity (FRAP) levels were measured using the technique described by Benzie and Strain (1996). TAC and FRAP results were expressed as nmol/g of protein.

## Ruminal environment

Ruminal fluid collected four hours after the morning feeding on days 15 and 30 postpartum using a 1.5-m, 11-mm diameter ororuminal tube (PROVAR®), was allocated to 100-mL universal collectors. Then, the pH was measured using a portable digital pH meter, model AK103 (TESTO®). Part of the remaining ruminal fluid was filtered through three layers of gauze, stored in 3 mL microtubes (EPPENDORF®), and frozen at –20 °C for subsequent analysis of volatile fatty acids (VFA). Another portion of the rumen fluid was used in the methylene blue reduction test to quantify bacterial activity in the fluid; for this methodology by Dirksen and Vannevel (1969), the fluid was filtered immediately after collection, then 10 mL were placed in two tubes of test, 500 µL of methylene blue was added to one of them. From there, the time it takes for the substance to be consumed until it returns to its original color when compared to the second aliquot is measured. The remaining unfiltered fluid was used to count protozoa, which was carried out by adapting methodology (Dehority 1993).

To determine short-chain fatty acids (SCFAs), fluid samples were thawed at 5 °C and manually shaken for homogenization. Aliquots of 1 mL of ruminal fluid supernatant were collected in polypropylene microtubes (2 mL) and centrifuged for 5 min (12,300 × *g*). Then, 250 µL of the supernatant was transferred to a new microtube containing 250 µL of formic acid. The mixture was manually shaken and centrifuged for 3 min. After centrifugation, 250 µL of the supernatant of the mixture was collected in another polypropylene tube previously containing 500 µL of 3-octanol solution (665 µg $mL^{-1}$ in methanol), used as an internal standard, homogenized, and centrifuged again; 600 µL of the sample was inserted into an injection vial and injected into a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3400, Palo Alto, USA) and an autosampler (Varian 8200CX, Palo Alto, USA); 1 µL of the extract was injected in split mode at 1:10. The carrier gas used was hydrogen at a constant pressure of 20 psi. The analytes (acetic, propionic, butyric, valeric, and isovaleric acids) were separated on a CP-Wax 52CB capillary column (60 m × 0.25 mm; 0.25 µm stationary phase thickness). The initial column temperature was set at 80 °C for 1 min and increased to 120 °C at a rate of 8 °C $min^{-1}$, then to 230 °C for 20 °C $min^{-1}$, where it remained for 1 min. The injector and detector temperatures were set at 250 °C. Method validation comprised the following parameters: selectivity, linearity, linear range, repeatability, precision, limit of detection (LOD), and limit of quantification (LOQ) for acetic, propionic, butyric, and isovaleric acids. Linearity was assessed by calculating a regression equation using the least squares method. LOD and LOQ values were obtained by sequential dilutions to signal-to-noise ratios of 3:1 and 6:1, respectively. Accuracy was assessed by analyzing the repeatability of six replicate samples. Accuracy was determined by recovering known amounts of standard substances added to a diluted sample. The results were expressed as mol 100 mol-1 of each SCFA in the rumen fluid.

## Milk analysis

Protein oxidation was measured by the advanced oxidation protein products (AOPP) technique described by (Witko-Sarsat et al. 1998), with results presented in µmol/g protein (AOPP). Antioxidant levels were measured by the ferric reducing capacity of plasma (FRAP) according to Benzie and Strain (1996), and the TAC measurement was described according to Erel (2004). These methodologies were standardized for milk, as Radavelli et al. (2016) described. FRAP results were expressed in mmol/g of protein and TAC in nmol Trolox equivalent/L.

The chemical composition, SPC, and SCC analyses were conducted at the State Milk Quality Laboratory – LABLEITE of Concórdia -SC accredited to the Ministry of Agriculture, Livestock and Supply by ordinance MAPA: Nº 212 of July 31, 2014. Fat quantification, protein,

lactose, total solids, and defatted dry extract were carried out using the Mid-Infrared Spectrometry Method, according to ISO 9622—IDF Standart 141:2013. The Total Bacterial Count was carried out using the Flow Cytometry Method, according to ISO 21187—IDF Standart 196 (2021), and the somatic cell count using the flow cytometry method, according to ISO 13366–2—IDF Standart 148–2 (2006).

To determine the fatty acid profile in milk, lipid extraction was performed using the method of Bligh and Dyer (1959) with adaptations. 1.5 g of samples, 0.8 mL of water, 5 mL of methanol, and 2.5 mL of chloroform were added to a 15 mL polypropylene tube, and mechanical stirring was carried out for 30 min. Next, 2.5 mL of chloroform solution and 1.5% NaSO4 were added to promote a two-phase system. This mixture was stirred for 2 min and then centrifuged for 15 min at 2000 rpm. The lipids obtained from the chloroform phase were subjected to fatty acid analysis.

FA methylation was performed using a transesterification method proposed by Hartman and Lago (1973). 1 mL of 0.4 M KOH methanolic solution was added to the extracted lipids in a test tube and vortexed for 1 min. The samples were kept in a water bath for 10 min at boiling temperature. Subsequently, they were cooled to room temperature, and 3 mL of 1 M methanolic $H_2SO_4$ solution were added, vortexed, and kept in a water bath for 10 min. After cooling, 2 mL of hexane was added and centrifuged at 2000 rpm for 10 min. Finally, hexane with fatty acid methyl esters (FAME) was subjected to chromatographic analysis.

A gas chromatograph model TRACE 1310 with a flame ionization detector (Thermo Scientific) was used to determine FAME. One microliter of samples was injected into a split/splitless injector, operated in split mode with a 1:20 ratio at 250 °C. Hydrogen was used as the carrier gas at a constant flow rate of 1.5 mL/min. Separation of FAMEs was performed using an RT 2560 chromatography column (100 m × 0.25 mm × 0.20 µm film thickness, Restek, USA). The initial oven temperature was programmed at 100 °C for 5 min and increased to 180 °C at an 8 °C/min rate. Then, increasing to 210 °C at a rate of 4 °C/min, and finally up to 250 °C, increasing to 20 °C/min, and maintaining for seven min in isothermal conditions. The detector temperature was kept constant at 250 °C. FAME compounds were identified by comparing the experimental retention time with those of the authentic standard (FAME Mix-37, Sigma Aldrich, St. Louis, MO). The results were presented as a percentage of each FA identified in the lipid fraction, considering the factor equivalent to the size of the FAME chain for FID and the ester conversion factor for the respective acid (Material Supplementary 1), according to Visentainer and Franco (2006).

### Statistical analysis

All data were analyzed using the SAS "MIXED procedure" (SAS Inst. Inc., Cary, NC, USA; version 9.4), with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed effects test. Milk production and feed intake were tested for the fixed effect of treatment using animal (treatment) as a random effect. Milk, rumen fluid, and blood data were analyzed as repeated measures and tested for treatment and treatment × day fixed effects using animal (treatment) as a random effect. D-30 results (30 days prepartum, "start of experiment") were included as an independent covariate. The D -30 scores were removed from the data set for these variables to generate the mean by treatment but were retained as a covariate. Means were separated using the PDIFF method, and all results were reported as LSMEANS followed by SEM. Significance was defined for treatment effects and treatment × day interaction when $p \leq 0.05$ and trend when $p > 0.05$ and $\leq 0.10$.

Considering FRAP levels, we calculated the post hoc power for the groups using Proc Power by SAS. We used the mean of each group in serum and milk, and its SEM with an alpha of 0.05, and we ended up with a reasonable power of 84.2% and 87.6, respectively.

## Results

### Performance

The variables of milk production, DMI, and milk efficiency in the pre- and postpartum periods are displayed in Table 3. There was no effect of the GSO treatment or treatment x

**Table 3** Productive performance and feed intake of primiparous Jersey cows fed grape seed oil (GSO) or soybean oil (SO)

| Variable | SO | GSO | SEM | P:treat[1] | P:treat x day[2] |
|---|---|---|---|---|---|
| Average milk production (L) | 18.7 | 18.1 | 0.39 | 0.89 | 0.68 |
| Feed intake (Kg/DM) | | | | | |
| Average: Pre-delivery | 6.58 | 6.81 | 0.11 | 0.86 | 0.81 |
| Average: Postpartum | 10.5 | 10.5 | 0.08 | 0.95 | 0.94 |
| Dairy efficiency (Kg/Kg) | 1.78 | 1.71 | 0.03 | 0.75 | 0.71 |

Note 1. Demonstrates the effect of treatment, where different letters in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and $\leq 0.1$) between groups

Note 2. Demonstrates the interaction between treatment × day, where different letters on the same line for "mean" show a significant difference ($P \leq 0.05$) and trend ($P > 0.05$ and $\leq 0.1$) between groups

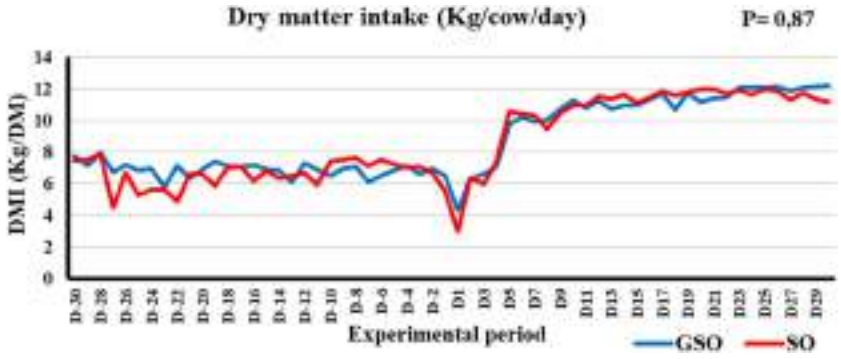



**Fig. 2** Dry matter intake (Kg/cow/day) of animals in the treatment and control groups throughout the experimental period (D1 = Parturition)

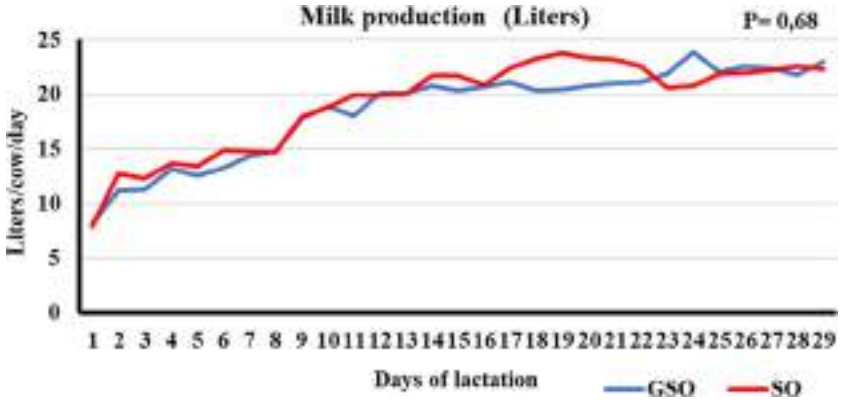



**Fig. 3** Growth in average daily milk production in the GSO and SO groups during the first 30 days of lactation

day interaction between the groups on the variables of milk production and milk efficiency, as well as in IMS, in the experimental period. Figure 2 shows the ingestive behavior of the animals throughout the period. There was a lower DMI during the pre-partum period, a decrease in intake in the days leading up to and following birth, and an increasing intake after the beginning of lactation. Accumulated milk production also showed no statistical difference over the first 30 days of lactation, as seen in Fig. 3.

## Hemogram

Table 4 presents the hemogram results of the animals during the experiment, with a treatment x day interaction for the lymphocyte count, which was significantly higher on D1 in the GSO group than the SO group ($P = 0.05$). The interaction was also observed on day 30, showing lower lymphocyte counts in the GSO group animals than in the SO group. There was no effect or interaction for the count of leukocytes, granulocytes, monocytes, total erythrocytes, and platelets, as well as for the percentage of hematocrit and hemoglobin concentration, MCV, MCH, MCHC, and RDW-SD between the groups.

## Serum biochemistries

The treatment had an effect, with a higher total cholesterol concentration observed in the GSO group ($P = 0.05$) than in the SO. There was a treatment x day interaction in D -15 where the concentration was lower in the GSO group ($P = 0.02$), different from the treatment x day interaction observed on days 5, 15, and 30 when the cholesterol concentration was higher in the GSO group than the SO group. There was no treatment and treatment x day interaction effect on blood glucose concentration, total protein, urea, albumin, and globulins (Table 5).

## Serum oxidative status

Figure 4 shows part of the oxidative status results in the cows' blood serum. TBARS levels tended to be lower in the GSO group compared to the SO ($P = 0.08$). Treatment x day interaction was observed for lower TBARS concentration on days 5 and 10 ($P = 0.01$). Total thiol levels were significantly higher in the GSO group compared to the SO group ($P = 0.03$). Treatment x day interaction on days -15, 1, 15, and 30 revealed higher levels of total thiols in the GSO group compared to the SO ($P = 0.01$).

The plasma ferric reducing capacity (FRAP) and TAC levels showed similar behavior, with the treatment effect,

**Table 4** Hematological parameters of primiparous Jersey cows fed grape seed oil (GSO) or soybean oil (SO)

| Variable | SO | GSO | SEM | P:treat[1] | P:treat x day[2] |
|---|---|---|---|---|---|
| Total leukocytes ($\times 10^3$/µL) | | | | 0.88 | 0.64 |
| | 6.96 | 6.75 | | | |
| Lymphocytes ($\times 10^3$/µL) | | | | 0.75 | **0.05** |
| D-30 | 4.54 | 6.96 | 1.95 | | |
| D-15 | 2.98 | 3.22 | 0.62 | | |
| D1 (Birth) | 4.32[b] | 5.53[a] | 0.35 | | |
| D5 | 3.28 | 3.84 | 0.27 | | |
| D10 | 2.8 | 3.14 | 0.27 | | |
| D15 | 2.95 | 3.04 | 0.28 | | |
| D30 | 5.37[a] | 4.06[b] | 0.31 | | |
| Granulocytes ($\times 10^3$/µL) | | | | 0.82 | 0.58 |
| | 2.18 | 2.13 | 0.12 | | |
| Monocytes ($\times 10^3$/µL) | | | | 0.73 | 0.46 |
| | 1.39 | 1.29 | 0.17 | | |
| Total erythrocytes ($\times 10^6$/µL) | | | | 0.94 | 0.86 |
| | 6.19 | 6.32 | 0.03 | | |
| Hemoglobin (g/dL) | | | | 0.88 | 0.74 |
| | 10.4 | 11.2 | 0.07 | | |
| Hematocrit (%) | | | | 0.45 | 0.36 |
| | 30.4 | 32.8 | 1.05 | | |
| MCV (fL) | | | | 0.28 | 0.18 |
| | 50.3 | 52.3 | 0.95 | | |
| MCH (pg) | | | | 0.9 | 0.85 |
| | 17.3 | 18.4 | 0.14 | | |
| MCHC (g/dL) | | | | 0.91 | 0.84 |
| | 34.1 | 35.2 | 0.16 | | |
| RDW-SD (fL) | | | | 0.89 | 0.83 |
| | 34.0 | 34.9 | 0.24 | | |
| Platelets ($\times 10^3$/µL) | | | | 0.18 | 0.11 |
| | 358 | 325 | 10.7 | | |

Note 1. Demonstrates the effect of treatment, where different letters in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and $\leq 0.1$) between groups

Note 2. Demonstrates the interaction between treatment × day where different letters ([a, b]) on the same line for "mean" show a significant difference ($P \leq 0.05$) and trend ($P > 0.05$ and $\leq 0.1$) between groups

**Table 5** Serum biochemistry parameters of primiparous Jersey cows fed grape seed (GSO) or soybean oil (SO)

| Variable | SO | GSO | SEM | P:treat | P:treat x day |
|---|---|---|---|---|---|
| Glucose (mg/dL) | | | | 0.89 | 0.71 |
| | 53 | 54.1 | 2.51 | | |
| Triglycerides (mg/dL) | | | | 0.62 | 0.46 |
| | 25.3 | 22.9 | 0.95 | | |
| Total cholesterol (mg/dL) | | | | **0.05** | **0.02** |
| D-30 | 102 | 102 | 3.98 | | |
| D-15 | 95[a] | 81.3[b] | 3.65 | | |
| D1 (Birth) | 74.7 | 85.7 | 3.65 | | |
| D5 | 76.3[b] | 106[a] | 3.59 | | |
| D10 | 77.3 | 87.3 | 3.62 | | |
| D15 | 107[b] | 126[a] | 3.58 | | |
| D30 | 122[b] | 147[a] | 3.42 | | |
| Total protein (g/dL) | | | | 0.79 | 0.65 |
| | 9.45 | 8.98 | 0.45 | | |
| Urea (mg/dL) | | | | 0.82 | 0.77 |
| | 41 | 40.2 | 1.23 | | |
| Albumia (g/dL) | | | | 0.21 | 0.13 |
| | 3.15 | 2.84 | 0.44 | | |
| Globulin (g/dL) | | | | 0.8 | 0.61 |
| | 6.17 | 6.12 | 0.51 | | |

Note 1. Demonstrates the effect of treatment, where different letters in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and $\leq 0.1$) between groups

Note 2. Demonstrates the interaction between treatment × day where different letters ([a, b]) on the same line for "mean" show a significant difference ($P \leq 0.05$) and trend ($P > 0.05$ and $\leq 0.1$) between groups

that is, higher levels in the GSO group compared to the SO ($P = 0.01$). Treatment x day interaction (days 5, 10, 15, and 30) was also observed when higher levels were detected in animals in the GSO group than in the SO group for FRAP ($P = 0.01$) and TAC (P = 0.03). Glutathione S-peroxidase (GST) activity showed a treatment x day interaction ($P = 0.01$) higher on D-15 in the GSO group than the SO. The treatment group observed lower GST activity on days 5, 15, and 30 ($P = 0.01$). There was no treatment effect on GST enzyme activity (Table 6).

## Ruminal environment

The variables for the ruminal environment are in Table 7. The methylene blue reduction test showed an effect of the treatment, smaller in the treated group than the control ($P = 0.05$); a treatment x day interaction was observed on day 15, with a significant decrease in reduction time ($P = 0.03$). The pH of the fluid showed a tendency toward a treatment effect, being lower in the treatment group ($P = 0.06$), and, was also significantly lower on day 15 ($P = 0.04$), characterizing an interaction. The protozoa count was higher in the treatment group than the control during the experiment ($P = 0.10$) and higher in the treatment group on day 30 ($P = 0.07$). The concentration of total SCFA showed a treatment x day interaction, which was higher on day 15 in the control group ($P = 0.05$), but there was no effect of treatment. The concentration and

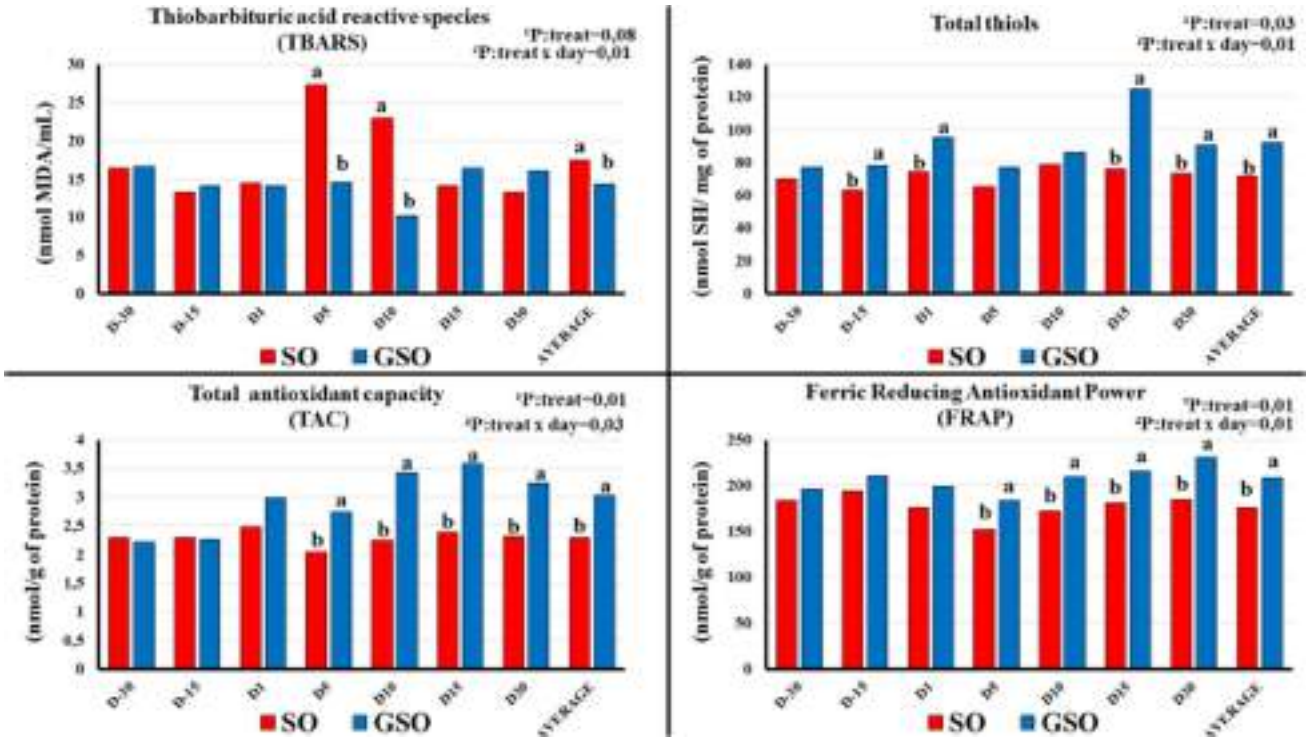



**Fig. 4** Serum antioxidant activity of primiparous Jersey cows fed grape seed oil (GSO) or soybean oil (SO). Note 1. Demonstrates the effect of treatment, where different letters in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and $\leq 0.1$) between groups. Note 2. Demonstrates the interaction between treatment × day, where different letters on the same line for "mean" show a significant difference ($P \leq 0.05$) and trend ($P > 0.05$ and $\leq 0.1$) between groups

percentage of propionic acid in the rumen was higher on day 15 in the treatment group, an effect that was seen in a treat x day interaction ($P = 0.05$), which corroborates the higher acetate/propionate ratio in the control group observed in the day 15 ($P = 0.05$). Treatment x day interaction for acetic acid concentration (day 15), being lower in the rumen fluid of cows that consumed grape seed oil; but when evaluating the proportion (%) of acetic acids, we did not observe the same difference. The concentration and percentages of butyric, valeric, and isovaleric acid did not differ between the control and treatment groups.

**Table 6** GST enzyme activity of primiparous Jersey cows fed grape seed oil (GSO) or soybean oil (SO)

| Variable | SO | GSO | SEM | P:treat | P:treat x day |
|---|---|---|---|---|---|
| GST (µmolCDNB/min/mg of protein) | | | | 0.25 | **0.01** |
| D-30 | 372 | 402 | 6.82 | | |
| D-15 | 364[b] | 412[a] | 6.72 | | |
| D1 (birth) | 377 | 384 | 6.75 | | |
| D5 | 392[a] | 364[b] | 6.21 | | |
| D10 | 403 | 393 | 6.55 | | |
| D15 | 550[a] | 480[b] | 7.01 | | |
| D30 | 493[a] | 448[b] | 6.86 | | |

Note 1. Demonstrates the effect of treatment, where different letters in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and $\leq 0.1$) between groups

Note 2. Demonstrates the interaction between treatment × day, where different letters ([a, b]) on the same line for "mean" show a significant difference ($P \leq 0.05$) and trend ($P > 0.05$ and $\leq 0.1$) between groups

## Composition, quality, and oxidative status of milk

The chemical and qualitative composition of the milk is presented in Table 8. There was no difference between the groups for the fat, protein, lactose, total solids, and defatted dry extract (DDE) concentration. There was a trend toward lower somatic cell counts (SCC) ($P = 0.09$) and lower standard plaque counts (SPC) ($P = 0.06$) in the treatment group compared to the control group. The oxidative status of the milk produced by the cows is shown in Fig. 5. An effect of the treatment was verified, with a higher concentration of the variables FRAP ($P = 0.03$) and TAC ($P = 0.05$) in the milk of cows in the treatment group. There was an effect of treatment for AOPP, that is, lower concentration in the milk of cows in the treatment group compared to the control ($P = 0.01$).

**Table 7** Variables of the ruminal environment of primiparous Jersey cows fed grape seed oil (GSO) or soybean oil (SO)

| Variable | SO | GSO | SEM | P:treat | P: treat x day |
|---|---|---|---|---|---|
| PRAM (seconds) | | | | **0.05** | **0.03** |
| D15 | 210[a] | 169[b] | 10.6 | | |
| D30 | 255 | 213 | 12.8 | | |
| pH | | | | **0.06** | **0.04** |
| D15 | 6.61[a] | 6.4[b] | 0.01 | | |
| D30 | 6.54 | 6.42 | 0.01 | | |
| Protozoa (n°$\times 10^4$/ mL) | | | | **0.10** | **0.07** |
| D15 | 22.3 | 25.3 | 2.6 | | |
| D30 | 16.3[b] | 21.5[a] | 2.14 | | |
| ***VFAs concentration,* nmol/L** | | | | | |
| Total VFA (mmol/L) | | | | 0.23 | **0.05** |
| D15 | 115[a] | 96.5[b] | 4.08 | | |
| D30 | 122 | 122 | 4.51 | | |
| Acetic acid (mmol/L) | | | | 0.15 | **0.01** |
| D15 | 80.3[a] | 68.0[b] | 1.74 | | |
| D30 | 83.9 | 86.6 | 1.72 | | |
| Propionic acid (mmol/L) | | | | 0.82 | **0.03** |
| D15 | 19.3 | 23.9 | 0.78 | | |
| D30 | 21.7 | 18.7 | 0.78 | | |
| Butyric acid (mmol/L) | 13.0 | 11.9 | 0.65 | 0.74 | 0.81 |
| Isovaleric acid (mmol/L) | 1.49 | 1.39 | 0.12 | 0.89 | 0.94 |
| Valeric acid (mmol/L) | 1.34 | 1.48 | 0.09 | 0.42 | 0.23 |
| ***VFA results in proportion, %*** | | | | | |
| Propionic acid (%) | | | | 0.15 | **0.05** |
| D15 | 16.3[b] | 18.5[a] | 0.15 | | |
| D30 | 15.3 | 16.7 | 0.15 | | |
| Acetic acid (%) | 69.2 | 69.6 | 0.51 | 0.94 | 0.97 |
| Butyric acid (%) | 10.9 | 10.8 | 0.12 | 0.92 | 0.95 |
| Isovaleric acid (%) | 1.25 | 1.27 | 0.03 | 0.95 | 0.97 |
| Valeric acid (%) | 1.33 | 1.33 | 0.04 | 0.96 | 0.98 |
| Acetic/propionic ratio | | | | 0.21 | **0.05** |
| D15 | 4.31[a] | 3.70[b] | 0.03 | | |
| D30 | 4.27 | 4.26 | 0.03 | | |

Note 1. Demonstrates the effect of treatment, where different letters in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and $\leq 0.1$) between groups

Note 2. Demonstrates the interaction between treatment × day, where different letters ([a, b]) on the same line for "mean" show a significant difference ($P \leq 0.05$) and trend ($P > 0.05$ and $\leq 0.1$) between groups

In the profile of fatty acids in milk, a trend toward lower concentration of oleic acid was observed in the treatment group ($P = 0.1$). The effect of the treatment was noticed in the increase in saturated fatty acids (SFA) in the treatment group ($P = 0.05$). Still, there was a decrease in unsaturated fatty acids (UFA) in the treated group ($P = 0.03$). The concentration of monounsaturated fatty acids (MUFA) was lower in the treatment group compared to the control ($P = 0.01$), and the UFA/SFA ratio was also significantly lower in the treatment group compared to the control ($P = 0.02$). The remaining fatty acids did not differ between groups. There was also no statistical difference in the concentration of ω3 and ω6 or the relationship of these fatty acids (Table 9).

**Table 8** Proximate composition, SPC, and SCC of primiparous Jersey cows fed grape seed oil (GSO) or soybean oil (SO)

| Variable | SO | GSO | SEM | P:treat[1] |
|---|---|---|---|---|
| Fat (g/kg) | 33.7 | 33.1 | 0.22 | 0.92 |
| Protein (g/kg) | 31.4 | 31.9 | 0.18 | 0.94 |
| Lactose (g/kg) | 46.7 | 46.3 | 0.09 | 0.97 |
| Total solids (g/kg) | 121 | 120 | 0.31 | 0.91 |
| DDE (g/kg) | 87.5 | 87.4 | 0.19 | 0.95 |
| SCC (×1000/mL) | 124[a] | 106[b] | 3.85 | **0.09** |
| SPC (CFU×1000/mL) | 49.4[a] | 34.1[b] | 2.36 | **0.06** |

Note 1. Demonstrates the effect of treatment, where different letters ([a, b]) in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and $\leq 0.1$) between groups

## Discussion

Milk production, DMI, and, consequently, milk efficiency of treated animals were not affected by treatment during the study. In a recent study, researchers reported no difference in the production and DMI of Holstein cows that received an infusion of oleic acid (Dos Santos Neto; Prom; Lock, 2023). Likewise, another study showed no effect on milk production in the first 21 days of lactation regardless of whether or not cows consumed ω3 fatty acids from flaxseed or fish oil (Kra et al. 2021). Researchers found a trend toward improvement in milk production in cows when supplemented with vitamin E in the transition period (Moghimi-Kandelousi et al. 2020). Supplementing extruded linseed to dairy cows that provide linoleic acid in the three weeks before calving increased milk production (Moallem et al. 2020), and the study used multiparous Israeli Holstein cows during lactation. As this was the first study using GSO in cows' diet, with interesting effects on the cow's health, it is essential to deepen the research, carrying out new studies that make it possible to test doses and thus define the optimal dose for cows—in a transition period, thinking about animal health.

According to the literature, the intake of ω3 fatty acids can alter the immune response because PUFA exerts changes in the activation of cells in both the innate and adaptive immune systems. ω3 fatty acids and their derivatives can act as signaling molecules (Gutierrez et al., 2019). As a result, in our study, we expected to have more significant treatment effects on immunological variables. However, only the lymphocyte count differed; that is, on the day of birth, the

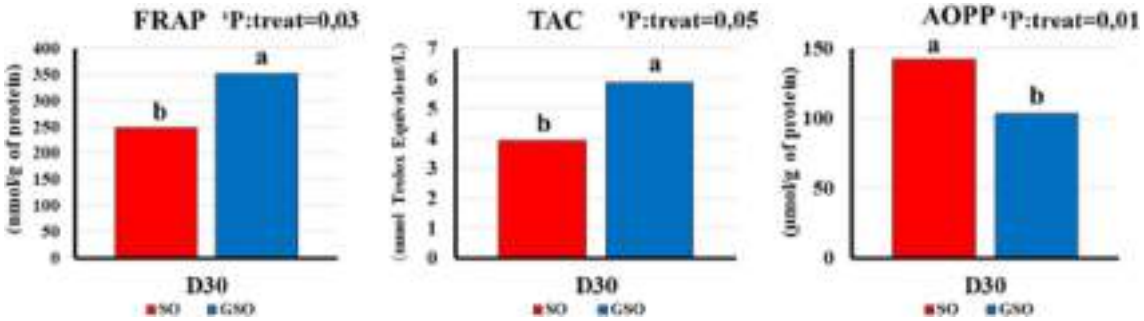



**Fig. 5** Oxidative status: Ferric reducing capacity of plasma (FRAP), total antioxidant capacity (TAC), and advanced oxidation protein products (AOPP) of milk from primiparous Jersey cows fed grape seed oil (GSO) or soybean oil (SO). Note 1. Demonstrates the effect of treatment, where different letters in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and $\leq 0.1$) between groups

**Table 9** Fatty acid profile of milk from primiparous Jersey cows fed grape seed oil (GSO) or soybean oil (SO)

| Fatty acid | SO | GSO | SEM | P-treat[1] |
|---|---|---|---|---|
| C6:0 (Caproic) | 0.4 | 0.6 | 0.12 | 0.28 |
| C8:0 (Caprilic) | 0.4 | 0.5 | 0.09 | 0.16 |
| C10:0 (Capric) | 1.3 | 1.5 | 0.17 | 0.43 |
| C11:0 (Undecanoic) | 0.0 | 0.1 | 0.03 | 0.82 |
| C12:0 (Lauric) | 1.8 | 2.1 | 0.11 | 0.25 |
| C14:0 (Myristic) | 8.2 | 9.5 | 0.24 | 0.46 |
| C14:1 (Myristoleic) | 0.4 | 0.4 | 0.07 | 0.68 |
| C15:0 (Pentadecanoic) | 0.6 | 0.7 | 0.13 | 0.92 |
| C16:0 (Palmitic) | 31.7 | 32.2 | 1.24 | 0.69 |
| C16:1 (Palmitoleic) | 1.6 | 1.2 | 0.16 | 0.11 |
| C17:0 (Heptadecanoic) | 0.7 | 0.7 | 0.04 | 0.95 |
| C17:1 (cis-10-Heptadecenoic) | 0.3 | 0.2 | 0.03 | 0.61 |
| C18:0 (Estearic) | 16.1 | 18.3 | 0.87 | 0.18 |
| C18:1n9c (Oleic) | 31.7[a] | 27.3[b] | 1.09 | **0.10** |
| C18:2n6c (Linoleic) | 2.7 | 2.7 | 0.03 | 0.93 |
| C20:0 (Arachidic) | 0.1 | 0.2 | 0.02 | 0.58 |
| C20:1n9 (cis-11-Eicosanoic) | 0.0 | 0.0 | 0.01 | 0.89 |
| C18:3n3 (a-Linolenic) | 0.2 | 0.2 | 0.01 | 0.92 |
| C21:0 (Heneicosanoic) | 0.4 | 0.5 | 0.02 | 0.86 |
| C22:0 (Beenic) | 0.0 | 0.1 | 0.00 | 0.77 |
| C20:3n6 (cis8.11.14 Eicosatrienoic) | 0.0 | 0.0 | 0.00 | 0.96 |
| C20:4n6 (Arachidonic) | 0.0 | 0.0 | 0.00 | 0.80 |
| C24:0 (Lignoceric) | 0.0 | 0.0 | 0.00 | 0.65 |
| C20:5n3 (cis-5.8.11.14.17- Eicosapentaenoic) | 0.0 | 0.0 | 0.00 | 0.21 |
| C24:1n9 (Nervonic) | 0.0 | 0.0 | 0.00 | 0.91 |
| ∑ AG saturated (SFA) | 62.5[b] | 67.5[a] | 1.63 | **0.05** |
| ∑ AG unsaturated (UFA) | 37.4[a] | 32.4[b] | 0.87 | **0.03** |
| ∑AG monounsaturated (MUFA) | 34.3[a] | 29.3[b] | 0.84 | **0.01** |
| ∑ AG polyunsaturated (PUFA) | 3.1 | 3.1 | 0.10 | 0.84 |
| UFA/SFA | 0.6[a] | 0.4[b] | 0.08 | **0.02** |
| ∑ ω6 | 2.8 | 2.8 | 0.01 | 0.95 |
| ∑ ω3 | 0.2 | 0.2 | 0.00 | 0.82 |
| ω6/ω3 | 10.3 | 9.6 | 0.41 | 0.73 |

Note 1. Demonstrates the effect of treatment, where different letters ([a, b]) in the same row show significant differences ($P \leq 0.05$) and trends ($P > 0.05$ and $\leq 0.1$) between groups

number of lymphocytes was higher in cows in the treatment group, but at the end of the research (DEL 30), the count was lower; however, these changes did not alter the concentrations and total protein and globulins. In the work carried out by Veshkini et al. (2023a, b), using abomasal infusion of essential fatty acids, it was observed that the leukocyte and lymphocyte count increased until delivery and then gradually decreased, similar to those observed in the present study. We do not have an explanation for this effect on cholesterol concentration since the diets were isoenergetic. Still, it is crucial to investigate which cholesterol is higher (LDL or HDL) when considering animal health.

Oxidative markers demonstrated beneficial results for cow health with a tendency to reduce lipid peroxidation, which was also observed by Santos et al. (2022) when prepartum Holstein cows consumed açaí seed oil, which has fatty acids and antioxidant properties similar to GSO. Antioxidant effects in animals supplemented with grape residue were also observed in the qualitative maintenance of lamb meat (Vieira et al. 2022). The formation of reactive oxygen species is normal and even more significant in the transition period (Basiricò et al. 2017); however, when it exceeds the neutralization capacity of antioxidants, it causes oxidative stress and tissue damage, reinforcing the importance of antioxidant levels suitable, especially in critical periods (Abuelo et al. 2015). The high antioxidant activity is confirmed by the increased serum and milk levels of FRAP and TAC and the consequent reduction in AOPP, corroborating an increase in the proportion of total thiols in the organism of treated animals. In agreement, Mu et al. (2020) concluded that grape seed proanthocyanidin supplementation in the diet of lambs had positive effects on antioxidant activity and liver function. The antioxidants in milk can benefit human health, although this is not yet a product pricing criterion. It is believed that the lower GST activity in the treatment group on days 5, 15, and 30 must be correlated with the reduction of oxidants in the blood serum, discouraging the action of the enzyme, which is essential in detoxifying, transporting, and eliminating oxidative stress products. (Dasari et al. 2018). In general, beneficial improvements to the health of the treated animals are observed.

We observed a discrete effect in the ruminal environment; considering that there was no impact on performance variables, it was expected that there would be no significant changes. Only on day 15 postpartum, cows that consumed grape seed oil had a lower concentration of total VFA due to lower levels of acetic acid; however, we do not have an explanation for the mechanism involved. The greater bacterial activity is positive, as well as the greater production of propionate, reducing the acetate/propionate ratio, as reported by Akhlaghi et al. (2022). However, there are few relationships since this supplemented lipid fraction is little used by rumen microorganisms; its structural lipid portion is around 10%, and more than 90% of environmental fatty acids are saturated (Kozloski 2019). Although the diets were isoenergetic and isoproteic, the pH was higher in the rumen fluid of the cows in the group treated with GSO, but it remained within acceptable parameters. For a more assertive conclusion that the treatment could harm the ruminal environment, collections would be necessary at more points in the post-treatment period.

In the literature, some authors cited changes in the proximate composition of milk, such as the reduction in milk fat synthesis in dairy cows (Bernard et al. 2018), with the maintenance of milk production and composition (Lock et al. 2008). There are also reports of decreased milk and lactose production after fatty acid supplementation (Bell and Kennelly 2003). In our study, the proximate composition did not differ. Still, the somatic cell count may have decreased due to the improvement in the animal's health, including considering the reduction in inflammatory cells, correlated with the reduction in TBARS, as found by Santos et al. (2022).

The administration of feed enriched with olive extract, a source of polyphenols, increased the PUFA ω6 to ω3 ratio from 3:1 to 4:1 (Di Meo et al. 2023). According to the same author, MUFA and SFA levels were maintained, resulting in a significant increase in PUFA after 15 days of administration (Di Meo et al. 2023). In our study, despite PUFA supplementation, SFA levels increased in milk compared to unsaturated milk. As the milk had a more significant amount of SFA, which means more stable lipids, this change may have influenced the stability of the milk, which had less oxidation and a more substantial amount of antioxidants. Fautex et al. (2016) also observed a challenge to the oxidative stability of milk by supplementing flaxseed oil in dairy cows, which was indicated by a marked increase in all oxidation products. These results show that GSO would not be desirable if we were looking to positively modulate milk quality to have more significant amounts of omegas in milk. It is essential to highlight that in recent years, society has been looking for increasingly healthier foods with lower amounts of saturated fat, as it is known that this type of fat, when consumed constantly, can compromise the functioning of the cardiovascular system in humans (Maki; Dicklin; Kirkpatrick, 2021).

In conclusion, grape seed oil did not affect the cows' feed intake throughout the experimental period. The consumption of GSO had beneficial effects on the cows' health in the transition period as a primary focus on the antioxidant activity, reducing oxidations in the whey and milk of the cows. The cows' intake of GSO discretely altered the ruminal environment. Still, the cows had lower pH, higher bacterial activity, and several protozoa associated with a lower concentration of VFA at 15 days in lactation. The additive did not affect the proximate composition of the milk. Still, the sum of SFA in the milk was higher in the cows of the group that received

GSO, while the sum of unsaturated fatty acids was lower in these animals. As this study used only one dose of the treatment, further studies should be carried out to define an ideal dose that is economically viable and guarantees the same benefits. Another point is to consider that the cost of extraction and purification of the oil adds value to the product, so in some situations, the use of the residue in natura in coarser forms can be considered.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11250-024-04064-1.

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**Authors' contributions** MH Signor, AL Dos Santos, and AS Da Silva contributed to the design and implementation of the research to analyze the results. MG Vitt, L Nora, NG Correa, RVP Lago, and GJ Wolschick participated in the execution of the experiment and collection of samples and data. ACH Xavier, B Bissacotti, and B Klein performed laboratory analyses. All authors discussed the results and contributed to the final manuscript.

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**Data availability** All data and materials used in the experiment are available and ready to be provided.

## Declarations

**Ethics statement** The UDESC Ethics Committee on the Use of Animals in Research approved the project (protocol number 4200270922).

**Consent to participate** All names on the author list were involved in various stages of experimentation or writing.

**Consent for publication** All authors agreed to submit the work for publication in *Tropical Animal Health and Production*.

**Conflict of interest** The authors declare no conflicts of interest.

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# Dehydrated Natural Feed for Cats: Influence on Apparent Digestibility Coefficient, Feces Scores, and Serum Immune, Antioxidant, and Metabolic Biomarkers

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## Abstract

The present study aimed to determine whether natural dehydrated feed (DNF) can replace dry extruded feed while preserving the required daily intake, nutrient digestibility and benefits for immunity and antioxidant response in cats. The animals were divided into two groups of seven animals each. One group received DNF, and the other consumed commercial extruded dry feed (DCF). The experiment lasted 45 days and we measured daily consumption and collected blood and feces samples. The animals in the DNF group showed greater digestibility of fiber and mineral matter than DCF. Cats in the DNF group had a higher volume of feces. DNF cats had lower leukocyte and granulocyte counts and cholesterol levels. Serum enzymatic antioxidant responses and non-enzymatic were higher in DNF cats. Cats in the DNF group showed lower serum acute phase proteins in addition to a tendency toward a higher concentration of transferrin than in the DCF group. Fecal *Escherichia coli* and total coliform counts of cats fed DNF were higher than DCF. We conclude that the daily consumption of cats is adequate. This feed had greater digestibility of fiber and mineral matter, in addition to an improvement in antioxidant and immunological responses.

**Keywords**

Alternative Feed, Antioxidant, Companion Animals, Health

## 1. Introduction

Recently, interest in the pet food market has become a research focus. A review of the pet food market revealed that in the last 18 years, there had been an increase of more than 250% in the publication of scientific articles in this area, with 2018 producing the highest number of publications [1]. Nutrition or meeting the requirements of pets is not the only focus of companies; that is, in the last 15 years, intensifying in the last five years, they seek to formulate and produce feed that is aligned with health, longevity, and (mainly) animal welfare [2]. The pet food market has numerous varieties of feed for the consumer; however, the modern owner experiences doubt regarding choice, as there is difficulty in differentiating among available commercial products [3].

The cat, in the context of modern anthropomorphism, is associated with characteristics distinct from its ancestors, becoming sweet and easily adaptable animals kept as family companions, with a disproportionate influence on the well-being of the guardian and the animal [4]. The feed industry has been adapting to the growing demand for production and formulation to meet the physiological and metabolic needs of the species, races, or stages of life; they are based on the available ingredients aiming at a high cost-benefit ratio [5]. However, cats have not changed the typical eating habits and requirements of their ancestors overtime; they are strictly carnivorous, and they need to ingest animal protein and relatively low levels of fat and carbohydrates [6] [7]. Felines can digest carbohydrates, despite the low enzymatic action; however, the type of carbohydrate and the thermal processing can affect digestion [8].

The pet food market is developing practical alternatives to facilitate the use of natural diets and meet the demands of consumers and their pets. The aim is to replace extruded dry feed, the market leader. However, the lack of adequate information to be passed on to the owner causes them to feed their cats potentially harmful diets, including unbalanced feeds in unhealthy proportions [9]. The market, in the quest to facilitate the use of alternative diets, has opted for dehydrated natural feed, and the dehydration process consists of removing moisture from the formulated and produced feed, leaving it with almost zero water activity, intending to maintain the quality, stability, and integrity [10], allowing its use in various situations. Furthermore, its use consists of being more practical and quicker compared to natural frozen diets, facilitating handling by the owner and promoting greater water consumption by the animals, since in most cases, dehydrated feeds are rehydrated (as occurs in the food used in the present study) at the time they are consumed. This process is one of the oldest food conservation methods [11]; however, it remains unexplored in feline diets. Therefore, the present

study aimed to determine whether whole dehydrated feed can replace extruded dry feed, preserve daily intake, nutrient digestibility, and metabolism, and confer benefits in immunity and antioxidant responses in cats.

## 2. Materials and Methods

### 2.1. Feed

For the supply of dehydrated natural feed, the following steps were followed: weighing the feed, and then rehydrating it with feed in warm water (72˚C), at a dose of 150 ml of water for each 100 g of feed used, then waiting for 10 minutes and delivered.

### 2.2. Animals and Experimental Design

The experiment was carried out on the premises of the experimental farm of the University of the State of Santa Catarina, located in the municipality of Guatambu-SC. Fourteen mongrel females were used, centered in an experimental cattery under controlled temperature (22˚C), containing fourteen experimental cages (1 × 1 m) and a communal area with two water sources available *ad libitum*, in addition to four hygienic boxes of sand/sawdust available for the cats, which were cleaned twice a day to avoid an accumulation of excrement. The cats were raised in this facility since they were kittens, keeping their vaccination protocol up to date; all tested negative for FIV and FELV and using a current antiparasitic protocol. The animals had access to an external area during the day, and during the night, they were enclosed in the internal area of the facility.

The animals were divided into two groups for the experimental period, with seven animals in each group; the DNF group: cats that consumed natural dehydrated feed, and the DCF group: cats that consumed commercial extruded dry feed. The cats underwent a five-day adaptation period to the feed until day 0 of the experiment, where the animals were fed twice a day in individual cages with availability for one hour, available, after this time the leftovers were quantified to determine average daily consumption. The amount of feed supplied daily was calculated and established according to the commercial dose indicated on the product label and according to the body weight of each animal, in addition to a comparison being made by calculating the determination of metabolizable energy (ME) requirements with based on the animal's metabolic weight (PCP). The cats had access to water ad libitum.

### *2.3.* Sampling

The weight of the cats was measured, and blood samples were collected on days 1, 20, and 40 of the experiment; in each experimental stage, the cats fasted for 12 h before blood collection. The cats were sedated with a mixture of two commercial sedatives (xylazine at 0.06 to 0.1 mL/kg; and ketamine at 0.1 mL/kg) at the dose indicated by the manufacturer; then, the neck was scraped to facilitate collection. Blood was collected through the jugular vein with a 3-ml syringe fitted

with a 25/7-G needle. The collected blood was placed in Eppendorf tubescontaining 20 µl of anticoagulant solution (EDTA) for hemogram and tubes without anticoagulant for biochemical analysis, antioxidants, and serum proteinogram. Samples were centrifuged (5500 rpm for 10 min) to obtain serum.

### 2.4. Chemical Composition of the Feed and Feces

Feed samples were collected during the experimental period and stored at −20˚C. A pool was made for composition analysis at the end of four collections at ten-day intervals. The feces were collected between days 41 to 45, total feces collection method was used, and all the excrement that the animal defecated was collected. Feed and feces were thawed, weighed, and placed in an oven to determine moisture (*i.e.* the percentage of dry matter). The analyses of dry matter, mineral matter, ether extract, crude protein, acid detergent fiber, and neutral detergent fiber were carried out following [12].

### 2.5. Apparent Digestibility Coefficients

Using the chemical composition of feces and feed and the proportion of feed consumed and feces excreted, the apparent digestibility coefficient (ADC) was calculated according to the equation described by [13]: ADC (%) = ((Nutr I (g) – Nutr E (g)) × 100)/Nutr I (g), where Nutr I = ingested nutrient, and Nutr E = nutrient excreted in the feces.

### 2.6. Hemogram

The number of erythrocytes and leukocytes, hemoglobin concentration, platelets, and hematocrit were obtained using an automatic counter (EquipVET 3000). Leukocyte differential counts were also performed using an automatic counter, as were the mean corpuscular volume, red blood cell count, hemoglobin concentration, mean corpuscular hemoglobin concentration, red blood cell width distribution, and erythrocyte size variability.

### 2.7. Serum Biochemistry

Total protein, albumin, glucose, cholesterol, triglycerides, and creatinine levels were measured using specific commercial kits (Analisa) and a semi-automatic analyzer (BioPlus 2000). Globin levels were mathematically obtained from the difference between total protein and albumin.

### 2.8. Oxidant/Antioxidant Status

Serum lipid peroxidation was measured as the amount of thiobarbituric acid reactive substances (TBARS), according to literature [14]. The reaction was read in a spectrophotometer at 535 nm. The result was expressed as nmoles malondialdehyde/ml of serum.

Glutathione S-transferase (GST) activity was measured according to researchers [15]. To measure non-protein thiols (NPSH), the method used to use 5,5-dithiobis-(2-nitrobenzoic) acid (Sigma) was based on literature [16]. The de-

termination of the NPSH content in the samples was measured after deproteinization with trichloroacetic acid (50%). The absorbance readings (405 nm) were performed using a spectrofluorometer (Biotek, Synergy HT).

### 2.9. Protein Electrophoresis

For the proteinogram, electrophoresis in a polyacrylamide gel containing sodium dodecyl sulfate was performed according to the technique described by researchers [17] using mini-gels (10 × 10 cm). The gels were stained with Coomassie blue and photographed to identify and quantify protein fractions using LabImage 1D software (Loccus Biotechnology). A standard containing fractions with molecular weight between 10 and 250 KD (Kaleidoscope-BIORAD) was used as a reference.

### 2.10. Fecal Score

The fecal score was determined between days 40 and 45 of the experiment through visual observation of the feces and their characteristics. We used a scale from 1 to 5 proposed by researchers [18]: score 1—very hard and dry feces with small dry pellets; score 2—hard, dry, firm, soft, and well-formed feces; score 3—soft, well-formed, moist feces with preserved shape; score 4—soft feces, with no defined shape; score 5—liquid feces.

### 2.11. Statistical Analysis

Each animal was considered the experimental unit for all analyses. Data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Levene tests, respectively. All data were analyzed using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4) with the Satterthwaite approximation to determine the denominator degrees of freedom for the test of fixed effects. The results of body weight (BW) changes were tested for fixed effects of treatment and using animal and period as a random variable. All other variables (BW, serum biochemistry, serum antioxidant responses, and hemogram) were analyzed as repeated measures and tested for fixed effects of treatment, treatment × day, and using animal and period as random variables. The results of day 1 were also included as independent covariates in each analysis but were removed from the model when $P > 0.10$. The covariance structures were selected according to the lowest Akaike information criterion. Means were separated using PDIFF, and all results were reported as LSMEANS followed by SEM. The variables related to the animals' behaviors with the firecracker test were analyzed using the Chi-square test. Significance was defined when $P \leq 0.05$, and the tendency to significance when $P > 0.05$ and $P \leq 0.10$.

## 3. Results

### 3.1. Chemical Composition of Feed

The chemical composition of the feeds is presented in **Table 1**. The difference

between the feeds tested in terms of chemical composition is small, a numerical increase for the contents of ether extract, crude protein, and acid detergent fiber for the dehydrated natural feed.

**Table 1.** Chemical composition of dehydrated natural diet and commercial extruded dry feed.

| Chemical composition | DNF group | DCF group |
|---|---|---|
| Dry matter (g/kg) | 928 | 944 |
| Mineral matter (g/kg of DM) | 62.3 | 69.2 |
| Crude protein (g/kg of DM) | 457 | 344 |
| Ether extract (g/kg of DM) | 173.1 | 128.6 |
| Neutral detergent fiber (g/kg of DM) | 161.2 | 242.5 |
| Acid detergent fiber (g/kg of DM) | 69.4 | 48.5 |

**Note**: DNF: Natural dehydrated feed; DCF: Commercial extruded dry feed; **DNF**: One super-premium commercial cat feed was purchased and used as a control. The feed included poultry offal flour, rice grits, wheat gluten, ground whole corn, pea hulls, corn gluten, chicken fat, pork fat, isolated pork protein flour, refined soybean oil, beetroot, dried brewer's yeast, dried egg, zeolite, borage oil, fructo-oligosaccharides, marigold extract, grape polyphenols, green tea 10%, dicalcium phosphate, calcium sulfate, potassium chloride, monosodium phosphate, sodium chloride (common salt), calcium carbonate, monocalcium phosphate, vitamins (A, C, E, D3, B1, B2, B6, B12, PP), pantothenic acid, biotin, folic acid, choline chloride, iron sulfate, sulfate copper, manganese oxide, zinc oxide, calcium iodate, sodium selenite, chicken liver, annatto natural coloring, taurine, L-lysine, DL-methionine, antioxidant (BHA), ground whole corn, and genetically engineered corn gluten modified by *Bacillus thurigiensis* and *Streptomyces viridochromogenes*; Refined soybean oil produced from soybeans genetically modified by *Agrobacterium* sp. **DCF**: We used natural dehydrated feed (SmilePET mix, Tectron). Its composition is based on pork fat, salmomega forte, medium rolled oats, linseed grain (brown), sesame, distilled yeast at alcohol 36.7%, brewer's yeast 40 PB, dehydrated chicken, dehydrated bovine heart, dehydrated chicken liver, bovine lung, dehydrated pork bone trimmings, cassava starch, dehydrated pumpkin in flakes, dehydrated sweet potato in cubes, dehydrated carrot in cubes, dehydrated peas, dehydrated broken peas, whole apple in cubes, meat broth, chicken broth, refined salt with an anti-wetting agent, tricalcium phosphate, guar gum (thickener), xanthan gum, natural antioxidant, sodium hexametaphosphate, L-lysine 80%, DL-methionine 98%, choline chloride 60%, taurine 98%, sodium bisulfate, *Curcuma longa*, liver protein hydrolyzate, probiotics, yucca schidigera extract, vit/min premix, and ACIDMAX.

### 3.2. Feed Intake and Apparent Digestibility Coefficients

To adapt the cats to the new diet, the DNF was mixed with extruded dry feed in increasing amounts according to the animals' acceptance. From the beginning, the new feed was well accepted by the animals. However, from day 15 of the experiment, one of the cats started to vomit the consumed feed partially. On day 16, she vomitedthe entire consumed diet. We reduced the amount of feed to normalize the situation, and the cat continued to expel what was consumed; as a result, we removed this animal from the treated group of the experiment.

The ADC is presented in Table 2. Lower ADC of the ether extract was found for natural dehydrated feed compared to commercial dry feed ($P < 0.05$). On the other hand, higher ADC for mineral material, fiber neutral detergent (NDF), and fiber acid detergent (ADF) were observed in the DNF group than in the DCF group (control) ($P < 0.05$). There was no significant difference for ADC of dry matter and crude protein ($P = 0.95$; $P = 0.58$, respectively).

**Table 2.** The apparent digestibility coefficient of feed consumed by cats natural dehydrated feed (DNF) and commercial extruded dry feed (DCF).

| Chemical composition | DNF group | DCF group | SEM | P-value |
|---|---|---|---|---|
| Dry matter (%) | 83.4 | 83.2 | 0.45 | 0.95 |
| Mineral matter (%) | 67.7[a] | 51.5[b] | 1.05 | **0.01** |
| Crude protein (%) | 82.2 | 84.6 | 0.93 | 0.58 |
| Ether extract (%) | 88.1[b] | 95.2[a] | 0.89 | **0.01** |
| Neutral detergent fiber (%) | 86.4[a] | 80.2[b] | 0.52 | **0.01** |
| Acid detergent fiber (%) | 82.1[a] | 39.5[b] | 1.06 | **0.01** |

**Note**: [a,b]Within a treatment, underwriting differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$).

### 3.3. Feces

The amount of feces excreted and the fecal score of cats fed with dehydrated natural feed and DCF are presented in Table 3. Animals fed natural chow had more feces excreted (more wet feces than commercial chow) ($P < 0.05$). Stool scores in the DNF group were higher, reaching an average score of 3 in most animals (rare scores of 4), while most in the DCF group had a score of 1 to 2 ($P < 0.05$).

**Table 3.** Body weight and feces score of cats receiving experimental diets.

| Variable | Treatments | | SEM | P-value | |
|---|---|---|---|---|---|
| | DNF group | DCF group | | Treat | Treat × day |
| ***Body weight* (*kg*)** | | | | 0.91 | 0.93 |
| Day 1 | 2.38 | 2.29 | 0.24 | | |
| Day 20 | 2.45 | 2.44 | 0.22 | | |
| Day 40 | 2.50 | 2.47 | 0.22 | | |
| ***Weight gain* (*g*)** | | | | 0.31 | - |
| Days 1 to 40 | 120 | 180 | 5.96 | | |
| ***Feces score*** | | | | **0.01** | - |
| Days 40 to 45 | 3.13[a] | 1.48[b] | 0.01 | | |
| ***Feces MN* (*g*)** | | | | **0.01** | - |
| Days 40 to 45 | 212[a] | 147[b] | 4.12 | | |
| ***Feces MS* (*g*)** | | | | 0.56 | - |
| Days 40 to 45 | 50.0 | 47.0 | 0.85 | | |

Continued

| | | | | | |
|---|---|---|---|---|---|
| ***Feed intake (g/ day)*** | | | | 0.98 | - |
| Day 1 to 40 | 52.5 | 52.6 | 1.09 | | |
| ***Feed efficiency*** | | | | 0.25 | - |
| Day 1 to 40 | 0.057 | 0.086 | 0.008 | | |

**Note**: [a,b]Within a treatment, underwriting differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$). To analyze the treatment effect, the results of the variables on days 20 and 40, which correspond to the experimental period, were used; the results were presented as an average.

### 3.4. Body Weight

Results are presented in Table 3. There was no effect of treatment and no interaction between treatment vs. day for BW, feed intake, and feed efficiency ($P > 0.10$).

### 3.5. Hematologic Analysis

The complete blood count results are shown in Table 4. There was no treatment and treatment vs. day interaction effect for hematocrit, hemoglobin concentration, erythrocyte, monocyte, lymphocyte, and platelet counts ($P > 0.10$). We verified the effects of treatment and treatment vs. day interaction (day 40) for total leukocyte count, with lower values observed in cats in the DNF group compared to the DCF group ($P < 0.05$). Following the same dynamics, the granulocyte count was lower in the cats in the DNF group (day 40) ($P < 0.05$) and had a significant trend between treatments, lower for DNF ($P < 0.10$).

Table 4. Complete blood counts of cats receiving experimental diets.

| | Treatments | | SEM | P-value | |
|---|---|---|---|---|---|
| | DNF group | DCF group | | Treat | Treat × day |
| Hematocrit (%) | | | | 0.95 | 0.93 |
| | 33.6 | 33.8 | 1.02 | | |
| Hemoglobin (g/dL) | | | | 0.92 | 0.88 |
| | 9.66 | 9.73 | 0.25 | | |
| Erythrocytes ($\times 10^6$ µL) | | | | 0.81 | 0.84 |
| | 7.18 | 7.50 | 0.14 | | |
| Leukocytes ($\times 10^3$ µL) | | | | **0.04** | **0.01** |
| D1 | 5.69 | 6.88 | 0.44 | | |
| D20 | 6.35 | 7.54 | 0.39 | | |
| D40 | 5.13[b] | 6.82[a] | 0.40 | | |
| Average | 5.74[b] | 7.18[a] | 0.36 | | |
| Granulocytes (/µL) | | | | **0.08** | **0.05** |
| D1 | 1.23 | 1.96 | 0.52 | | |

**Continued**

| | | | | | |
|---|---|---|---|---|---|
| D20 | 2.07 | 2.41 | 0.50 | | |
| D40 | 1.76[b] | 2.55[a] | 0.32 | | |
| Average | 1.91[b] | 2.48[a] | 0.38 | | |
| Lymphocytes (/μL) | | | | 0.65 | 0.49 |
| | 3.31 | 3.93 | 0.41 | | |
| Monocytes (/μL) | | | | 0.71 | 0.56 |
| | 0.52 | 0.77 | 0.35 | | |

**Note**: [a,b]Within a treatment, underwriting differs (P ≤ 0.05) or tends to differ (P ≤ 0.10). [a,b]In lines without common subscripts, there are differences (P ≤ 0.05) or tendencies to differ (P ≤ 0.10). To analyze the treatment effect, the results of the variables on days 20 and 40, which correspond to the experimental period, were used; the results were presented as an average.

### 3.6. Serum Biochemical Analysis

The serum biochemistry results are presented in Table 5. There was a treatment effect on serum cholesterol concentrations; animals in the DNF group had lower serum levels of this biomarker compared to the control (DCF group) (P < 0.05). Glucose, triglycerides, creatinine, total protein, albumin, and globulin levels did not differ between groups (P > 0.10).

Table 5. Serum biochemistry and oxidative profile of cats receiving experimental diets.

| | Treatments | | SEM | P-value | |
|---|---|---|---|---|---|
| | DNF group | DCF group | | Treat | Treat × day |
| Glucose (mg/dL) | | | | 0.19 | 0.26 |
| | 105.9 | 91.4 | 5.14 | | |
| Triglycerides (mg/dL) | | | | 0.95 | 0.92 |
| | 43.9 | 44.7 | 2.41 | | |
| Cholesterol (mg/dL) | | | | **0.01** | 0.11 |
| | 91.2[b] | 104.0[a] | 2.84 | | |
| Total protein (g/dL) | | | | 0.89 | 0.93 |
| | 7.01 | 6.73 | 0.10 | | |
| Albumin (g/dL) | | | | 0.96 | 0.93 |
| | 2.22 | 2.25 | 0.05 | | |
| Globulin (g/dL) | | | | 0.83 | 0.78 |
| | 4.78 | 4.48 | 0.09 | | |
| Creatinine (mg/dL) | | | | 0.94 | 0.95 |
| | 1.03 | 1.10 | 0.02 | | |
| GST (μmol CDNB/min) | | | | **0.01** | **0.01** |
| D1 | 585 | 609 | 9.36 | | |

**Continued**

| | | | | | |
|---|---|---|---|---|---|
| D20 | 734[a] | 672[b] | 8.54 | | |
| D40 | 687[a] | 591[b] | 8.42 | | |
| Average | 710[a] | 632[b] | 8.14 | | |
| Total tiois (nmol SH/mg) | | | | 0.52 | **0.05** |
| D1 | 103 | 102 | 5.36 | | |
| D20 | 88.2 | 90.2 | 4.97 | | |
| D40 | 92.1[a] | 75.1[b] | 4.84 | | |
| Average | 90.2 | 82.9 | 4.68 | | |
| TBARS (nmol MDA/mL) | | | | 0.97 | 0.95 |
| | 16.4 | 16.0 | 1.03 | | |

**Note**: [a,b]Within a treatment, underwriting differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$). [a,b]In lines without the common subscript, there are differences ($P \leq 0.05$) or tendencies to differ ($P \leq 0.10$). To analyze the treatment effect, the results of the variables on days 20 and 40, which correspond to the experimental period, were used; the results were presented as an average.

### 3.7. Oxidant and Antioxidant Status

The results of the oxidant and antioxidant profile are presented in Table 5. We observed treatment effect and treatment vs. day interaction (day 20; 40) for GST levels, with the DNF group being more significant than the DCF group ($P < 0.05$). For total thiols, significance was found in the treatment vs. day interaction (day 40), also more significant for the DNF group ($P < 0.05$). There was no difference in lipid peroxidation (TBARS) in the serum of cats ($P > 0.10$).

### 3.8. Proteinogram

The results of the serum proteinogram are presented in Table 6. We verified a treatment effect for IgA concentrations, with higher values observed in cats in the DNF group compared to the DCF group ($P < 0.05$). Different from what was found for ceruloplasmin levels, both between treatments and in the interaction between treatment and day (day 40), the DNF group was lower compared to the DCF group ($P < 0.05$). Following the same situation, C-reactive protein levels were lower for the DNF group ($P < 0.05$). Transferrin concentrations were higher in cats in the DNF group (day 40) ($P < 0.05$), and there was still a significant trend between treatments, being higher for DNF ($P < 0.10$). No significant difference was observed for heavy chain IgG concentrations ($P > 0.05$).

### 3.9. Microbiological Feces

The results of the microbiological feces are presented in Figure 1. We found a higher bacterial count in the feces of cats fed natural dry feed both for *E. coli* ($P < 0.05$) and a trend toward higher total coliform counts when compared to the DCF group ($P < 0.10$).

**Table 6.** Serum proteinogram of cats receiving experimental diets.

| Variable[1] | Treatments | | SD | P-value | |
|---|---|---|---|---|---|
| | DNF group | DCF group | | Treat | Treat × day |
| IgA (g/dL) | | | | **0.01** | 0.12 |
| Average[2] | 0.94[a] | 0.80[b] | 0.02 | | |
| Heavy chain IGG (g/dL) | | | | 0.14 | 0.13 |
| Average[2] | 1.25 | 1.08 | 0.05 | | |
| Ceruloplasmin (g/dL) | | | | **0.05** | **0.03** |
| D1 | 0.62 | 0.64 | 0.07 | | |
| D20 | 0.57 | 0.65 | 0.08 | | |
| D40 | 0.48[b] | 0.65[a] | 0.08 | | |
| Mean[2] | 0.52[b] | 0.65[a] | 0.07 | | |
| C-reactive protein (g/dL) | | | | **0.05** | 0.11 |
| Mean[2] | 0.27[b] | 0.37[a] | 0.04 | | |
| Transferrin (g/dL) | | | | **0.10** | **0.05** |
| D1 | 0.25 | 0.28 | 0.03 | | |
| D20 | 0.29 | 0.27 | 0.03 | | |
| D40 | 0.33[a] | 0.28[b] | 0.03 | | |
| Mean[2] | 0.31[a] | 0.27[b] | 0.02 | | |

**Note**: [1]Immunoglobulin A (IgA), heavy chain immunoglobulin G (IgG). [2]To analyze the treatment effect, the results of the variables on days 14 and 28, which correspond to the experimental period, were used; the results were presented in an average. [a,b]Within a treatment, the subscript differs ($P \leq 0.05$) or tends to differ ($P \leq 0.10$) in the same column. [a,b]In the same line, without the common subscript, there are differences ($P \leq 0.05$) or tendencies to differ ($P \leq 0.10$) between treatments.

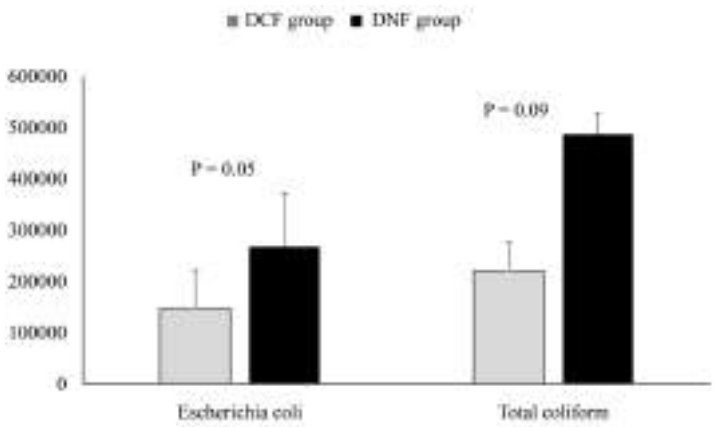



**Figure 1.** *Escherichia coli* counts and total coliforms in the feces of cats receiving experimental diets. Results presented in colony forming units (CFU).

## 4. Discussion

The study of pet food comparisons is very controversial due to the discrepancy

of feeds that each product can offer; however, to maintain the integrity of the study, we tried to maintain a similar nutritional base in the feeds tested. The dehydrated natural feed didn't affect the cat feed intake, as they themselves went through an adaptation period so that they could get used to consuming the new diet, highlighting that only an animal had to be removed from the experiment as it didn't accept the new diet. However, numerous factors modify the nutrients in a feed, as we observed that the dehydrated natural feed had higher values of protein and ether extract and lower values of fiber than the extruded feed. This fact is positive because cats are highly demanding animals and can control their intake according to the quality of the feed, consuming feed in several portions during the day [19]. Researchers [20] demonstrated that cats could select and prefer diets with higher values of protein and fat, with protein being the most critical factor in this decision, as it is the nutrient that this species requires nutritionally, in particular, a point that may have been beneficial since the natural feed used had these characteristics.

The digestibility of some nutrients differed between groups of cats, something expected; however, there was an important finding: Even though the feed had a lower value of the mineral matter, the digestibility of this nutrient was more significant for the group that consumed a natural dehydrated diet. However, the diet had higher values of this component for the ether extract, and its digestibility was significantly lower than the extruded dry feed. Regarding digestibility, several factors can interfere because, according to literature [21], the ADC may differ by the composition of ingredients used, the type of processing that the feed may be subjected to, in addition to aspects specific to the animal, such as the stage of life of gastrointestinal flora. However, nutrients have their particularities, including fat, which cats digest well; however, they depend on their saturation load [22], the amount of calcium present in the diet that can cause fat hydrolysis, age of the animal and also losses of endogenous fecal fat [23]. These factors may have beneficially influenced the study, based on the assumption that self-fat levels can harm cats' health in the long term.

Little importance is given to fiber in the diet of cats, but in the present study, the ingredients in the diet directly influenced the existing amount of NDF and ADF in the feed, demonstrating low levels for the natural dehydrated feed, which in turn proved to be highly digestible, as pointed out in the present study. Cats are strictly carnivores by nature; however, a minimal amount of fiber in the diet of these animals can have a positive effect on certain diets that require specific care, such as obesity, and intestinal problems, among other situations that fiber can contribute to this species [24]. This situation is not the case in the present study; however, this finding may influence and stimulate further research to determine the benefits in animals with certain particularities. Protein digestibility was similar for both feeds, remembering that protein levels were higher in the natural dehydrated diet; thus, similar digestibility can be interpreted as beneficial due to the greater availability of amino acids. The lower di-

gestibility of the ether extract may have directly affected blood cholesterol levels, decreasing its concentration in the group that consumed the dehydrated natural feed. Researchers [25] point out that high cholesterol levels can predispose to obesity in dogs and cats, so considering a long-term feeding characteristic, the use of NDF can be metabolically beneficial for the animal predisposed to obesity.

Cats from the DNF group showing wetter feces and consequently in greater quantity, which we believe is related to two factors: a) the dehydrated feed had a smaller amount of fiber, in addition to the type of dietary fiber used in the diet, which aligns with Kerr *et al*. [26], who claim that different types of fiber for felines directly influence the production, consistency, and type of fecal fermentation; another study describes cellulose as the main component of fiber to adjust fecal consistency [24], a component that proved to be highly digestible in the diet in question in the present study; b) another highly relevant factor is that the DNF needs to be hydrated to be supplied; adding water to the diet can directly influence the amount and consistency of the animals' feces.

White blood cell counts differed across diets because feed acts directly in modulating the function of the immune system, in addition to the fact that the components present in the diet can help increase or decrease resistance to possible infections [27]. We highlight the lower presence of white blood cells responsible for our body's defense against infectious agents, being able to associate this result with the serum proteinogram, which showed that the cats that consumed DNF had lower concentrations of inflammatory response biomarkers. We also noticed a higher IgA antibody concentration in the cats, an immunoglobulin that protects the mucosa [28]. It is essential to emphasize that the animals used in the study were healthy. Hence, the differences between diets allow us to believe that the natural diet can improve the immune response without concomitantly generating an inflammatory response.

The diet influenced the antioxidant status, in which the cats in the DNF group had higher levels of enzymatic and non-enzymatic antioxidants, that is, the endogenous activity of GST and those coming from the feed itself (thiols). Antioxidants can delay lipid oxidation and characterize a feed as rancid; however, commercial feeds often use synthetic antioxidants to maintain feed stability and prevent lipid peroxidation [29]; thus, our study demonstrates that dehydrated natural feed, which is rich in natural antioxidants, can beneficially influence protecting the animal organism from possible damage by free radicals and future lipid oxidation of the feed [30]. We hypothesize that it is a cascade effect, where the entire antioxidant system benefits from the positive effects of a natural diet.

We highlighted small changes in acute phase proteins produced by the liver, which are the most critical and sensitive marker of inflammatory response in the body of animals [31]. Lower concentrations of ceruloplasmin were observed for the DNF group, a protein responsible for transporting blood copper in healthy individuals. Ceruloplasmin levels of 0.48 to 0.65 g/dL are considered healthy. This was also seen with C-reactive protein, which was lower in the DNF group;

this protein is typically stimulated by tissue damage linked to inflammatory processes and modulates neutrophil function [32]. Low levels demonstrate that animals are in a normal state, associated with low white blood cell values in the present study. The liver can synthesize the primaryiron-transporting protein, transferrin; in our study, the NDF group had higher levels than DCF. Naturally, blood iron derives almost entirely from erythrocytes that macrophages have phagocytosed; very little blood iron comes from feed [33], and virtually all lost iron is equivalent to how much was absorbed by the diet [34]. Bohn [33] stated that if iron is deficient in the body, transferrin may increase, requiring the iron to be revolved several times by the circulation; however, it is a typical case of a healthy animal unless it is associated with chronic liver disease.

Finally, cats that receiving dehydrated natural feed demonstrated a higher bacterial count measured in *E. coli* and a trend toward the superiority of total coliform counts. One of the factors driving consumers to not adhere to a completely natural diet (which is focused on raw feed) is the fear of bacterial cross-contamination linked to antimicrobial resistance to pathogens [35]. However, a factor to be taken into account is that the natural diet had higher levels of fiber and greater digestibility, and cats will ferment this fiber in the large intestine, thus, it is assumed that there was a higher fermentation rate for digestion and absorption, increasing bacterial levels present in the fecal cake [36]. Even so, when talking about the feed dehydration process, it is assumed that drying the feed, leaving its water activity at minimum levels, can denature the bacterial proteins, alter the bacterial membrane, and cause its extravasation and destruction; *E. coli* is particularly susceptible to this type of damage [10]. Studies focusing on numerous bacterial species, microbiological quality of feed processing and animal excretion can elucidate consumer perspectives regarding dry feed.

During the experiment period, approximately 15 days after using the dehydrated natural feed, one of the animals began to expel all the feed consumed, even though the cat consumed it completely. Vomiting in felines can be a clinical sign indicative of a possible gastrointestinal disease or specific intoxication arising from feed or even an expansion of the stomach caused by feed that leaves it with digestion difficulties [37], the latter cause being more likely to be accepted in the present study, due to the amount of feed increased by the age of the animals. However, the animal was healthy, without characteristic signs of illness or contractions, it only expelled the feed shortly after consuming it. We can also characterize it by a possible regurgitation, which is usually passive expulsion of feed still present in the pharynx or esophagus, which according to literature [38], can happen soon after feed ingestion. We removed this animal from the treatment group and returned to supplying extruded feed and thus the animal's situation immediately normalized.

## 5. Conclusion

The cats that consumed the dehydrated natural feed had a higher digestibility of

mineral matter, neutral detergent fiber and acid detergent fiber but a lower digestibility coefficient of the ether extract. Cats that consumed natural feed stimulated the antioxidant system, where antioxidants were higher, which may favor the process of controlling oxidative reactions in the body. Lower levels of acute phase proteins, leukocytes, and granulocytes are seen in the blood of cats that have consumed DNF. Therefore, we conclude that both feeds are nutritionally appropriate for growing cats, much like extruded dry feed; moreover, the ingestion of dehydrated natural feed has nutraceutical effects, which are beneficial to animal health.

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## Ethics Approval

The ethics committee on the use of animals in research of the Universidade do Estado de Santa Catarina approved all procedures for this project under protocol number 5167240422 and all procedures followed the rules issued by the National Council for Control of Animal Experimentation (CONCEA).

## Data Availability Statement

Raw data are held by the authors and may be available upon request.

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## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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# Combination of Phytoactives in the Diet of Lactating Jersey Cows: Effects on Productive Efficiency, Milk Composition and Quality, Ruminal Environment, and Animal Health

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**Simple Summary:** Adding the phytoactive mixture to the dairy cows' diet positively affected animal nutrition and health, improving Jersey production efficiency. The intake of phytogenics improved milk quality, reduced somatic cell counts, and increased unsaturated fatty acids. The intake of phytogenics per cow protected the mammary gland from bacteria *Streptococcus* spp. The phytoactive mixture directly affected the digestibility and modulation of the volatile fatty acid profile. The phytoactive mixture positively affected immune and antioxidant responses and anti-inflammatory action.

**Abstract:** This study's objective was to evaluate whether adding a combination of phytoactive (microencapsulated essential oils, minerals, turmeric extract, tannin, prebiotic, and probiotic) to the feed of lactating Jersey cows positively affects the production, composition, and quality of milk, rumen environment, and animal health. Fourteen Jersey cows were divided into two groups (control and phytogenic) for an experiment with two lactation phases of 45 days each (early lactation and mid-lactation). During the experiment, milk production was higher at various times in cows that consumed phytoactive, and these animals had the best feed efficiency. In mid-lactation, phytoactive intake increased nutrient digestibility. The number of lymphocytes in the blood is reduced when cows consume phytoactive substances. Globulin levels increased in these cows fed with the additive, which may be related to a higher concentration of immunoglobulins, especially IgA. Cows fed phytoactives had lower ceruloplasmin and haptoglobin concentrations. Lower serum lipid peroxidation, associated with greater glutathione S-transferase activity, is a good health indicator in cows that consume phytoactive substances. The higher concentration of volatile fatty acids was due to the higher proportion of acetic acid in the ruminal fluid combined with lower butyric acid. Somatic cell counts in milk were lower in cows that consumed phytoactives during mid-lactation, as well as the effect of the treatment on *Streptococcus* spp. (lower in cows that consumed the additive). We conclude that consuming the additive benefits cows' health modulates rumen fermentation and nutrient digestibility, and positively affects milk production and quality.

**Keywords:** dairy cattle; phytobiotic; nutraceutical; immunity; *Streptococcus*; animal health

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## 1. Introduction

Milk production has grown considerably over the last ten years [1]; however, the number of cows milked has not seen a representative increase in productivity, which shows that dairy farms are becoming more efficient. Dairy cow nutrition influences milk production and quality, cows' health, and the system's environmental impacts. The symbiosis between ruminants and microorganisms is fundamental; the microorganisms degrade the feed consumed and provide the animals mainly with fatty acids and microbial protein; however, in this process, heat, ammonia, and methane are produced, which are considered protein and energy losses [2]. To reduce these losses and consequently enhance animal performance by improving efficiency in using feed, alternatives have been explored, such as additives in the diet [3].

Cows at peak production have high metabolic demands and are more prone to disorders such as physiological oxidative stress and decreased immune response [4]. Natural feed additives are on the rise due to their numerous benefits to animals, including antioxidant, anti-inflammatory, antibacterial, antiviral, coccidiostats, and anthelmintic actions [5–7]. In addition to having great immunological functionality, some natural compounds act in rumen fermentation to improve nutrient absorption and increase ruminant productivity [8,9]. These actions can improve cows' health by avoiding or minimizing metabolic disorders and infectious diseases such as mastitis, thus maintaining constant productivity. In cases of mastitis, due to the antimicrobial action, there has been a record of positive effects of cinnamon and oregano essential oils [10,11] and curcumin [12], which are ingredients of the phytogenic used in the present study.

It is essential to note that the work carried out has shown the efficiency of phytogenics in a mixed and individual way. Despite this, research is needed to adjust indications, dosages, and supply modes so adverse reactions do not occur, according to the literature [13]. Commercial products, defined as blends, have gained space in the animal nutrition market, where combinations of essential oils, plant extracts, organic minerals, prebiotics, and probiotics in search of synergism and health and productive efficiency are becoming an increasingly common practice. However, in these combinations, little is known scientifically about the real benefits of including these additives in the diet for lactating cows; at the same time, there is a need to avoid or minimize health problems in high-producing cows, which are constantly challenged.

We hypothesized that cows that consume the additive produce more milk and milk with lower somatic cell count (SCC) because the synergism between the ingredients present in the additive would trigger an antioxidant and modulate ruminal fermentation. Therefore, this study's objective was to evaluate whether adding a combination of phytoactives to the feed of lactating primiparous Jersey cows positively affects milk production, composition and quality, rumen environment, and animal health.

## 2. Materials and Methods

### 2.1. Additive

The product used was a commercial additive (Phytomast® Concentrado, Tecphy, Canelinha, Brazil). Its formulation is based on cinnamon and oregano essential oil, chelated amino acid chromium (min. 200 mg/kg), selenium proteinate (min. 250.00 mg/kg), inactivated *Saccharomyces cerevisiae* (min. 270 g/kg), *S. cerevisiae* (min. $5.0 \times 10^{11}$ CFU/kg), turmeric extract, and tannic acid. The dosage used was 20 g per animal day, as recommended by the manufacturer.

### 2.2. Animals and Facilities

The experiment was conducted in the ruminant sector—dairy cattle farming at the Experimental Farm (FECEO) of the State University of Santa Catarina (UDESC) located in Guatambu/SC, Brazil. Fourteen primiparous Jersey cows, $27 \pm 0.5$ months old and weighing $410 \pm 12.6$ kg, with 30 days in milk (DIM), were used. The cows used in the research were daughters of the same bull (Swoosh, STgenetics, Indaiatuba, SP, Brazil) to

have a consistent genetic standard for the experiment. Animals were acquired by the university shortly after birth, where they were managed and fed similarly until the current experimentation phase. At the experiment's beginning, the cows' body score was 3.0 to 3.5, indicating a homogeneous herd.

The herd was housed in a compost barn-type sawdust bed confinement system, with 20 $m^2$ per animal. The animals were milked using two types of milking systems: (a) a mechanical system in a herringbone milking parlor (45 days) and (b) a robotic milking system in a guided flow system (45 days).

In the pre-partum period, 60 days before the start of the experiment, the animals received a vaccine for clostridiosis and underwent parasite control (ticks, flies, and helminths). Pre-partum, the animals received an anionic diet. Still, in the first stage of the experiment, one of the cows in the control group became ill (bovine parasitic disease), and despite treatment, the cow died. Thus, 13 cows (6 and 7) remained for the control and treated groups, respectively.

### *2.3. Experimental Design and Diets*

#### 2.3.1. Early Lactation—Peak Lactation

The early lactation phase lasted 45 days (15 days of adaptation + 30 days of data collection), during which the animals were randomly divided into two groups of seven cows: phytogenic, where the concentrate had 20 g of Phytomast® (feed additive) added, and control, which received the basal concentrate without added additives. To form the groups, we considered the body score, body weight (control: 412 ± 12.8 kg; phytogenic: 408 ± 11.9 kg), and the average milk production of these cows over the last seven days before the beginning of this experiment (control group: 19.8 ± 0.56 kg of milk; phytogenic group: 20.0 ± 0.52 kg of milk).

The diets were formulated according to the nutritional requirements of the animals [14] with a 52 V:48 C ratio, considering the following feeds: concentrate, Tifton 85 hay, and corn silage, which were mixed and supplied in the form of a total mixed ration (TMR) in individual feeders (with the animals restrained), divided into three daily treatments (06:00, 11:00, and 16:00). Water was provided ad libitum in the shed and during feeding when cows were contained by a cage (that is, a drinking trough separated the feeders) (Supplementary Materials S1). It is essential to make it clear that each cow was fed in its feeder, i.e., the feeding was individual for each animal despite being in a compost barn system. The composition of the concentrate and the feeds used in the diet are shown in Table 1.

In early lactation, the cows were milked mechanically at 7:00 a.m. and 4:30 p.m., with three milking sets using automatic extraction [model AMI 5550 (Boumatic®, Moncton, NB, Canada)].

**Table 1.** Chemical composition of feed and total diet fed to dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM).

| | Silage | Basal Concentrate | TMR Control | TMR Phytogenic | Robot Feed—Pelleted |
|---|---|---|---|---|---|
| Early lactation: DIM 30–75 | | | | | |
| Dry matter | 27.35 | 86.06 | 48.6 | 48.81 | - |
| Crude protein | 10.56 | 26.33 | 16.83 | 16.86 | - |
| Ethereal extract | 2.45 | 6.79 | 3.77 | 3.83 | - |
| Ash | 5.91 | 8.84 | 9.26 | 8.71 | - |
| NDF | 57.85 | 29.77 | 43.75 | 43.5 | - |
| ADF | 26.43 | 17.21 | 22.29 | 21.36 | - |

**Table 1.** *Cont.*

| | Silage | Basal Concentrate | TMR Control | TMR Phytogenic | Robot Feed—Pelleted |
|---|---|---|---|---|---|
| Mid-lactation: DIM 90–135 | | | | | |
| Dry matter | 25.09 | 84.07 | 43.8 | 41.8 | 88.75 |
| Crude protein | 9.88 | 26.15 | 16.8 | 15.8 | 20.87 |
| Ethereal extract | 2.9 | 2.77 | 2.69 | 3.32 | 5.51 |
| Ash | 3.74 | 7.6 | 9.17 | 8.49 | 10.74 |
| NDF | 40.35 | 22.5 | 39.24 | 40.35 | 30.06 |
| ADF | 19.62 | 12.82 | 19.17 | 20.5 | 13.86 |

Concentrate (Soybean meal, corn, soybean hulls, wheat bran, mineral, and sodium bicarbonate); TMR: in the total mixed ration; NDF: neutral detergent fiber; ADF: acid detergent fiber; DIM: days in milk.

#### 2.3.2. Mid-Lactation

After completing the first stage, the animals continued to consume the same diet as in early lactation for 15 days (with and without the phytogenic, according to their group). This interval was not foreseen in the initial project but was necessary because the installation of the robotic milking system had yet to be completed.

Next, mid-lactation began, which also lasted 45 days (15 days of adaptation + 30 days of data collection), where the division of animals was the same as in early lactation, with seven cows in each group: phytogenic, where the concentrate included 20 g of Phytomast® (feed additive); control, which received the basal concentrate without added additives. It is essential to highlight that the same animals were always kept in each group; therefore, the cows in the phytogenic group consumed the feed additive for 105 continuous days (early lactation + 15-day interval + mid-lactation).

At this stage, the cows had a DIM of approximately 90 days; they would have the robot's feed, and a new diet was formulated according to the nutritional requirements of the animals [14] with a 52 V:48 C ratio, considering the following feeds: concentrate, Tifton 85 hay and silage of corn. These feeds were mixed and fed into individual feeders, which were also divided into three daily treatments. Water was supplied ad libitum, as in early lactation. The composition of the concentrate and feeds used in the diet are also shown in Table 1.

In mid-lactation, the cows were milked using a robotic milking system with guided flow at two times: 5:30 a.m. and 3:30 p.m. Therefore, when milking was not scheduled, the robot remained in the "manual" function, with doors closed. At these milking start times, the "automatic" function was switched. After milking, the animals entered the feeding lane, waiting for feed to be supplied.

### *2.4. Productive Performance*

Daily milk production was measured using a digital production marker coupled to the mechanical milking system during early lactation, and the output was recorded on a spreadsheet. In mid-lactation, automatic milking determines the daily production of each cow, with data stored on a computer connected to the robot. In two lactation phases, the amount of feed provided was measured, and after feeding, the amount of feed left over was weighed to determine daily consumption. Based on these data, dairy efficiency is calculated: milk production/feed consumption.

Feed samples were pre-dried in a forced ventilation oven at 55 °C for 72 h, then removed from the oven and weighed again to determine the partial dry matter content, followed by grinding in a Wiley-type mill (Marconi, model: MA340, Piracicaba, SP, Brazil) using a 1 mm mesh sieve. The pre-dried and ground samples were heated at 105 °C to obtain the MS and the mineral material in a muffle furnace at 600 °C [15]. The micro-Kjeldahl method determined the N content (Method 984.13, AOAC, 1997), which made it possible to predict the CP content through mathematical calculation. To determine the neutral detergent fiber (NDF) content, samples were placed in polyester bags [16]

and treated with a neutral detergent solution in an autoclave at 110 °C for 40 min [17]. Acid detergent fiber (ADF) concentrations were determined according to AOAC (1997, method 973.18). The ether extract portion of the samples was quantified using an automatic SER158 Fat Extractor (VELP® Scientifica, Usmate Velate, MB, Italy).

### *2.5. Sampling and Sample Collection*

Blood samples were collected at intervals of 15 days during early lactation and mid-lactation (DIM 30, 45, 60, and 75 and DIM 90, 105, 120, and 135, respectively). Samples were collected in tubes with clot activator (4 mL) to separate blood serum and in tubes with 10% EDTA anticoagulant (4 mL) for blood count analysis. The material was stored in a thermal box until processing, which took up to 3 h.

In both milking systems, milk collectors allow a homogeneous, individual sample of the entire milking. Milk for proximate composition analysis was collected during morning milking at DIM 30, 45, 60, and 75 in early lactation and DIM 90, 105, 120, and 135 in mid-lactation. TSCC and proximate composition in early lactation and mid-lactation were measured at strategic moments. Milk collection for fatty acid profile was carried out at DIM 60 and 75 in early lactation and DIM 120 and 135 in mid-lactation. To evaluate the microbiota of the mammary/milk gland at three moments (DIM 30, 75, and 135), the teats were cleaned using a pre-dipping solution and dried with paper towels. The first three jets were removed from each teat using gloves to reduce environmental contamination. This material was frozen at −80 °C until the end of the experiment. Afterward, it was thawed and homogenized, and a sample was collected using a specific commercial kit for transporting and preserving biological material to the laboratory.

Ruminal fluid was collected at the end of each experimental period, with DIM 75 in early lactation and DIM 135 in mid-lactation. For collection, a silicone esophageal probe was connected to a vacuum system, allowing the collection of material with pH measured in less than one minute after collection; then, it was placed in plastic pots and kept cool in an ice box.

Feces were collected to determine nutrient digestibility coefficients on the last three days of the experiment: DIM 73, 74, and 75 in early lactation and DIM 133, 134, and 135 in mid-lactation. Feces were collected directly from the rectal ampoule three times daily.

### *2.6. Hemogram*

Hematologic variables were obtained using EDTA blood tubes and analyzed with the VET3000 automatic hematologic analyzer (EQUIP®), which determines the number of leukocytes, lymphocytes, granulocytes, monocytes, erythrocytes, and platelets, as well as determining the concentration of hemoglobin (g/dL) and hematocrit (%).

### *2.7. Serum Biochemistry and Proteinogram*

The samples collected in tubes without anticoagulant were centrifuged in a QUIMIS® tube centrifuge at 8000 revolutions per minute (rpm) for ten minutes. Subsequently, the serum was stored in microtubes at −20 °C until analysis. In the blood serum, serum levels of total protein, albumin, urea, C-reactive protein (CRP), cholesterol, and fructosamine were evaluated, and the activity of the enzymes aspartate aminotransferase (AST) and gamma-glutamyltranspeptidase (GGT) was determined. For this, ANALISA® kits were used in EQUIPIVET equipment (Zybio EXC-200®, Shenzhen, China). The concentration of globulins was calculated as total protein—albumin.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed using mini gels (10 × 10 cm), as described by researchers [18]. The gels were stained with Coomassie blue and photographed to identify and quantify protein fractions using Labimage 1D software (Loccus Biotechnology, Cotia, SP, Brazil). A standard containing fractions with molecular weight between 10 and 250 KD (Kaleidoscope—Bio-Rad, Maynard, MA, USA) was used as a reference.

### *2.8. Oxidative Status*

Firstly, the concentration of proteins in serum samples was determined using the literature methodology [19], using bovine albumin as the standard. A calibration curve was previously constructed with albumin, and subsequently, the absorbance of the samples was measured at 595 nm using cuvettes in a spectrophotometer. Then, the quantification of total proteins in the serum samples was performed, and the data were used to calculate the results of the oxidative biomarkers described below.

Serum superoxide dismutase (SOD) activities were evaluated spectrophotometrically as described by researchers [20], based on the superoxide anion inhibition reaction in the presence of pyragolol. Enzymatic activity was expressed in SOD units per mg of protein.

Serum GST activity was measured according to the literature [21] with minor modifications. GST activity was measured using the rate of formation of dinitrophenyl-S-glutathione at 340 nm in a medium containing 50 mM potassium phosphate, pH 6.5, 1 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, and tissue supernatants (approximately 0.045 mg protein). Results were calculated and expressed as U per mg of protein.

Levels of TBARS (thiobarbituric acid reactive substances) were assessed to determine lipid peroxidation levels using the method described by Jentzsch et al. [22]. The TBARS results were obtained using a spectrophotometer at 535 nm and expressed as nmol MDA per mL.

Carbonines were measured using the reaction method of dinitrophenylhydrazine (DNPH) with protein carbonyls forming hydrazones, which are measured spectrophotometrically at 360 nm. For each sample, another tube was used, where the total protein of the sample was measured using HCl instead of DNPH; 20% TCA and then 0% TCA were added to both tubes to precipitate the carbonyls and proteins; the precipitate was washed with ethanol and ethyl acetate to remove free DNPH and contaminating lipids. The final precipitate was dissolved in guanidine and read at 360 nm, while protein concentration was read at 280 nm. Results were expressed in nM/mg protein.

### *2.9. Biomarkers in Rumen Liquid*

The rumen fluid was collected four hours after feeding using a 1.5 m long, 11 mm diameter ororuminal probe and was allocated to 100 mL universal collectors. Part of the remaining rumen fluid was filtered through three gauzes, stored in 3 mL microtubes (Eppendorf®, Hamburg, Germany), and frozen at −20 °C for subsequent analysis of volatile fatty acids (VFAs).

To determine short-chain fatty acids (SCFAs), the fluid samples were thawed at 5 °C and manually shaken for homogenization. Aliquots of 1 mL of the ruminal fluid supernatant were collected into polypropylene microtubes (2 mL) and centrifuged for five minutes (12,300× *g*). Then, 250 µL of the supernatant was transferred to a new microtube containing 250 µL of formic acid. The mixture was manually shaken and centrifuged for three minutes. After centrifugation, 250 µL of the supernatant of the mixture was collected in another polypropylene tube previously containing 500 µL of 3-octanol solution (665 µg $mL^{-1}$ in methanol) used as an internal standard. The mixture was homogenized and centrifuged again. Then, 600 µL of the sample was inserted into an injection vial and injected into a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3400, Palo Alto, CA, USA) and an autosampler (Varian 8200CX, Palo Alto, CA, USA). One microliter of the extract was injected in split mode at 1:10. The carrier gas used was hydrogen at a constant pressure of 20 psi. The analytes (acetic, propionic, butyric, valeric, and isovaleric acids) were separated using a CP-Wax 52CB capillary column (60 m × 0.25 mm; 0.25 µm stationary phase thickness). The initial column temperature was set at 80 °C for one minute and increased to 120 °C at a rate of 8 °C $min^{-1}$, then to 230 °C at 20 °C $min^{-1}$, where it remained for one min. The injector and detector temperatures were set at 250 °C.

Method validation comprised the following parameters: selectivity, linearity, linear range, repeatability, precision, limit of detection (LOD), and limit of quantification (LOQ) for acetic, propionic, butyric, and isovaleric acids (Supplementary Materials S2). Linearity

was assessed by calculating a regression equation using the least squares method. LOD and LOQ values were obtained by performing sequential dilutions until signal-to-noise ratios of 3:1 and 6:1 were achieved, respectively. Accuracy was assessed by analyzing the repeatability of six replicate samples. Accuracy was determined by recovering known amounts of standard substances added to a diluted sample. Results were expressed as mol 100 $mol^{-1}$ of each SCFA in ruminal fluid.

### *2.10. Milk*

#### 2.10.1. Composition and Quality

Analyses of chemical composition, standard plate count of bacteria, and SCC were carried out by the State Milk Quality Laboratory—LABLEITE of Concórdia—SC, which is accredited to the Ministry of Agriculture, Livestock and Supply by the MAPA ordinance: No. 212 of 31 July 2014. The quantification of fat, protein, lactose, total solids, and the defatted dry extract was quantified using the Mid-Infrared Spectrometry Method, according to ISO [23]. The Total Bacterial Count was carried out using the Flow Cytometry Method, according to ISO [24], and the SCC was measured using the Flow Cytometry Method, according to ISO [25].

#### 2.10.2. Fatty Acid Profile

To determine the fatty acid profile in milk, lipid extraction was performed using a specific method [26], but with adaptations; 1.5 g of samples, 0.8 mL of water, 5 mL of methanol, and 2.5 mL of chloroform were added to a 15 mL polypropylene tube, and mechanical stirring was carried out for 30 min. Next, 2.5 mL of chloroform solution and 1.5% $NaSO_4$ were added to promote a two-phase system. This mixture was stirred for 2 min and then centrifuged for 15 min at 2000 rpm. The lipids obtained from the chloroform phase were subjected to fatty acid analysis.

FA methylation was performed using a transesterification method proposed by researchers [27]. One milliliter of 0.4 M KOH methanolic solution was added to the extracted lipids in a test tube and vortexed for one minute. The samples were kept in a water bath for ten minutes at boiling temperature. Subsequently, they were cooled to room temperature, and 3 mL of 1 M methanolic $H_2SO_4$ solution was added and vortexed for chromatographic analysis.

A gas chromatograph model TRACE 1310 with a flame ionization detector (Thermo Scientific, Milan, Italy) was used to determine FAME. One microliter of the sample was injected into a split/splitless injector, operated in split mode with a 1:20 ratio at 250 °C. Hydrogen was used as the carrier gas at a constant flow rate of 1.5 mL/min. Separation of FAMEs was performed using an RT 2560 chromatography column (100 m × 0.25 mm × 0.20 µm film thickness, Restek, Bellefonte, PA, USA). The initial oven temperature was programmed at 100 °C for five min and increased to 180 °C at 8 °C/min, followed by an increase to 210 °C at a rate of 4 °C/min, and finally up to 250 °C at 20 °C/min, maintaining it for 7 min under isothermal conditions. The detector temperature was kept constant at 250 °C. FAME compounds were identified by comparing the experimental retention time with those of the authentic standard (FAME Mix-37, Sigma Aldrich, St. Louis, MO, USA). Results were presented as a percentage of each FA identified in the lipid fraction, considering the factor equivalent to the size of the FAME chain for FID and the ester conversion factor for the respective acid, according to the literature [28]. Fatty acid profile results in TMR are presented in Supplementary Materials S3.

#### 2.10.3. Milk Microbiota

On days 30 (pool all cows—also day 0 of the experiment), 75, and 135 of lactation, milk was collected and stored in 3M™ Quick Swabs for transport to the laboratory, which reduced the qualitative and quantitative detection of microorganisms using metagenomics by sequencing the 16S rRNA gene, as performed by the laboratory BPI—Biotechnology Research and Innovation®, Sao Paulo, Brazil.

Total DNA was extracted from 200 mg (wet weight) of samples with the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, Tustin, CA, USA). Primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) were selected to amplify the V4 region of the bacterial 16S rRNA gene via the polymerase chain reaction [29].

Libraries were quantified using qualitative polymerase chain reaction with the Kapa Library Quantification Kit (Illumina, San Diego, CA, USA), following the manufacturer's recommendations. Samples were normalized to a final concentration of 2 nM and sequenced with an Illumina MiSeq for 250 cycles from each end.

### *2.11. Apparent Digestibility Coefficients*

Researchers described using indigestible neutral detergent fiber (iNDF) to determine apparent digestibility [30]. Feed and feces samples were incubated in bovine rumen for 288 h, then washed and dried in a forced ventilation oven. NDF and ADF concentrations were determined to calculate digestibility [31].

### *2.12. Statistical Analysis*

Data were tested for normality and homogeneity of variance using the Shapiro–Wilk and Levene tests, respectively. All data were analyzed using the SAS "MIXED procedure" (SAS Inst. Inc., Cary, NC, USA; version 9.4). The Satterthwaite approximation was used to determine the denominator degrees of freedom for the fixed effects test. Feed efficiency was tested for the fixed effect of treatment using animal (treatment) as a random effect. The remaining data were analyzed as repeated measures and tested for fixed effects of treatment and treatment × day, using animal (treatment) as a random effect. Day-one results were included as an independent covariate. Means were separated using the PDIFF method (Student's test), and all results were reported as LSMEANS followed by SEM. Significance was defined when $p \leq 0.05$, and trend when $p > 0.05$ and $\leq 0.10$.

Sequence data were processed using Mothur v.1.39.5 [32], which aligns with the Mothur MiSeq SOP [33]. Taxonomy was assigned by querying the representative sequence of each oligotype against the SILVA database (release 132) [34] and Greengenes [35]. Closed-reference clustered operational taxonomic unit data were exported for analysis with Phyloseq v1.41 [36] in R 4.3.1 (R Core Team, 2023). The Permanova test was used to compare the abundance of specific taxons between groups. Taxonomic alpha diversity was estimated using Chao1 richness and the Shannon, Simpson, and Fisher indices, which account for both richness and evenness. Beta diversity was assessed using the Bray–Curtis distance, and taxonomic dissimilarity among samples was explored using principal coordinates analysis. Differential abundance of taxa was calculated using a negative Binomial method implemented in the DESeq2 package [37] to identify individual virtual taxa that were more or less abundant when comparing phytobiotic or control conditions. All figures were generated using the ggplot2 package v3.2.1 [38].

## 3. Results

### *3.1. Milk Production, Feed Intake and Feed Efficiency*

Data on milk production, dry matter intake (DMI), and feed efficiency from early lactation and mid-lactation are shown in Table 2. Milk production was not affected by the treatment in the two lactation phases. However, a treatment × day interaction was detected in both production moments, sometimes more significant in cows that consumed the additive (Supplementary Materials S4). There was no effect of treatment and treatment × day interaction on DMI in early lactation and mid-lactation. The feed efficiency of cows in the phytogenic group was higher compared to the control group during early lactation. At the same time, in mid-lactation, there was only a trend toward greater feed efficiency in the treated animals compared to the control.

**Table 2.** Productive performance of dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM).

| Exp. Period | Group | Production (kg) of Milk/Cow/Day | Consumption (kg) Feed/Cow/Day | Feed Efficiency |
|---|---|---|---|---|
| Early lactation | | | | |
| DIM 30 to 75 | CON | 23.3 | 14.34 | 1.62 [b] |
| | PHY [1] | 24.8 | 14.01 | 1.75 [a] |
| | SEM | 0.48 | 0.19 | 0.03 |
| | P treat | 0.25 | 0.82 | 0.05 |
| | P treat × day | 0.01 | 0.74 | - |
| Mid-lactation | | | | |
| DIM 90 to 135 | CON | 19.7 | 15.17 | 1.29 [b] |
| | PHY [1] | 20.8 | 15.21 | 1.36 [a] |
| | SEM | 0.42 | 0.18 | 0.07 |
| | P treat | 0.52 | 0.92 | 0.09 |
| | P treat × day | 0.05 | 0.95 | - |

Note: [a,b] Significance differences between groups (column) were defined when $p \leq 0.05$, and trend when $p > 0.05$ and $\leq 0.10$; DIM: days in milk; kg: kilograms. [1] The phytogenic additive is a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

### *3.2. Hematologic Changes*

The cows' blood count results are described in Table 3. During early lactation or mid-lactation, there was no effect of treatment and treatment × day interaction on the variables of erythrocytes, hemoglobin, hematocrit, granulocytes, monocytes, and platelets. Treatment × day interaction was also not verified for the WBC variables. Nevertheless, during the peak lactation period (early lactation), there was an effect of treatment on the total leukocyte count, with the number of cells being lower in animals in the treatment group; this effect was related to a tendency for lower lymphocyte counts in these cows compared to the control. In mid-lactation, there was a tendency toward a treatment effect on the total leukocyte count (lower in cows that consumed the additive), which was due to an effect of treatment on lymphocyte count, as the number of cells was lower in cows in the treatment group compared to the control. There was no treatment effect on the granulocyte and monocyte variables between treatments.

**Table 3.** Blood count of dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM).

| Exp. Period | Group | ERY | HEM | HT | LEU | LYM | GRA | MON | PLA |
|---|---|---|---|---|---|---|---|---|---|
| Early lactation | | | | | | | | | |
| DIM 30 to 75 | CON | 5.63 | 9.56 | 26.6 | 5.34 [a] | 3.00 [a] | 1.49 | 1.10 | 443 |
| | PHY [1] | 5.12 | 8.88 | 25.5 | 4.29 [b] | 2.40 [b] | 1.15 | 0.88 | 391 |
| | SEM | 0.14 | 0.18 | 0.38 | 0.32 | 0.16 | 0.09 | 0.08 | 27.9 |
| | P treat | 0.38 | 0.45 | 0.64 | 0.05 | 0.09 | 0.21 | 0.48 | 0.55 |
| | P treat × day | 0.24 | 0.32 | 0.41 | 0.11 | 0.17 | 0.12 | 0.56 | 0.31 |
| Mid-lactation | | | | | | | | | |
| DIM 90 to 135 | CON | 5.85 | 9.88 | 27.7 | 6.30 [a] | 3.44 [a] | 1.64 | 1.22 | 318 |
| | PHY [1] | 5.51 | 9.79 | 27.1 | 5.28 [b] | 2.97 [b] | 1.33 | 0.99 | 255 |
| | SEM | 0.16 | 0.29 | 0.69 | 0.34 | 0.13 | 0.09 | 0.06 | 27.6 |
| | P treat | 0.87 | 0.93 | 0.95 | 0.07 | 0.05 | 0.52 | 0.74 | 0.40 |
| | P treat × day | 0.92 | 0.90 | 0.96 | 0.16 | 0.12 | 0.20 | 0.53 | 0.21 |

Note: [a,b] Significance differences between groups (column) were defined when $p \leq 0.05$, and trends when $p > 0.05$ and $\leq 0.10$. DIM: days in milk; ERY: erythrocytes ($\times 10^6$ µL); HEM: hemoglobin (mg/dL); HT: hematocrit (%); LEU: leukocytes ($\times 10^3$ µL); LYM: lymphocyte ($\times 10^3$ µL); GRA: granulocyte ($\times 10^3$ µL); MON: monocyte ($\times 10^3$ µL); PLA: platelets ($\times 10^3$ µL). [1] The phytogenic additive is a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

### *3.3. Serum Biochemistry, Proteinogram, and Oxidative Status*

The serum biochemistry results are shown in Table 4. The groups had no treatment effect or treatment × day interaction for albumin, fructosamine, CRP, total protein, and urea variables. In the first period of mechanical milking (early lactation), treatment affected cholesterol levels, which was lower in cows in the phytogenic group compared to the control group. At the same time, there was no difference between treatments in mid-lactation. In early lactation, treatment affected the GGT enzyme, with its activity being lower in cows in the phytogenic group compared to the control; however, this effect was not verified in mid-lactation. In early lactation, there was no effect of treatment and no treatment × day interaction for AST activity. Still, in mid-lactation, AST activity impacted the treatment, being lower in the serum of cows in the phytogenic group compared to the control. There was no treatment × day interaction for globulins, but there was an effect of the treatment; that is, in early lactation, higher levels of globulins were identified in cows in the phytogenic group compared to the control, different from what occurred in mid-lactation, when the level of globulins tended to be lower in the serum of cows in the phytogenic group.

**Table 4.** Serum biochemistry of dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM).

| Exp. Period | Group | ALB | CHO | FRU | GGT | CRP | TP | AST | URE | GLO |
|---|---|---|---|---|---|---|---|---|---|---|
| Early lactation | | | | | | | | | | |
| DIM 30 to 75 | CON | 3.25 | 163 [a] | 239 | 28.5 [a] | 2.96 | 7.39 | 85.9 | 50.0 | 4.14 [b] |
| | PHY [1] | 3.16 | 144 [b] | 236 | 23.8 [b] | 2.84 | 7.78 | 96.6 | 52.9 | 4.62 [a] |
| | SEM | 0.06 | 5.40 | 5.02 | 1.53 | 0.03 | 0.08 | 3.07 | 1.43 | 0.05 |
| | P treat | 0.68 | 0.03 | 0.91 | 0.05 | 0.46 | 0.69 | 0.24 | 0.46 | 0.05 |
| | P treat × day | 0.84 | 0.15 | 0.97 | 0.10 | 0.65 | 0.75 | 0.22 | 0.32 | 0.13 |
| Mid-lactation | | | | | | | | | | |
| DIM 90 to 135 | CON | 3.24 | 149 | 239 | 33.4 | 2.97 | 7.51 | 108 [a] | 45.7 | 4.27 [a] |
| | PHY [1] | 3.31 | 155 | 235 | 32.5 | 2.97 | 7.27 | 97.3 [b] | 48.4 | 3.96 [b] |
| | SEM | 0.02 | 2.85 | 3.00 | 3.01 | 0.03 | 0.06 | 3.48 | 0.94 | 0.06 |
| | P treat | 0.85 | 0.74 | 0.95 | 0.88 | 0.97 | 0.89 | 0.09 | 0.39 | 0.08 |
| | P treat × day | 0.89 | 0.62 | 0.92 | 0.91 | 0.94 | 0.91 | 0.12 | 0.24 | 0.37 |

[a,b] Significance differences between groups (column) were defined when $p \leq 0.05$, and trends when $p > 0.05$ and $\leq 0.10$. DIM: days in Milk; ALB: albumin (g/dL), CHO: cholesterol (mg/dL), FRU: fructosamine (µmol/L); GGT: gamma-glutamyl transferase (U/L), reactive-C protein (CRP) (g/dL), TP: total protein (g/dL), AST: aspartate aminotransferase (U/L), URE: urea (mg/dL), GLO: globulin (g/dL). [1] The phytogenic additive is a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

The protein profile values from electrophoresis are described in Table 5. There was no effect of treatment and treatment × day interaction on the concentration of heavy chain immunoglobulins, transferrin, ferritin, and serum amyloid during the two periods of the experiment. There was an effect of treatment and treatment × day interaction (DL 45 and 60) for IgA levels in the period I of the experiment, being higher in the serum of cows that consumed the additive compared to the control ($p \leq 0.05$); however, this effect was not verified in mid-lactation (Supplementary Materials S5). In early lactation and mid-lactation phases, there was an effect of treatment (Table 5) and treatment × day interaction for ceruloplasmin and haptoglobin levels measured at lower levels in cows that consumed the additive compared to the control (Supplementary Materials S5).

The results for oxidative and antioxidant biomarkers are presented in Table 6. Only during early lactation was there an effect of treatment and treatment × day interaction on SOD activity, which was lower in the serum of animals in the phytogenic group compared to the control (Supplementary Materials S6). GST activity was affected by treatment and interaction in both lactation phases (Supplementary Materials S6); the serum activity

was more significant when the cows consumed the phytogenic additive. There was an effect of treatment (Table 6) and treatment × day interaction on TBARS concentration in both research moments (Supplementary Materials S6), with lower levels in cows from the phytogenic group. For carbolines, only treatment × day interaction was observed in early lactation, with the concentration being lower in animals in the phytogenic group (Supplementary Materials S6).

**Table 5.** Serum proteinogram of dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM): control (CON) vs. phytogenic (PHY).

| Exp. Period | Group | IgA | Ig-HC | CERU | TRAN | FER | HAPT | SE-A |
|---|---|---|---|---|---|---|---|---|
| Early lactation | | | | | | | | |
| DIM 30 to 75 | CON | 0.78 $^{b}$ | 0.99 | 0.76 $^{a}$ | 0.35 | 0.42 | 0.25 $^{a}$ | 0.21 |
| | PHY [1] | 0.84 $^{a}$ | 1.07 | 0.45 $^{b}$ | 0.33 | 0.42 | 0.19 $^{b}$ | 0.20 |
| | SEM | 0.02 | 0.04 | 0.02 | 0.02 | 0.03 | 0.02 | 0.01 |
| | P treat | 0.05 | 0.35 | 0.01 | 0.65 | 0.95 | 0.01 | 0.92 |
| | P treat × day | 0.02 | 0.12 | 0.01 | 0.73 | 0.90 | 0.01 | 0.97 |
| Mid-lactation | | | | | | | | |
| DIM 90 to 135 | CON | 0.77 | 0.94 | 0.80 $^{a}$ | 0.29 | 0.41 | 0.31 $^{a}$ | 0.23 |
| | PHY [1] | 0.81 | 0.98 | 0.52 $^{b}$ | 0.31 | 0.37 | 0.20 $^{b}$ | 0.25 |
| | SEM | 0.02 | 0.04 | 0.02 | 0.02 | 0.04 | 0.01 | 0.02 |
| | P treat | 0.19 | 0.82 | 0.01 | 0.52 | 0.13 | 0.01 | 0.77 |
| | P treat × day | 0.13 | 0.89 | 0.01 | 0.63 | 0.24 | 0.01 | 0.55 |

$^{a,b}$ Significance differences between groups (column) were defined when $p \leq 0.05$, and trends when $p > 0.05$ and $\leq 0.10$. DIM: days in Milk; IgA: immunoglobulin A (g/dL), Ig-HC: Ig heavy chain (g/dL), CERU: ceruloplasmin (g/dL), TRAN: transferrin (g/dL); FER: ferritin (g/dL), HAPT: haptoglobin (g/dL), SE-A: serum amyloid (g/dL). [1] The phytogenic additive is a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

**Table 6.** Serum oxidative status of dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM: control (CON) vs. phytogenic (PHY).

| Exp. Period | Group | SOD | GST | TBARS | CAB-P |
|---|---|---|---|---|---|
| Early lactation | | | | | |
| DIM 30 to 75 | CON | 14.0 $^{a}$ | 16.8 $^{b}$ | 38.3 $^{a}$ | 5.15 |
| | PHY [1] | 11.7 $^{b}$ | 23.1 $^{a}$ | 23.2 $^{b}$ | 4.19 |
| | SEM | 0.17 | 1.38 | 1.24 | 0.45 |
| | P treat | 0.04 | 0.03 | 0.01 | 0.24 |
| | P treat × day | 0.01 | 0.01 | 0.01 | 0.03 |
| Mid-lactation | | | | | |
| DIM 90 to 135 | CON | 11.3 | 15.5 $^{b}$ | 20.9 $^{a}$ | 7.76 |
| | PHY [1] | 11.5 | 22.7 $^{a}$ | 13.0 $^{b}$ | 7.64 |
| | SEM | 0.12 | 0.84 | 1.39 | 0.29 |
| | P treat | 0.89 | 0.03 | 0.05 | 0.91 |
| | P treat × day | 0.93 | 0.01 | 0.02 | 0.95 |

$^{a,b}$ Significance differences between groups (column) were defined when $p \leq 0.05$, and trends when $p > 0.05$ and $\leq 0.10$. DIM: days in Milk; SOD: superoxide dismutase (U SOD/mg protein), GST: glutathione S-transferase (U GST/mg protein), TBARS: lipid peroxidation (nmol MDA/mL); CAB-P: carbonyl protein (nmol of carbonyl groups/mg of protein). [1] The phytogenic additive is a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

### *3.4. Ruminal Environment*

The results of the profile of VFA in the ruminal environment are described in Table 7. The effect of treatment was observed for SCFA levels, which were higher in animals in the phytogenic group at DIM 75 and 135. The impact of treatment on acetic acid proportion was

observed only at DIM 135 when higher percentages were found in cows in the phytogenic group compared to the control. The effect of treatment on butyric acid was lower at both moments (DIM 75 and 135) in the ruminal fluid of cows that consumed the phytogenic additive compared to the control. The effect of treatment on the concentration of valeric acid was observed on days 75 and 135, being lower in cows that consumed the additive compared to the control. The acetic acid/propionic acid ratio tended to be higher in the phytogenic group compared to the control. There was no treatment effect on propionic acid and isovaleric acid percentages between treatments in this experiment.

**Table 7.** Profile of short-chain fatty acids in the rumen liquid of cows in each experimental period (early lactation—DIM 75 and mid-lactation—DIM 135): control (CON) vs. phytogenic (PHY).

| | CON | PHY [1] | SEM | P: Treat |
|---|---|---|---|---|
| VFA, mmol/L | | | | |
| DIM 75 | 84.18 [b] | 96.71 [a] | 0.43 | 0.01 |
| DIM 135 | 72.68 [b] | 82.83 [a] | 0.41 | 0.01 |
| Acetic acid (%) | | | | |
| DIM 75 | 65.09 | 68.64 | 0.35 | 0.02 |
| DIM 135 | 63.68 [b] | 69.85 [a] | 0.36 | 0.01 |
| Propionic acid (%) | | | | |
| DIM 75 | 16.60 | 15.48 | 0.14 | 0.67 |
| DIM 135 | 16.5 | 15.51 | 0.13 | 0.62 |
| Butyric acid (%) | | | | |
| DIM 75 | 16.59 [a] | 13.47 [b] | 0.15 | 0.01 |
| DIM 135 | 15.30 [a] | 12.00 [b] | 0.14 | 0.02 |
| Isovaleric acid (%) | | | | |
| DIM 75 | 1.44 | 1.31 | 0.12 | 0.52 |
| DIM 135 | 1.42 | 1.33 | 0.12 | 0.58 |
| Valeric acid (%) | | | | |
| DIM 75 | 1.52 [a] | 1.15 [b] | 0.05 | 0.01 |
| DIM 135 | 1.61 [a] | 1.33 [b] | 0.05 | 0.05 |
| Acetic/propionic | | | | |
| DIM 75 | 4.30 | 4.59 | 0.17 | 0.65 |
| DIM 135 | 3.66 [b] | 5.00 [a] | 0.18 | 0.07 |

Note: [a,b] Significance differences between groups (line) were defined when $p \leq 0.05$, and trends when $p > 0.05$ and $\leq 0.10$. DIM: days in milk. [1] The phytogenic additive is a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

### *3.5. Apparent Digestibility Coefficient*

Table 8 describes the results of the apparent digestibility coefficient (ADC). During the mechanical milking period (early lactation), the treatment had no effect on the ADC of DM and nutrients (OM, CP, NDF, ADF, and EE). In the robotic milking period (mid-lactation), the treatment had an impact, with a higher ADC for MS, MO, PB, NDF, FDA, and EE in cows that consumed the additive compared to the control.

**Table 8.** Apparent nutrient digestibility coefficient of dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM: control (CON) vs. phytogenic (PHY).

| Exp. Period | Group | Dry Matter | Organic Matter | Crude Protein | NDF | ADF | EE |
|---|---|---|---|---|---|---|---|
| Early lactation | | | | | | | |
| DIM 73,74, 75 | CON | 0.68 | 0.71 | 0.68 | 0.59 | 0.57 | 0.83 |
| | PHY [1] | 0.67 | 0.70 | 0.67 | 0.60 | 0.56 | 0.82 |
| | SEM | 0.01 | 0.01 | 0.02 | 0.02 | 0.02 | 0.01 |
| | P treat | 0.97 | 0.96 | 0.95 | 0.95 | 0.94 | 0.96 |

**Table 8.** *Cont.*

| Exp. Period | Group | Dry Matter | Organic Matter | Crude Protein | NDF | ADF | EE |
|---|---|---|---|---|---|---|---|
| Mid-lactation | | | | | | | |
| DIM 133, 134, 135 | CON | 0.56 [b] | 0.60 [b] | 0.57 [b] | 0.51 [b] | 0.55 [b] | 0.65 [b] |
| | PHY [1] | 0.71 [a] | 0.73 [a] | 0.74 [a] | 0.69 [a] | 0.73 [a] | 0.81 [a] |
| | SEM | 0.04 | 0.03 | 0.06 | 0.05 | 0.05 | 0.06 |
| | P treat | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |

[a,b] Differs ($p \leq 0.05$) between treatments (column). DIM: days in Milk; NDF: neutral detergent fiber; ADF: acid detergent fiber; EE: ethereal extract. [1] The phytogenic additive is a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

### *3.6. Milk Analyses*

Table 9 presents the results of the milk composition. No treatment effect was observed for the percentage of fat, protein, lactose, total solids, standard plate count, defatted dry extract, and casein between experimental groups. In early lactation, the treatment had no effect; however, in two lactation phases, there was a tendency for a treatment × day interaction for SCC, with SCC being lower in the milk of cows in the phytogenic group. In mid-lactation, the effect of the treatment on SCC was smaller in the phytogenic group ($p > 0.05$).

**Table 9.** Chemical composition in the milk of dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM: control (CON) vs. phytogenic (PHY).

| Exp. Period | Group | Fat (g/100 g) | Protein (g/100 g) | Lactose (g/100 g) | TS (g/100 g) | SCC (×1000/mL) | DDE (g/100 g) | Urea (mg/dL) | Casein (g/100 g) |
|---|---|---|---|---|---|---|---|---|---|
| Early lactation | | | | | | | | | |
| DIM 30 to 75 | CON | 4.47 | 3.27 | 4.66 | 13.3 | 68.2 | 8.84 | 17.0 [b] | 2.58 |
| | PHY [1] | 4.10 | 3.24 | 4.62 | 12.9 | 50.8 | 8.78 | 19.3 [a] | 2.50 |
| | SEM | 0.28 | 0.04 | 0.03 | 0.27 | 8.64 | 0.05 | 0.61 | 0.04 |
| | P treat | 0.82 | 0.95 | 0.97 | 0.78 | 0.14 | 0.95 | 0.06 | 0.89 |
| | P treat × day | 0.71 | 0.92 | 0.98 | 0.86 | 0.07 | 0.91 | 0.17 | 0.93 |
| Mid-lactation | | | | | | | | | |
| DIM 90 to 135 | CON | 2.96 | 3.56 | 4.70 | 12.2 | 72.5 [a] | 9.20 | 14.1 [b] | 2.85 |
| | PHY [1] | 3.16 | 3.51 | 4.50 | 12.1 | 44.9 [b] | 8.94 | 16.7 [a] | 2.81 |
| | SEM | 0.21 | 0.04 | 0.03 | 0.23 | 12.5 | 0.06 | 0.41 | 0.04 |
| | P treat | 0.74 | 0.92 | 0.85 | 0.94 | 0.04 | 0.88 | 0.03 | 0.95 |
| | P treat × day | 0.81 | 0.95 | 0.91 | 0.9 | 0.09 | 0.81 | 0.11 | 0.95 |

[a,b] Significance differences between groups (line) were defined when $p \leq 0.05$, and trends when $p > 0.05$ and $\leq 0.10$. DIM: days in Milk; ST: total solids; SCC: somatic cell count; DDE: defatted dry extract. [1] The pPhytogenic additive is a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

In this early lactation, a trend toward a treatment effect was observed for the concentration of urea in milk ($p < 0.05$–>0.10), being higher in the milk of cows in the phytogenic group. In mid-lactation, the urea concentration was significantly higher in the milk of cows that consumed the additive.

Table 10 describes the results of the milk fatty acid profile in early lactation (mechanical milking). An effect of treatment and treatment × day interaction was observed, with lower percentages of palmitic acid and henicosanoic acid being measured in the cows' milk in the phytogenic group ($p > 0.05$). There was an effect of treatment and a treatment × day interaction for oleic acid, where a higher concentration of oleic acid was found in the milk of cows that consumed the additive compared to the control group. For the sum of acids, there was an effect of treatment for saturated fat (lower in milk from the phytogenic group) and a treatment × day interaction (DIM 60), which was lower in the phytogenic group compared to the control. While the sum of unsaturated and monounsaturated fats was

higher in the phytogenic group, there was also a treatment × day interaction, with higher levels at DIM 60. The unsaturated/saturated fat ratio was affected by treatment and a treatment × day interaction (DIM 60 and 75), which was higher in the cows that consumed the additive. There was no effect on treatment and treatment interaction for the other fatty acids presented in Supplementary Materials S7.

**Table 10.** Profile of fatty acids in milk from dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM: control (CON) vs. phytogenic (PHY).

| Fatty Acid | CON | PHY [1] | SEM | P Treat | P Treat × Day |
|---|---|---|---|---|---|
| Early lactation | | | | | |
| C16:0 (Palmitic) | | | | 0.05 | 0.01 |
| DIM 60 | 38.7 [a] | 34.6 [b] | 0.720 | | |
| DIM 75 | 41.3 [a] | 39.0 [b] | 0.770 | | |
| C18:1n9c (Oleic) | | | | 0.01 | 0.01 |
| DIM 60 | 18.5 [b] | 24.7 [a] | 0.640 | | |
| DIM 75 | 16.9 [b] | 20.0 [a] | 0.640 | | |
| C21:0 (Henicosanoic) | | | | 0.01 | 0.02 |
| DIM 60 | 0.78 [a] | 0.65 [b] | 0.026 | | |
| DIM 75 | 0.80 [a] | 0.64 [b] | 0.025 | | |
| ∑ Saturated fatty acids (SFA) | | | | 0.05 | 0.01 |
| DIM 60 | 77.0 [a] | 70.5 [b] | 0.895 | | |
| DIM 75 | 78.6 | 75.6 | 0.908 | | |
| ∑ Unsaturated fatty acids (UFA) | | | | 0.02 | 0.01 |
| DIM 60 | 22.4 [b] | 29.4 [a] | 0.908 | | |
| DIM75 | 21.3 | 24.2 | 0.903 | | |
| ∑ Monounsaturated fatty acids (MUFA) | | | | 0.01 | 0.01 |
| DIM 60 | 20.1 [b] | 26.4 [a] | 0.878 | | |
| DIM 75 | 18.6 | 21.7 | 0.872 | | |
| UFA/SFA | | | | 0.05 | 0.01 |
| DIM 60 | 0.30 [b] | 0.42 [a] | 0.017 | | |
| DIM 75 | 0.27 [b] | 0.32 [a] | 0.016 | | |
| Mid-lactation | | | | | |
| C16:0 (Palmitic) | | | | 0.25 | 0.02 |
| DIM 120 | 50.78 | 49.53 | 0.68 | | |
| DIM 135 | 51.69 [a] | 48.5 [b] | 0.65 | | |
| C18:1n9c (Oleic) | | | | 0.11 | 0.03 |
| DIM 120 | 15.68 | 15.76 | 0.29 | | |
| DIM 135 | 14.68 [b] | 16.75 [a] | 0.27 | | |

[a,b] Differs ($p \leq 0.05$) between treatments (lines). DIM: days in milk; ∑sum of fatty acids; ω: omega. [1] The phytogenic additive is a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

The results of the mid-lactation of the experiment are described in Table 10. A treatment × day interaction was verified for palmitic and oleic acid ($p > 0.05$), with palmitic acid being lower and oleic acid being higher in the cows that consumed the additive at DIM 135. The sum of saturated and unsaturated fatty acids did not differ between treatments, as the other fatty acids detected in chromatography and presented in Supplementary Materials S7.

Figure 1 presents the results of microbiota abundance in cows' milk. We found that the primary bacteria were identified as *Acinetobacter* spp., *Escherichia-Shigella* spp., *Lactococcus* spp., *Staphylococcus* spp., and *Streptococcus* spp., as well as the family (*Enterobacteriaceae*) and order (*Lactobacillales*).

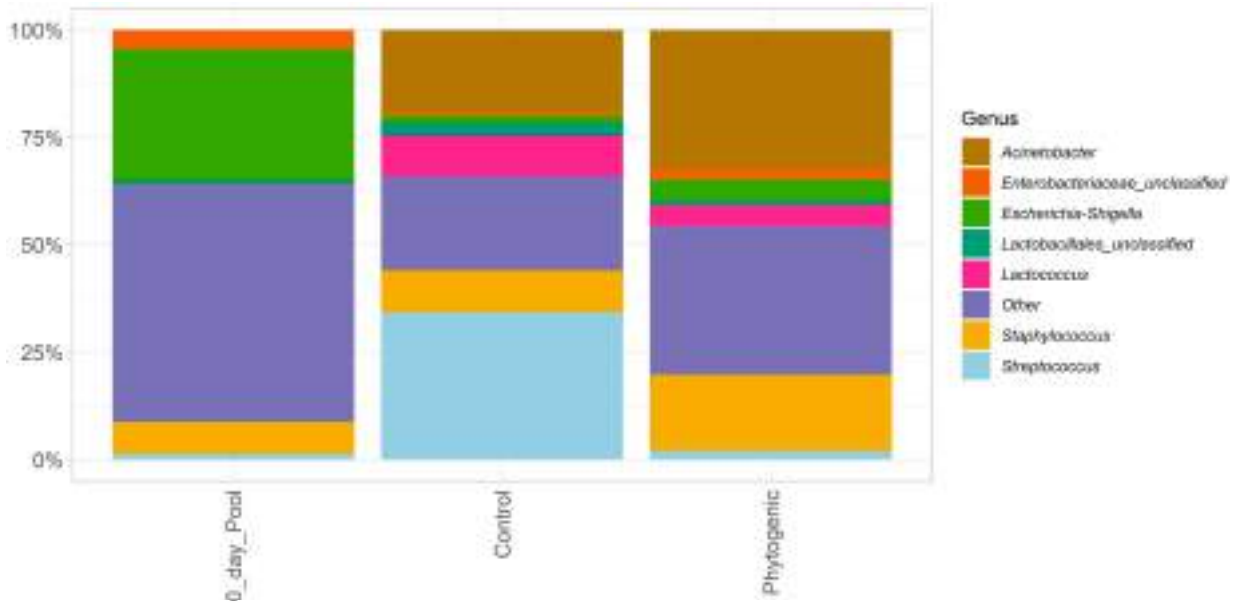



**Figure 1.** Percentage abundance of microorganisms present in cows' milk before the beginning of the experiment (pool of all cows on day 0 of the experiment = DIM 30) and at the end of each experimental stage (DIM 75 and DIM 135). Note: phytogenic group: animals' intake of an additive formulated with a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

Statistically, a lower abundance of *Streptococcus* spp. was found in the milk of cows that consumed the phytogenic additive (Figure 2). Alpha diversity results (Figure 3) showed that the richness indices (Chao1 and Fisher) were significant, while the evenness indices (InvSimpson and Shannon) were not statistically different. Beta diversity (Supplementary Materials S8) demonstrated individual variability between animals, regardless of treatment, as seen in the seven most abundant taxa presented individually at both moments (DIM 75 and 135).

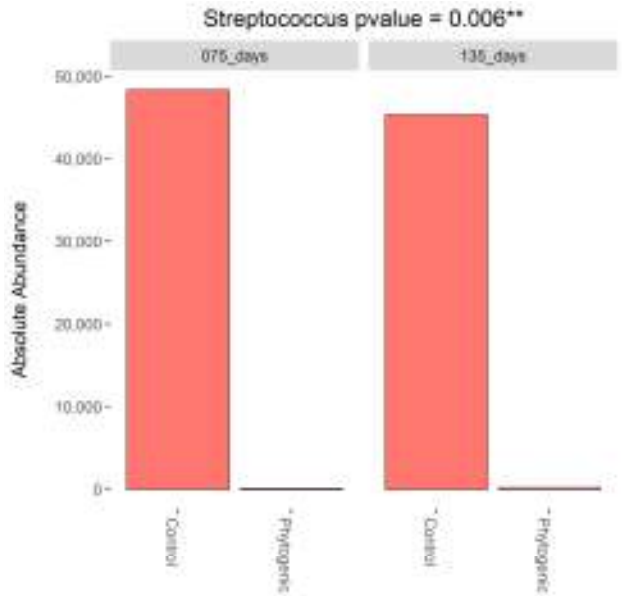



**Figure 2.** Several sequences of *Streptococcus* were observed in milk at 75 (peak lactation stage) and 135 (middle lactation stage) days of lactation. Note: phytogenic group: animals' intake of an additive formulated with a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid. Marked with two (**) if $p$-value < 0.01.

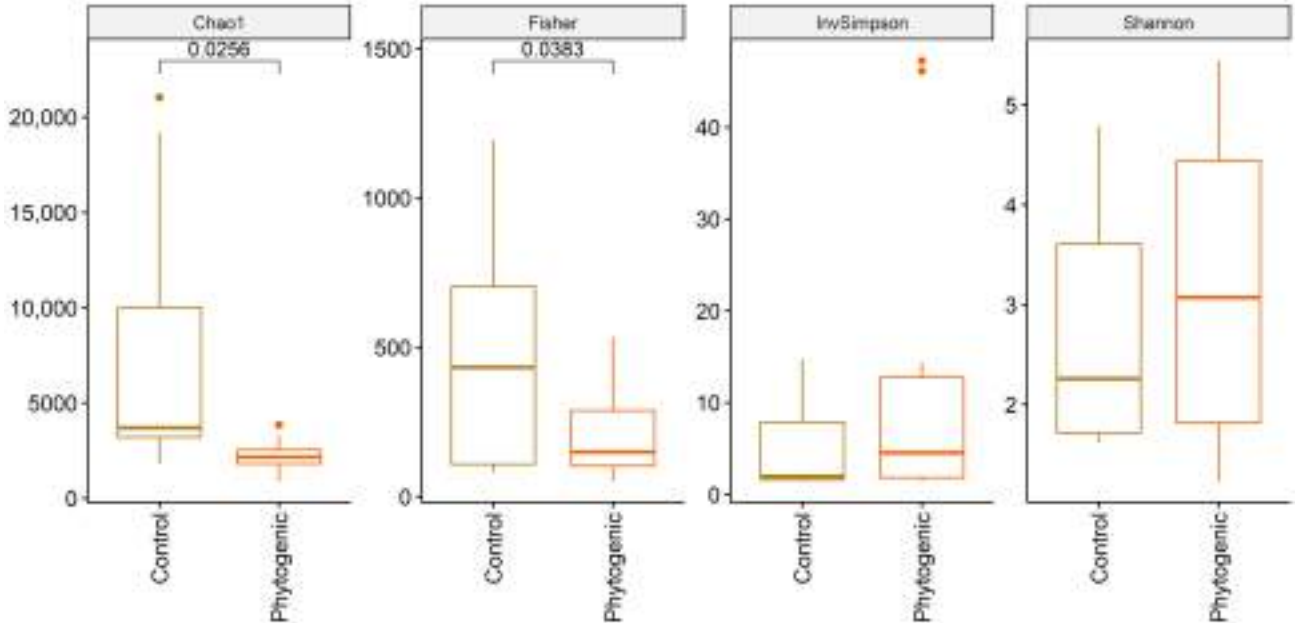



**Figure 3.** Alpha diversity of microorganisms in milk from dairy cows during early lactation (30–75 DIM) and mid-lactation (90–135 DIM) assessed using three data analysis models (Chao1, Fisher, InvSimpson and Shannon): control (CON) vs. phytogenic (PHY). Note: phytogenic group: animals' intake of an additive formulated with a combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid. If there are values that fall above or below the end of the whiskers, they are plotted as dots.

## 4. Discussion

The sum of milk produced by cows that consumed the phytogenic product was higher, but not significantly higher, during the two periods. However, at some points in the study, production was higher in the cows that consumed the additive. In early lactation, we found that after 25 days of ingesting the additive, the milk production of cows in the phytogenic group was higher. In mid-lactation, we observed a drop in the productivity of animals in both groups, a factor related to the change in the milking system, as already demonstrated by other researchers [39–41]. However, after the adaptation period of this mid-lactation, the cows that consumed phytogenics produced more milk daily compared to the control group at various times, notably during the last seven days of the experiment. However, the change in the milking system (from mechanical to robotic) influenced the productivity of cows, as they needed to adapt to the new one (robotic milking system), and thus during the adaptation period and beginning of lactation in the second phase of this study, we observed no difference in milk production between experimental groups. This is a possible limitation of the effect of the tested additive due to interference from the milking management. Despite this, the treatment did not affect feed consumption, but feed efficiency was more significant in cows that consumed phytogenics. At the peak of lactation, greater milk production is related to better cow health, as the feed additive, when consumed, stimulates an immune, anti-inflammatory, and antioxidant response. Already in the mid-lactation phase, in addition to these serum results, productivity improvements may result from the more significant amount of VFAs in the ruminal liquid and the greater digestibility of nutrients when consuming the additive. Therefore, it is clear that phytogenics directly affects nutrition, providing better absorption of nutrients.

The more significant amount of VFA in the cows' ruminal environment is a consequence of higher concentrations of acetic acid and valeric acid in the animals that consumed the phytogenic mixture. These results lead us to understand that the additive was capable of modulating the rumen environment, just as it tended to increase the acetate/propionate ratio. This is the first study with this phytogenic combination of essential oils (free and

encapsulated), plant extracts, minerals, and microorganisms. Still, the isolated nutritional effects of these blend ingredients are already well known. Using oregano and cinnamon essential oil changes the acetate/propionate ratio and the amount of VFA [42]. In an in vitro study carried out in [43], when essential oils were used, there was a tendency to increase VFAs, as well as when using a blend of essential oils (oregano and cinnamon) in the diet of sheep [44], they had changes in rumen fermentation and increased milk production. Using a blend of phytogenics with ingredients similar to those in this study, authors [45] found an increase in acetic acid in Holstein steers. Researchers [46] also described an increase in acetic acid in dairy cows when using *Saccharomyces cerevisiae*, which is another additive present in our blend. Researchers found a stimulation of nitrogen retention induced by curcumin in cattle, which was considered a potentially positive effect. However, a decrease in the apparent digestibility of ADF may limit forage utilization [47], unlike a study with dairy sheep with greater NDF digestibility [9]. Tannic acid supplementation in cattle reduced the percentages of butyrate, isobutyrate, and valerate; however, it increased the proportion of acetate and the acetate/propionate ratio [48], which demonstrates that tannins alter the profile of VFA in the rumen environment, similar to what was observed in this study with the feed additive.

VFA is directly linked to the digestibility of feed; in our study, it was found that digestibility had a positive effect on the phytogenic group only at the end of the experiment (after consuming the additive for more than 100 days), with higher digestibility coefficients observed for dry matter, organic matter, protein, fiber, and fat. However, it is essential to highlight that the diets during early lactation and mid-lactation differed due to the inclusion of concentrate available in the robot during milking. This difference in TMR between the two phases is probably the reason for increasing the digestibility coefficients when the cows were in a stable condition (mid-lactation), different from a cow at peak production, and still under the effects of metabolic disorders arising from the transition phase. However, these results on digestibility need to be further elucidated. Similar effects have already been described in sheep that used curcumin, where it had greater digestibility of DM and NDF [9,49]. Cinnamon essential oil in cattle diets increased the digestion of OM [50], and the addition of *Saccharomyces cerevisiae* to the diets of sheep and goats increased the digestibility of DM, OM, CP, NDF, and ADF [51,52]. Researchers [53] used a mixture of phytogenic additives in dairy cows and obtained results similar to ours, with an increase in nutrient digestibility. Therefore, it is suggestive that in phase 2 of this experiment (mid-lactation), the additive ingredients used here have high potential in animal nutrition and are nutritionally efficient in enhancing animal production; however, in phase 1 (peak lactation), it is believed that productivity was higher in cows that consumed the additive as an indirect effect, that is, it improved the cow's sweat, reducing energy expenditure on inflammation and oxidative reactions.

We observed a lower leukocyte count due to fewer lymphocytes in cows that consumed the additive. These variables can be used as markers of stress [54] and inflammatory processes [55,56]. This reduction in white blood cells was also observed when curcumin was used in dairy sheep [12] and dairy calves [57], as well as when using microencapsulated cinnamon and oregano oil in lambs [58]. Still related to the hypothesis that the additive provides an anti-inflammatory response, we had lower concentrations of acute phase proteins such as ceruloplasmin and haptoglobin, which are biomarkers known as indicators of a pro-inflammatory process [59,60]. This is likely related to the anti-inflammatory action of turmeric [61], cinnamon, and oregano essential oil, which has anti-inflammatory properties [62,63]. All the factors mentioned above are directly related to the lower SCC in milk presented by cows in the phytogenic group in our study, showing that when the ingredients are used together, they do not lose their effects. It is important to highlight that turmeric extract and cinnamon and oregano essential oils are some of the ingredients used in the production of the feed additive supplied to cows, as well as probiotics and minerals (selenium and chromium), also well-known as immune response stimulators [64,65]. In addition to reducing inflammation, the feed additive stimulated the IgA immune response,

similar to the study by authors [45], when a blend of phytogenics similar to the additive used here was used. The combination of these results related to the immune system was beneficial to the cow's health and, consequently, to milk production, combined with the nutritional effects already discussed.

The oxidative biomarkers were influenced by the consumption of the feed additive by the cows, highlighting lower SOD activity and lower TBARS levels, which shows the antioxidant potential of the phytogenic mixture. Antioxidant actions are found in animals that consumed curcumin [66], selenium, and chromium [64,65,67] and highlighted that cinnamon and oregano essential oils have components with the capacity to absorb oxygen radicals [68], which may justify the lower lipid peroxidation observed here. GST activity was higher in the group of cows that consumed the phytogenic mixture because the additive has a liver detoxification effect, and this antioxidant enzyme is the most important in this tissue and localized detoxification process. This effect is similar to that found when using curcumin in sheep [12] and a phytogenic blend in steers [45]. Therefore, the combination of ingredients in the production of the phytogenic mixture was efficient in stimulating the antioxidant response and consequently reducing oxidative reactions, which, when exacerbated, harmed the cow's health and interfered with milk production. Therefore, this desirable antioxidant effect may have indirectly contributed to enhancing milk production and reducing SCC in the milk.

The fatty acid profile of milk is entirely linked to the cow's diet and, therefore, can vary according to the feed consumed [69]. In our study, we observed a lower concentration of palmitic and heneicosanoic acids in the milk of cows that consumed the phytoactive mixture. Consequently, there was a lower amount of saturated fatty acids. We assessed that cows that consumed the phytogenic mixture had a higher proportion of oleic acid, contributing to a more significant sum of monounsaturated fatty acids. When using curcumin in dairy sheep, similar results were observed: lower presence of palmitic acid in the treated group and a more significant treatment effect for oleic acid and monounsaturated fatty acids [9]. The use of tannins in goats also resulted in an increase in oleic acid [70]. These results show that using additives is a way to positively modulate milk quality to have significant amounts of omegas in the milk and lower somatic cell counts. Ingesting feeds with lower amounts of saturated fatty acids is a recommendation from nutritionists, as saturated fat can deposit in blood vessels and, over time, cause cardiovascular problems [71].

The consumption of the phytogenic additive by cows affected the abundance of *Streptococcus spp*. in milk, reducing its occurrence. Furthermore, our research identified the genera *Acinetobacter* spp., *Escherichia-Shigella* spp., and *Lactococcus* spp. as the main microorganisms in milk. Moreover, *Streptococcus* spp. is a genus of bacteria commonly responsible for causing mastitis [72–74]. In in vitro studies, cinnamon essential oil has demonstrated antibiofilm potential and inhibitory activity against bacteria of the genus *Streptococcus* spp. [75]. It also disrupts the membrane's integrity and stops pathogens' ATP production [76]. According to [77], curcumin associated with hydrogen peroxide has shown an antimicrobial effect against *Streptococcus* spp. In a recent study, cows were infected with *Streptococcus uberis* in a mammary quarter and fed with *Saccharomyces cerevisiae* in their diet; this resulted in lower SCC and temperature of the infected quarter; further, this resulted in lower CCS and infected room temperature—furthermore, the prebiotic improved mammary genes related to the antibacterial function of cells [78]. As observed in our study, these phytogenics could inhibit or recover animals from problems caused by *Streptococcus* spp.

## 5. Conclusions

The results allow us to conclude that adding the phytoactive mixture to the dairy cows' diet positively affected animal nutrition and health, improving the production efficiency of Jersey cows. We also concluded that the intake of phytogenics improved milk quality, reduced SCC, increased unsaturated fatty acids in milk, and protected the mammary gland from the bacteria *Streptococcus* spp. It is believed that consumption of the phytoactive mixture

during mid-lactation had direct effects on digestibility and the modulation of the volatile fatty acid profile while also having an indirect impact by stimulating an immune and antioxidant response combined with anti-inflammatory action in two experimental phases.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14172518/s1, S1: Illustration of the feeders and drinkers used during the cows' holding period; S2: Standardization of methodology for evaluating fatty acids in the rumen of cows that consumed the feed additive; S3: Profile of fatty acids in the Total Mixed Ration (TMR) of dairy cows during early lactation (30–75 days in milk) and mid-lactation (90–135 days in milk); S4: Treatment × day interaction for milk production (mean and standard deviation) of dairy cows during early lactation (30–75 days in milk) and mid-lactation (90–135 days in milk). Note: Phytogenic group: animals' intake an additive formulated with the combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid. * $p \leq 0.05$; S5: Treatment × day interaction for immunoglobulin A (IgA), ceruloplasmin (CERU) and haptoblobin (HATP) of dairy cows during early lactation (30–75 days in milk) and mid-lactation (90–135 days in milk): Control (CON) vs. Phytogenic (PHY); S6: Treatment × day interaction for oxidative status (SOD: superoxide dismutase; GST: Glutathione S-transferase; TBARS: lipid peroxidation; CAB-P: cabonyl protein) of dairy cows during early lactation (30–75 days in milk) and mid-lactation (90–135 days in milk): Control (CON) vs. Phytogenic (PHY); S7: Profile of fatty acids in milk of dairy cows during early lactation (30–75 days in milk) and mid-lactation (90–135 days in milk: Control (CON) vs. Phytogenic (PHY); S8: Beta diversity of microorganisms in the milk of dairy cows during early lactation (30–75 days in milk) and mid-lactation (90–135 days in milk: Control (CON) vs. Phytogenic (PHY). Note: Phytogenic group: animals' intake an additive formulated with the combination of cinnamon and oregano essential oil, chelated amino acid chromium, selenium proteinate, inactivated *Saccharomyces cerevisiae*, *S. cerevisiae*, turmeric extract, and tannic acid.

**Author Contributions:** Conceptualization and formal analysis investigation M.G.d.V. and A.S.D.S., methodology, validation and data curation writing—original draft preparation M.G.d.V., A.S.D.S., M.H.S., N.G.C., M.B., G.J.W., B.K., L.E.L.S., C.T.K.J. and G.S.S.; and resources, writing—review and editing, visualization, supervision, project administration, and funding acquisition A.S.D.S., R.W., G.V.K., M.M.B. and C.F.M. All authors have read and agreed to the published version of the manuscript.

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# Adding red propolis to the diet of Lacaune lambs: Effects on animal health, ruminal environment, performance, and meat quality

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ABSTRACT

This study determined the influence of red propolis ethanolic extract in lambs' diets on the ruminal environment, animal health, and growth. Two experiments were carried out to achieve these objectives. Pilot study: Twenty-four female Lacaune lambs were divided into four groups, with three replicates and two animals per replicate (pen): T0 (Control), TP1.3, TP2.7, and TP5.3, representing 0, 1.3, 2.7, and 5.3 mL of propolis extract per animal/day, respectively. The highest daily weight gain was observed in lambs that consumed 2.7 mL of propolis extract per day, and they also had the lowest feed conversion rate compared to the control. Lower total leukocyte counts were observed in lambs that consumed propolis extract compared to control due to lower granulocyte and lymphocyte counts. Ceruloplasmin and C-reactive protein levels were lower in the serum of lambs that consumed propolis compared to the control. Lambs that consumed 1.3 and 2.7 mL of propolis extract had lower TBARS levels than the control. The effect of treatment was observed for ruminal pH, which was higher in lambs that consumed propolis extract than in control. A lower concentration of short-chain fatty acids was observed in lambs that consumed propolis than the control due to lower concentrations of acetic and butyric acid. In experiment II, 36 male Lacaune lambs were selected and divided into two groups: MONENSIN (23.45 ppm of monensin per kg/dry matter (DM)) and PROPOLIS (3.8 mL of propolis per animal/day). The animals that consumed propolis extract had greater body weight, weight gain, and average daily gain. These also had a shorter time to reduce methylene blue, thus demonstrating more significant bacterial activity in the rumen. Furthermore, they had lower counts of *Escherichia coli*, total coliforms, and Enterobacteria. The meat's fatty acid profile in animals fed the red propolis extract showed higher concentrations of monounsaturated fatty acids. In conclusion, adding red propolis extract to the concentrate increased weight gain, average daily gain, and oxidative balance with an antimicrobial and anti-inflammatory effect.

## 1. Introduction

Ionophores are growth promoters in ruminant breeding that improve energy and protein utilization and decrease methane emissions from ruminants (Russell and Strobel, 1988; Wischer et al., 2013), but are banned in many parts of the world due to food safety concerns and the potential development of antimicrobial resistance. Not all ionophores are antibiotics, there are ionophores that are mainly used as research tools to study mechanisms of ion transport across cell membranes (Callaway et al., 2003). However, there is controversy over the use of

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antibiotics in animal diets due to the risk of transferring residues to meat and milk and promoting the development of resistant bacterial strains (Mathew et al., 2001). These concerns have promoted the search for alternative natural feed additives, such as propolis (Oeztuerk and Sagmanligil, 2009; Morsy et al., 2013). Propolis includes resinous, gummy, and balsamic substances collected by bees from buds, flowers, and plant exudates and mixed with their salivary secretions, wax, and pollen (Berretta et al., 2017). Its chemical composition differs considerably according to the geographic region where the resins were collected due to regional differences in flora, and different plants are resin sources (Sforcin and Bankova, 2011). Propolis is active against gram-negative and gram-positive bacteria, viruses, fungi, oxidants, inflammation, tumors, and parasites and may act as an immunomodulator (Alencar et al., 2007; Morsy et al., 2013).

According to Mirzoeva and Calder (1996), the mechanism of action of propolis on gram-positive bacteria is probably related to changes in the bioenergetic state of the bacterial membrane, which inhibits its motility; this action is similar to that of ionophores. Furthermore, propolis affects fermentation and methanogenesis in continuous microbial cultures in ruminants; it decreases the protozoa population and increases propionate levels by 10.3 % (Brodiscou et al., 2000). Recent studies have mentioned a general improvement in the health and immunity of animals fed with propolis. Oral administration of ethanolic extract of Brazilian red propolis to Santa Inês sheep (3 g/sheep/day) during the flushing period increased the concentrations of total leukocytes, total proteins, and globulins while decreasing triglycerides, glutamate oxaloacetate transaminase, and glutamate pyruvate transaminase (Morsy et al., 2013).

Regardless of the type or place of collection, Brazilian red propolis and Egyptian brown propolis were incubated in vitro at different concentrations (125, 250, and 500 g per 500 mg of DM in the diet) (Morsy et al., 2015). The authors attributed an increase in total short-chain fatty acids ([SCFAs] mainly to a massive increase in acetate production (the main contributor to SCFAs) by 45 % compared to monensin (Morsy et al., 2015). Brazilian red and Egyptian brown propolis extracts seemed to combat ruminal methanogenesis, and the authors attributed this effect to their antiprotozoal properties since protozoa are known to provide hydrogen as a substrate for methanogens (Morsy et al., 2015). The color of propolis depends on the vegetation available near the hive (Koru et al., 2007). This variation directly affects its pharmacological properties (Afrouzan et al., 2018). Therefore, the influence of propolis extracts on ruminal fermentation may differ according to their origin and the extraction method (Morsy et al., 2015).

Recently, Paixão et al. (2022) conducted a study with Santa Inês lambs fed with red propolis extract (300 g/L), where they used doses of 0, 7, 14, 21, and 28 mL of propolis per animal/day. These doses can be considered high and economically unfeasible due to the high cost of red propolis in Brazil (*ca.* 400 dollars per kg), considering that Cécere et al. (2021) obtained positive results by feeding Lacaune lambs with 1.8 mL of green propolis per animal/day (150 μL of propolis/kg body weight/day). Therefore, a pilot study was carried out with doses similar to Cécere et al. (2021) to direct the research; then, an experiment was conducted to evaluate the efficacy of propolis extract as a potential substitute for monensin as a growth promoter. Economically, it is challenging to compete with this ionophore due to its low cost, but the prohibition it in food in several countries has opened up the market for substitutes. Therefore, the general objective of this work was to evaluate whether a low dose of ethanolic extract of red propolis in diets for confined lambs has positive effects on the rumen environment, health, animal performance, and meat quality.

## 2. Materials and methods

Two experiments were conducted to answer the objectives. The first was identified as a pilot study, essential to defining the dose of the second experiment.

The experiment was carried out in the sheep sector of the Experimental Farm of the *Centro de Educação Superior do Oeste* of the *Universidade do Estado de Santa Catarina* in Guatambu, SC, Brazil (27° 09' 09.2" S 52° 47' 18.8 "W). The UDESC animal use research committee approved the study, protocol number 2517210622.

### 2.1. Propolis

The ethanolic extract of red propolis was prepared according to Wang et al. (2013). Propolis produced by *Apis mellifera* (the honey bee) was purchased from the Bee Propolis (Minas Gerais). Raw propolis was ground in a porcelain mortar under liquid nitrogen to obtain small fragments and then ground in a hammer. A propolis solution of 100 g $L^{-1}$ 70 % ethanol was prepared. The samples were placed in a water bath at 40 °C for 1 h, and the supernatants were collected and filtered through Whatman No. 4 paper. The residue was subjected to three such extractions. The supernatants were placed in a rotary evaporator under reduced pressure at 50 °C to eliminate ethanol.

### 2.2. Determination of the chemical profile of propolis via GC-MS

To determine the general chemical profile of the propolis extract, the method described by Bayram et al. (2020) was used with some modifications. 5 mg of lyophilized propolis with 75 μL of dry pyridine and 50 μL of derivatizing N, O-bis(trimethylsilyl)trifluoroacetamide was homogenized, and kept at 80 °C for 20 min. The supernatant was analyzed using GC-MS (Shimadzu, GC-MS-QP2010 Ultra) in triplicate using pyridine and the derivatizer as the blank sample. The column oven temperature was initially held at 50 °C for one minute, then programmed to increase to 280 °C at 10 °C/min, and held for 2 min with a split ratio of 100.

### 2.3. Total phenolic compounds and antioxidant activity of propolis extract

Determining total phenolic compounds (TPC) and antioxidant activity by scavenging radicals by 2,2-diphenyl-1-picrylhydrazylhydrate (DPPH) was performed. 0.5 g of each sample was dissolved in 50 mL of distilled water for extraction. The mixture was placed in an ultrasonic bath (70 W) for 3 h and remained in the dark for another 3 h. The supernatants were filtered with quantitative filter paper (Whatman #40) and stored in 100-mL volumetric flasks wrapped in aluminum foil. The extracts were then stored in microtubes and maintained at -80 °C until analysis (Larrauri et al., 1997; Bertoletti et al., 2018). TPC quantification was performed using the Folin-Ciocalteu colorimetric method, modified by Bonoli et al. (2004). An aliquot of each diluted sample was mixed with 0.5 mL of the Folin-Ciocalteu reagent and stirred for one min. Then, 2 mL of sodium carbonate (20 %) was added to the mixture and stirred for 30 s. After 2 h of incubation, absorbance was read at 750 nm in a digital spectrophotometer Kasuaki IL-593-S. The standard curve was prepared using various concentrations of gallic acid in methanol. The TPC concentration was calculated using an equation derived from the standard curve of gallic acid (expressed in mg of gallic acid equivalent per g of dry sample; mg GAE/g). Data were expressed as mean ± standard deviation of triplicate experiments (Supplementary material 1).

The evaluation of the free radical scavenging activity in the extracts was determined to be the antioxidant capacity of reducing the DPPH radical, according to Brand-Williams et al. (1995). Five dilutions of each extract were prepared in test tubes, and 0.3 mL of each diluted extract was added to 2.7 mL of DPPH radical (40 μg/mL). After incubation for 1 h in the dark, absorbance was read at 515 nm. Free radical scavenging activity was reported as $IC_{50}$ (μg/mL), the antioxidant concentration required to scavenge 50 % of the DPPH in the test solution. All tests were performed in triplicate, and $IC_{50}$ values were expressed as mean ± standard deviation of triplicate experiments.

### 2.4. Experiment 1: pilot study

Twenty-four Lacaune lambs (90 days old; 20.1 ± 3.85 kg) were acquired from Cabanha 3-Leites – Lageado Grande, SC, Brazil. The experiment lasted 75 days, 15 of which corresponded to the adaptation phase to the diet and installation. The animals were accommodated in 1.2 m x 2.5 m pens with wooden slats and equipped with feeders and drinkers. The animals were separated into pens according to body weight. The distribution of the pens in the respective treatments in the installation was sequential to avoid environmental interference.

The basal diet was a total ration formulated to meet the requirements of the animals, according to the National Research Council (2007). The animals received corn silage, hay, and concentrate (2 % of body weight; Table 1). The animals assigned to each treatment were given doses of 2.2, 4.4, and 8.8 mL of propolis extract (10 % concentration solution) per kg concentrate, mixed during production. These doses allowed the lambs to be given four doses of propolis/animal/day, as detailed below. The animals were divided into four groups, with three repetitions and two animals per repetition (pen): T0 (Control), TP1.3, TP2.7, and TP5.3 representing 0, 1.3, 2.7, and 5.3 mL of propolis per animal/day, respectively. These doses in the pilot study were defined based on the experiment carried out by Cécere et al. (2021) using green propolis. To guarantee the total consumption of propolis, the concentrate was first supplied to the lambs; after 100 % of its consumption, the roughage (hay and silage) was supplied. Corn silage, hay, and concentrate samples were collected to determine chemical composition.

The animals were weighed individually at the beginning of the experiment and then every 7 days. The average daily gain (ADG) was calculated as the regression coefficient of individual live weight on time (Yuste et al., 2020). All weighings were performed in the morning, with the animals fasting, using a digital electronic scale. Leftover feed was collected throughout the experiment to determine the dry matter intake (DMI) per pen. Based on this information, feed conversion (DMI/weight gain - kg/kg) was calculated. It is essential to clarify that the pen was used as an experimental unit for these zootechnical variables.

The animals were restrained manually for blood sample collection on the first, fifteenth, forty-fifth, and seventy-fifth day through jugular vein puncture using vacuum tubes containing ethylenediaminetetraacetic acid to perform the erythrograms and leukograms. Tubes without anticoagulants were used to obtain serum for biochemical analysis. Fecal samples were collected on days 15, 45, and 75 directly from the rectal ampulla into sterile bags, stored in a refrigerated thermal box for transport, and analyzed within 2 h to determine total counts of coliforms, *Escherichia coli*, and Enterobacteria. Ruminal fluid (100 mL per animal) was collected 3 h after the morning feeding on days 15, 45, and 75 using an oro-ruminal silicone probe connected to a vacuum pump. The pH was measured using a portable digital pH meter (TESTO 205®). Immediately after measuring pH, the functional activity of the ruminal microbiota was measured using the methylene blue reduction test (MBRT) described by Dirksen (1993). A subsample of ruminal fluid was reserved for determination of SCFAs. The animal was the experimental unit for blood, ruminal fluid, and feces variables.

### 2.5. Experiment 2

Thirty-six male Lacaune lambs (30 days old; 18.4 ± 3.80 kg body weight) were used from a commercial farm in southern Brazil. The experiment lasted 75 days, with 15 days of adaptation to the diet and installation. The animals were housed in 1.2 m x 2.5 m pens with wooden slatted floors and equipped with feeders and drinkers. The animals were divided into two groups, with six repetitions and three per repetition (pen), totaling 18 animals for each treatment. The groups were MONENSIN and PROPOLIS, representing the dose of 23.45 ppm of monensin per kg/DM of the diet and the dose of 5 mL of red propolis extract/kg of concentrate, respectively. The groups received their treatments monensin and propolis in the concentrate, mixed during its production.

The animals received corn silage and concentrate (2.5 % of body weight; Table 2). The basal diet was a total ration formulated to meet the animals' requirements, according to National Research Council (2007) for an estimated gain of 300 g/day. Food leftovers were collected throughout the experiment to determine DMI. Corn silage and concentrate samples were collected to determine chemical composition.

The animals were weighed individually at the beginning of the experiment and every 15 days after that. The average daily gain (ADG) was calculated as the regression coefficient of individual live weight on time (Yuste et al., 2020). All weighings were carried out in the morning, with the animals fasting, using a digital electronic scale.

They were also manually restrained to collect blood samples on days 0, 15, 45, and 75 through jugular vein pulsing using vacuum tubes containing ethylenediaminetetraacetic acid to perform erythrogram and leukogram, and in tubes without anticoagulant to obtain serum for biochemical analysis. Fecal samples were collected on days 15, 45, and 75 directly from the rectal ampoule in sterile bags, stored in a

**Table 1**
Experiment 1: Pilot study - Ingredients (g/kg) and chemical composition of ingredients and experimental feeds (g/kg DM).

| **Ingredients** | **Composition** |
|---|---|
| Corn silage | 418.2 |
| Hay: Tifton 85 | 63.6 |
| Concentrate[a] | 518.2 |

| | | **Concentrate** | | | | |
|---|---|---|---|---|---|---|
| **Chemical composition**[b] | **Corn silage** | **Hay** | **Control**[c] | **TP1.3** | **TP2.7** | **TP5.3** |
| **DM** | 308.4 | 803.3 | 868.4 | 868.4 | 868.4 | 868.4 |
| **MM** | 40.0 | 60.9 | 81.6 | 81.6 | 81.6 | 81.6 |
| **CP** | 89.9 | 83.0 | 205.3 | 205.3 | 205.3 | 205.3 |
| **NDF** | 334.1 | 694.6 | 170.2 | 170.2 | 170.2 | 170.2 |
| **ADF** | 180.3 | 328.6 | 49.1 | 49.1 | 49.1 | 49.1 |
| **EE** | 33.8 | 14.0 | 31.0 | 31.0 | 31.0 | 31.0 |

Note: Total phenolic compounds (mg/kg) of concentrates. Control: 253.9; TP1.3: 266.8; TP2.7: 276.6; TP5.3: 285.0. $IC_{50}$ (mg/mL - the concentration responsible for reducing cellular viability by half) of concentrates. Control: 0.033; TP1.3: 0.152; TP2.7: 0.196; TP5.3: 0.255.
Control treatment and treatments TP1.3, TP2.7, and TP5.3 represent 0, 1.3, 2.7, and 5.3 mL of propolis per animal/day, respectively.

[a] Composition of the concentrate, production of 1000 kg: corn meal (540 g/kg), soybean meal (200 g/kg), wheat bran (200 g/kg), calcitic limestone (10 g/kg), sodium bicarbonate (4 g/kg) and 50 g/kg of premix (Calcium (min./max.) 175/245 g; Phosphorus (min.) 40 g; Sulfur (min.) 25 g; Magnesium (min.) 12 g; Sodium (max.) 100 g; Mineral Matter (max.) 980 g; Cobalt (min.) 40 mg; Iron (min.) 1800 mg; Iodine (min.) 80 mg; Manganese (min.) 1400 mg; Selenium (min.) 12 mg; Zinc (min.) 4000 mg; Vit. A (min.) 300,000 IU; Vit. D3 (min.) 60,000 IU; Vit. E (min.) 600 IU; Fluoride (max.) 600 mg; Monensin Sodium 600 mg).

[b] DM (dry matter), MM (mineral matter), CP (crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), and EE (ether extract).

[c] Basal concentrate - used in all groups. The chemical composition of the concentrate of the four groups was identical; that is, the phytogenic curvature did not alter the chemical composition of the concentrate ingested by the lambs.

**Table 2**
Experiment 2 - Ingredients (g/kg) and chemical composition of ingredients and experimental feeds (g/kg DM).

| **Ingredients** | **Composition, kg** | | |
|---|---|---|---|
| Corn silage | 315,4 | | |
| Concentrate[a] | 684,6 | | |
| | **Concentrate** | | |
| **Chemical composition**[b] | **Corn silage** | **Control**[c] | **Propolis** |
| **DM** | 308.4 | 873.0 | 873.0 |
| **MM** | 40.0 | 82.8 | 82.8 |
| **CP** | 89.9 | 228.5 | 228.5 |
| **NDF** | 334.1 | 147.8 | 147.8 |
| **ADF** | 180.3 | 46.7 | 46.7 |
| **EE** | 33.8 | 31.1 | 31.1 |

Note: Total phenolic compounds (mg/kg) of concentrates. Control: 243.07; TP138: 269.51. $IC_{50}$ (mg/mL - the concentration responsible for reducing cellular viability by half) of concentrates. Control: 0.206; TP138: 0.253.
The control and propolis treatments represent animals that received 23.45 ppm/kg/DM of monensin and 3.8 mL of animal propolis/day, respectively.

[a] Composition of the concentrate, production of 1000 kg: corn meal (525 g/kg), soybean meal (300 g/kg), wheat bran (117 g/kg) calcitic limestone (10 g/kg) and 50 g/kg of premix (Calcium (min./max.) 190/260 g; Phosphorus (min.) 20 g; Sulfur (min.) 40 g; Magnesium (min.) 20 g; Sodium (min.) 62 g; Mineral Matter (max.) 980 g; Cobalt (min.) 8 mg; Iron (min.) 1200 mg; Iodine (min.) 32 mg; Manganese (min.) 800 mg; Selenium (min.) 6 mg; Zinc (min.) 1.200 mg; Vit. A (min.) 200,000 IU; Vit. D3 (min.) 40,000 IU; Vit. E (min.) 400 IU.

[b] DM (dry matter), MM (mineral matter), CP (crude protein), NDF (neutral detergent fiber), ADF (acid detergent fiber), and EE (ether extract).

[c] Basal concentrate - used in all groups. The chemical composition of the concentrate of the four groups was identical; that is, the phytogenic curvature did not alter the chemical composition of the concentrate ingested by the lambs.

refrigerated thermal box for transport, and analyzed within 2 h to determine total coliforms/*Escherichia coli* and Enterobacteria count. Furthermore, 100 mL of rumen fluid per animal was collected after 3 h of morning feeding on days 15, 45, and 75, using an oro-ruminal probe connected to a vacuum pump. The pH was measured using a portable digital pH meter (TESTO 205®), then the functional activity of the rumen microbiota was measured using the MBRT, described by Dirksen (1993). A subsample of ruminal fluid was reserved for determination of SCFAs. These samples after collected were immediately submitted to freezing to avoid SCFAs degradation but adding an acid to allow sample deproteinizing wasn't used at this moment.

Slaughter was carried out following the end of the experiment (day 76) in a specific slaughterhouse for sheep, following current regulations and accompanied by a veterinarian from the municipal inspection. After the stunning, bleeding, skin removal, and evisceration procedures, muscle pH was measured in the longissimus dorsus muscle. Next, a gluteus medius muscle sample was taken to analyze the chemical composition and fatty acid profile. A piece of liver was also collected for antioxidant analysis.

### 2.6. Hematology, serum biochemistry, and proteinogram

Hemoglobin, erythrocyte, and total leukocyte counts, hematocrit, and leukocyte differentials were performed immediately upon sample arrival at the laboratory using an automatic hematologic analyzer (three parts EQUIP VET 3000®). The tubes without anticoagulant were centrifuged (7000 rpm for ten minutes) to separate the serum. The supernatants were transferred to 1.5-mL microtubes, identified, and stored at -20 °C. Total protein, albumin, triglycerides, cholesterol, glucose, aspartate aminotransferase (AST), and gamma-glutamyltranspeptidase (GGT) concentrations were evaluated in a semiautomatic analyzer (Bio-2000 BioPlus®) and commercial kits (Analisa®, Belo Horizonte, Brazil). Globin levels were calculated using the formula total protein – albumin.

For the proteinogram, electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate was performed according to Fagliari et al. (1998) using mini-gels (10 ×10 cm). Gels were stained with Coomassie blue and photographed to identify and quantify protein fractions using Labimage 1D software (Loccus Biotechnology). A standard containing fractions with molecular weight between 10 and 250 kD (Kaleidoscope – Bio-Rad) was used as the reference. The total protein content using the biuret technique was used as a reference for quantification (Layne, 1957).

### 2.7. Oxidant status

To determine oxidant status, serum glutathione S-transferase (GST) activity was measured based on Habig et al. (1974) and expressed as μmol 1-Chloro-2,4-dinitrobenzene (CDNB)/min/mg serum protein. According to Ellman (1959), protein thiols were measured, and the results were expressed as nmol thiols/mg of protein.

Serum lipid peroxidation was measured as the amount of TBARS, according to Jentzsch et al. (1996). The reaction was read in a spectrophotometer Kasuaki IL-593-S at 535 nm. The results were expressed as nmol of malondialdehyde (MDA)/mL of serum.

### 2.8. E. coli, total coliforms, and Enterobacteriaceae counts in stool

Counting was performed with 1 g of each stool sample, diluted in 9 mL of buffered peptone water in sterile test tubes, and homogenized in a vortex mixer, resulting in a dilution of $10^{-1}$, until reaching a final dilution of $10^{-4}$. Then, 1 mL of the final dilution was inoculated into 3 M™ Petrifilm™ EC 6414 Plates for counting *E. coli* and total coliforms. For counting Enterobacteria, 1 mL of each sample was inoculated into 3 M™ Petrifilm™ EB 6420 Plates. Subsequently, the plates were incubated in a bacteriological oven at 37 ± 1 °C for 24 h. Then, the colony count was performed according to the corresponding 3 M™ Petrifilm™ interpretation guide for each plate type. The results were expressed as colony-forming units per gram of feces (CFU/g).

### 2.9. Analysis of the chemical composition of silage and concentrate

Feed samples were previously pre-dried in a forced ventilation oven at 55 ºC for 72 h. Then, the samples were weighed to determine the DM content of the original sample, followed by grinding in a Wiley-type mill (Marconi, model: MA340) using a 1-mm mesh sieve. The pre-dried and ground samples were heated at 105 °C for 24 h to obtain the DM, and the ashes in a muffle at 600 °C (Silva and Queiroz, 2002).

Diets were analyzed according to Association of Official Analytical Chemists (2000): DM, method 930.15; crude protein (CP), method 976.05; ether extract, method 920.39 and ash, method 942.05. The method described by Van Soest et al. (1991) without adding sodium sulfite and alpha-amylase was chosen for neutral and acid detergent fiber analysis.

To determine TPC, 0.5 g of each concentrate (control, TP1.3, TP2.7, and TP5.3) was weighed in a Falcon tube, and 5 mL of ethanol solvent (60 %) was added. The mixture was stirred and kept for 20 min in a heated bath (96 °C). Then, they remained for 15 min in an ultrasound bath (180 W) and centrifuged for 15 min (4000 x *g*). The supernatants were filtered, and the extracts were analyzed.

### 2.10. Determination of short-chain fatty acids

Ruminal fluid samples were thawed at 5 °C and manually shaken for homogenization. Aliquots of 1 mL of the supernatant of ruminal fluid samples were collected in polypropylene microtubes (2 mL) and centrifuged for five minutes (12,300 × *g*). Then, 250 μL of the supernatants were removed and transferred to a new microtube containing 250 μL of formic acid. The mixture was shaken manually and centrifuged again for three min. Then, 250 μL of the supernatant was collected in an injection vial, and 500 μL of 3-octanol solution (665 μg $mL^{-1}$ in

methanol) was added, used as an internal standard (Zahedi et al., 2023) and homogenized. The samples were analyzed in a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3600, Palo Alto, USA) and an automatic sampler (Varian 8200CX, Palo Alto, USA). One microliter of the extract was injected in split mode at 1:10 at 250 °C. The carrier gas used was hydrogen at a constant pressure of 20 psi. The analytes (acetic, propionic, butyric, valeric, and isovaleric acids) were separated by a CP-WAX 52CB capillary column (60 m x 0.25 mm; 0.25 μm stationary phase thickness) (Supplementary material 2). The initial column temperature was set at 80 °C for one min and increased to 120 °C at a rate of 8 °C $min^{-1}$, then to 230 °C for 15 °C $min^{-1}$, where it remained for one min. The flame ionization detector temperature was set at 250 °C. The results were expressed in mmol $L^{-1}$ of each short-chain fatty acids (SCFAs) in the ruminal fluid.

### 2.11. Fatty acid profile and chemical composition of meat

Extraction was carried out using the method of Bligh and Dyer (1959) with some modifications. Fractions of 1.5 g of samples, 0.8 mL of water, 5 mL of methanol, and 2.5 mL of chloroform were added to a 15 mL polypropylene tube, and mechanical stirring was carried out for 30 min. Next, 2.5 mL of chloroform and 1.5 % $Na_2SO_4$ solution were added to promote a two-phase system. This mixture was stirred for 2 min and then centrifuged for 15 min at 2000 rpm. The lipids obtained from the chloroform phase were subjected to fatty acid analysis.

Fatty acid (FA) profile methylation was performed using a transesterification method proposed by Hartman and Lago (1973). 1 mL of 0.4 M KOH methanolic solution was added to the extracted lipids in a test tube and vortexed for one min. The samples were kept in a water bath for ten minutes at boiling temperature. Subsequently, they were cooled to room temperature, and 3 mL of 1 M methanolic $H_2SO_4$ solution were added, vortexed, and kept in a water bath for ten min. After cooling, 2 mL of hexane was added and centrifuged at 2000 rpm for ten min. Finally, hexane with fatty acid methyl esters (FAME) was subjected to chromatographic analysis. A gas chromatograph model TRACE 1310 with a flame ionization detector (Thermo Scientific) was used to determine FAME. One microliter of samples was injected into a split/splitless injector, operated in split mode with a 1:20 ratio at 250 °C. Hydrogen was used as the carrier gas at a constant flow rate of 1.5 mL/min. Separation of FAMEs was performed using an RT 2560 chromatography column (100 m × 0.25 mm × 0.20 μm film thickness, Restek, USA). The initial oven temperature was programmed at 100 °C for five min and increased to 180 °C at an 8 °C/min rate. Then, it increases to 210 °C at a rate of 4 °C/min, and finally up to 250 °C, increases to 20 °C/min, and maintains it for 7 min in isothermal. The detector temperature was kept constant at 250 °C. FAME compounds were identified by comparing the experimental retention time with those of the authentic standard (FAME Mix-37, Sigma Aldrich, St. Louis, MO). The results were presented as a percentage of each FA identified in the lipid fraction, considering the factor equivalent to the size of the FAME chain for FID and the ester conversion factor for the respective acid, according to Visentainer and e Franco (2006). For the chemical composition of the meat (DM, ether extract, CP and organic matter), the values were obtained using a near-infrared reflection spectrometer, model Spectra Star 2600 XT series of near-infrared analyzers (Unity Scientific®).

### 2.12. Statistical analysis

Data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene tests, respectively. Blood counts, stool bacterial counts, and GST activity results required transformation to achieve normality and homogeneity and then were retransformed to the original units for description.

All data were analyzed using the SAS 'MIXED procedure' (SAS Inst. Inc., Cary, NC, USA; version 9.4), with the Satterthwaite approximation to determine the denominator degrees of freedom for the fixed effects tested. In both experiments (pilot and experiment 1), in the experimental model we used treatment, sampling day, and the interaction between treatment and sampling day as fixed effects. The random effect was the "pen" per treatment (for data without repeated measurements over time, e.g.: growth performance data and postmortem meat data; exception for body weight), and the "animal" per treatment (for all repeated measurements over time, example: hematology, biochemistry, fatty acids and immunity).

In experiment 1, the means were compared using orthogonal contrasts at 5 % of significance. The first contrast (C1) represented the effect of propolis addition [control versus treatments (TP1.3 + TP2.7 + TP5.3)]; second contrast (C2): [Control versus TP1.3]; third contrast (C3): [(Control versus TP2.7), and four contrast (C4): [control versus TP5.3]. Regression analysis considering daily weight gain was performed to determine the ideal dose of propolis (mL/animal/day). In experiment 1 and 2, all data obtained on day 1 for each variable were included as covariates in each analysis, and the initial weight was included as a variable in the model. The first-order autoregressive covariance structure was selected according to the lowest Akaike information criterion. Means were separated using the PDIFF method, the t-test was selected, and all results were reported as means with standard errors. Significance was defined when $P \leq 0.05$.

## 3. Results

### 3.1. Chemical composition of red propolis extract

In red propolis extract used in the pilot study, the total phenolic compound content of the extract was 93.07 ± 0.780 mg gallic acid equivalent/g, and the antioxidant activity ($IC_{50}$) was 0.735 ± 0.170 mg/mL. The chemical profiles of the extract compounds using GC/MS are displayed in Supplementary material 1.

In red propolis used in experiment 2, the content of TPC in the propolis extract was 90.50 ± 3.8 mg gallic acid equivalent/g, and the antioxidant activity (IC50) was 0.735 mg/mL.

### 3.2. Experiment 1: pilot study

Results of the growth performance of the lambs were presented in Table 3. No effect of propolis treatment on body weight and feed consumption was observed. Greater daily weight gain was observed in lambs that consumed 2.7 mL of propolis extract per day compared to the control; as well as the animals in this group had the lowest feed conversion compared to the control. Regression analysis considered the ADG showed a trend ($p = 0.07$) toward an ideal dose of 3.81 mL/animal/day ($y = -0.001x^2 + 0.276x + 183.3$; $R^2 = 0.562$).

Results of the blood count of the lambs were presented in Table 4. There was no effect of treatment with propolis extract on red blood cells (erythrocytes, hematocrit and hemoglobin). However, lower total leukocyte counts were observed in lambs that consumed propolis extract compared to controls, due to lower granulocyte and lymphocyte counts. There was no difference between groups for monocyte count. No effect of day and interaction between treatment x day ($P \geq 0.05$) for the variable's erythrocytes, hematocrit, hemoglobin, leukocyte, monocytes and granulocyte. However, the lymphocyte count variable showed a significant day effect for all groups ($P = 0.05$) and treatment x day interaction ($P = 0.01$); the animals in the control treatment had higher counts on days 45 and 75 than the animals in the TP2.7 and TP5.3 groups (Supplementary material 3).

Serum biochemistry results are shown in Table 4. Lower triglyceride concentrations were observed in the serum of lambs in the TP5.3 group compared to control. Higher concentration of total cholesterol was found in the serum of TP2.7 and TP5.3 animals when compared to the control. There was no effect of treatment with propolis extract for the variables of AST, GGT, total protein, albumin, globulin and glucose.

**Table 3**
Experiment 1: Pilot study - Zootechnical performance of lambs fed red propolis extract mixed with the concentrate.

| Items | Treatments[a] | | | | SEM | *P-value*[b] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CONT | TP1.3 | TP2.7 | TP5.3 | | C1 | C2 | C3 | C4 |
| Average daily gain (ADG), kg/day | 0.178 | 0.182 | 0.194 | 0.184 | 0.01 | 0.18 | 0.80 | **0.03** | 0.36 |
| Dry matter intake (DMI), kg/day | 0.95 | 0.91 | 0.96 | 0.92 | 0.08 | 0.86 | 0.92 | 0.94 | 0.89 |
| Feed conversion (DMI/ADG), kg/kg | 5.33 | 5.00 | 4.94 | 5.00 | 0.05 | **0.05** | 0.21 | **0.03** | 0.18 |

[a] Control treatment and treatments TP1.3, TP2.7, and TP5.3 represent 0, 1.3, 2.7, and 5.3 mL of propolis per animal/day, respectively.
[b] Probabilities for contrast effect: C1 = [CONT vs (TP1.3+TP2.7+TP5.3)]; C2 = CONT vs TP1.3; C3 = CONT vs TP2.7; C4 = CONT vs TP5.3.

**Table 4**
Experiment 1: Pilot study – Hemogram, seric biochemistry, proteinogram and oxidative status of lambs fed red propolis extract mixed with the concentrate.

| Variables | Treatments[1] | | | | SEM | *P*-value[2] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CONT | TP1.3 | TP2.7 | TP5.3 | | C1 | C2 | C3 | C4 |
| ***Hematology*** | | | | | | | | | |
| Erythrocytes (x$10^6$ µL) | 9.65 | 9.52 | 9.63 | 8.76 | 0.14 | 0.89 | 0.85 | 0.87 | 0.72 |
| Hematocrit (%) | 28.7 | 29.1 | 28.5 | 27.4 | 1.35 | 0.92 | 0.90 | 0.89 | 0.91 |
| Hemoglobin (g/dL) | 10.9 | 10.9 | 10.8 | 10.3 | 0.27 | 0.94 | 0.91 | 0.92 | 0.90 |
| Leukocytes (x$10^3$ µL) | 8.10 | 7.27 | 6.47 | 6.72 | 0.81 | **0.02** | 0.26 | **0.01** | **0.01** |
| Granulocytes (x$10^3$ µL) | 1.39 | 0.95 | 0.95 | 1.02 | 0.19 | **0.05** | **0.04** | **0.04** | 0.091 |
| Lymphocytes (x$10^c$ µL) | 6.45 | 5.73 | 5.19 | 5.34 | 0.36 | **0.02** | 0.28 | **0.03** | **0.05** |
| Monocytes (x$10^3$ µL) | 0.48 | 0.42 | 0.28 | 0.31 | 0.09 | 0.16 | 0.84 | 0.13 | 0.23 |
| ***Serum biochemistry***[3] | | | | | | | | | |
| AST (U/L) | 94.2 | 99.8 | 94.5 | 101 | 3.74 | 0.92 | 0.89 | 0.80 | 0.87 |
| GGT (U/L) | 87.0 | 89.6 | 86.3 | 80.0 | 2.95 | 0.86 | 0.93 | 0.91 | 0.29 |
| Total protein (g/dL) | 7.72 | 7.21 | 7.52 | 7.30 | 0.36 | 0.21 | 0.87 | 0.79 | 0.61 |
| Albumin (g/dL) | 2.31 | 2.50 | 2.35 | 2.48 | 0.14 | 0.56 | 0.45 | 0.82 | 0.80 |
| Globulin (g/dL) | 5.41 | 4.71 | 5.17 | 4.81 | 0.20 | 0.12 | 0.08 | 0.67 | 0.35 |
| Triglycerides (mg/dL) | 35.1 | 34.8 | 31.3 | 27.3 | 1.87 | 0.65 | 0.94 | 0.75 | **0.04** |
| Cholesterol (mg/dL) | 73.7 | 75.5 | 79.7 | 78.1 | 2.08 | 0.58 | 0.92 | **0.04** | **0.05** |
| Glucose (mg/dL) | 68.7 | 69.7 | 66.7 | 66.1 | 3.01 | 0.85 | 0.96 | 0.89 | 0.90 |
| ***Proteinogram*** | | | | | | | | | |
| IgA (g/dL) | 0.74 | 0.69 | 0.72 | 0.73 | 0.04 | 0.95 | 0.90 | 0.95 | 0.96 |
| IgG heavy chain (g/gL) | 0.89 | 0.95 | 0.96 | 0.94 | 0.04 | 0.20 | 0.29 | 0.32 | 0.27 |
| Ceruloplasmin (g/dL) | 0.54 | 0.47 | 0.44 | 0.39 | 0.02 | **0.03** | 0.25 | **0.05** | **0.03** |
| Transferrin (g/dL) | 0.36 | 0.34 | 0.33 | 0.30 | 0.02 | 0.80 | 0.86 | 0.83 | 0.09 |
| C-reactive protein (g/dL) | 0.19 | 0.20 | 0.13 | 0.11 | 0.01 | 0.42 | 0.95 | **0.05** | **0.03** |
| Haptoglobin (g/dL) | 0.28 | 0.27 | 0.27 | 0.25 | 0.02 | 0.88 | 0.94 | 0.92 | 0.59 |
| ***Oxidative status*** | | | | | | | | | |
| TBARS (nmol MDA/mL) | 17.8 | 13.9 | 13.6 | 16.8 | 0.41 | 0.25 | **0.05** | **0.05** | 0.91 |
| Total thiols | 79.1 | 82.3 | 83.5 | 82.1 | 2.14 | 0.76 | 0.81 | 0.85 | 0.81 |
| GST (µmol CDNB/min/mg protein) | 430[a] | 394[b] | 395[b] | 423[a] | 6.87 | 0.35 | **0.02** | **0.02** | 0.92 |

1Control treatment and treatments TP1.3, TP2.7, and TP5.3 represent 0, 1.3, 2.7, and 5.3 mL of propolis per animal/day, respectively. 2Probabilities for contrast effect: C1 = [CONT vs (TP1.3+TP2.7+TP5.3)]; C2 = CONT vs TP1.3; C3 = CONT vs TP2.7; C4 = CONT vs TP5.3. 3Aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), immunoglobulin A (IgA) and G (IgG), thiobarbituric acid reactive substances (TBARS) and glutathione s-transferase (GST).

**Table 5**
Experiment 1: Pilot study - Ruminal indicators and feces microbiology of lambs fed with red propolis extract mixed with the concentrate.

| Variables | Treatments[b] | | | | SEM | *P*-value[5] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | CONT | TP1.3 | TP2.7 | TP5.3 | | C1 | C2 | C3 | C4 |
| *Ruminal liquid*[c] | | | | | | | | | |
| Rumen pH | 6.44 | 6.61 | 6.64 | 6.65 | 0.01 | 0.05 | 0.16 | 0.09 | 0.12 |
| MBRT[a] (s) | 232 | 160 | 235 | 191 | 28.9 | 0.21 | 0.07 | 0.92 | 0.59 |
| Total short-chain fatty acids mmol/L | 79.3 | 74.8 | 73.0 | 72.8 | 0.31 | 0.01 | 0.10 | 0.08 | 0.05 |
| Acetic acid, mmol/L | 52.9 | 50.5 | 48.8 | 49.0 | 0.85 | 0.05 | 0.25 | 0.12 | 0.18 |
| Propionic acid, mmol/L | 16.5 | 15.4 | 15.7 | 15.06 | 0.452 | 0.36 | 0.43 | 0.52 | 0.39 |
| Butyric acid, mmol/L | 7.85 | 7.08 | 6.65 | 6.66 | 0.212 | 0.01 | 0.26 | 0.03 | 0.09 |
| Isovaleric acid, mmol/L | 1.21 | 1.05 | 1.11 | 1.34 | 0.17 | 0.38 | 0.45 | 0.66 | 0.78 |
| Valeric acid, mmol/L | 0.76 | 0.72 | 0.73 | 0.67 | 0.03 | 0.74 | 0.82 | 0.80 | 0.41 |
| *Feces*[d] | | | | | | | | | |
| *Escherichia coli* (x $10^4$) | 49.2 | 40.6 | 39.5 | 42.4 | 3.12 | 0.08 | 0.10 | 0.23 | 0.28 |
| Total coliforms (x $10^4$) | 78.8 | 52.8 | 61.7 | 94.7 | 5.75 | 0.62 | 0.05 | 0.35 | 0.22 |
| *Enterobacteria* (x $10^4$) | 83.0 | 59.6 | 67.5 | 74.8 | 8.23 | 0.40 | 0.12 | 0.66 | 0.75 |

[5] Probabilities for contrast effect: C1 = [CONT vs (TP1.3+TP2.7+TP5.3)]; C2 = CONT vs TP1.3; C3 = CONT vs TP2.7; C4 = CONT vs TP5.3.
[a] MBRT (methylene blue reduction test).
[b] Control treatment and treatments TP1.3, TP2.7, and TP5.3 represent 0, 1.3, 2.7, and 5.3 mL of propolis per animal/day, respectively.
[c] Values expressed in mmol $L^{-1}$ of each short-chain fatty acid (SCFA) in the ruminal fluid.
[d] Values expressed in log CFU/g of feces.

There is also no day effect and treatment x day interaction (P ≥ 0.05) for triglycerides, cholesterol, AST, GGT, total protein, albumin, globulin, or glucose.

Protein profile results by electrophoresis are in Table 4. Effect of treatment with propolis extract was observed on ceruloplasmin levels, being lower in the serum of lambs that consumed propolis compared to the control. Lower concentration of C-reactive protein in the serum of lambs that consumed 2.7 and 5.3 mL of propolis extract compared to the control. No treatment effect was observed between groups for immunoglobulins, transferrin and haptoglobin. Also, there was no treatment x day interaction and day effect for concentrations of IgA, IgG heavy chain, transferrin, ceruloplasmin and C-reactive protein. But haptoglobin levels were influenced by the interaction between treatment x day (P = 0.05); no effect on the day (P = 0.67).

Oxidative status data are in Table 4. Lambs that consumed 1.3 and 2.7 mL of propolis extract had lower TBARS levels compared to the control. These same animals (TP1.3 and TP2.7) showed lower GST activity compared to the control. There was no effect of treatment on the concentration of total thiols. Also, there was no treatment x day interaction and day effect for concentrations of TBARS and total thiols, as well as GST activity differed between groups.

Results of biomarkers in ruminal fluid were presented in Table 5. Effect of treatment was observed for ruminal pH, being higher in lambs that consumed propolis extract compared to control. There was no difference between groups for bacterial activity (MBRT). Lower concentration of SCFAs was observed in lambs that consumed propolis compared to the control due to lower concentrations of acetic and butyric acid. There was no treatment effect for propionic, isovaleric and valeric acid. Acetic acid showed a significant interaction between treatment x day interaction (P = 0.01), and the animals in the TP2.7 and TP5.3 treatments showed a higher concentration on day 15. On day 75, the animals in the control group showed concentrations of acetic acid higher than those in the TP1.3 and TP2.7 groups. The concentrations of propionic acid showed a difference only on day 15 for the control group, which was higher than the TP5.3 group (P = 0.01 for the interaction between treatment x day). Butyric acid showed concentrations a treatment x day interaction (P = 0.001) i.e., on day 15, the TP2.7 and TP5.3 groups had lower concentrations than the control group; however, on day 75, the animals in the control group only outperformed the TP2.7 group. The concentration of valeric acid was higher in the control, TP1.3, and TP2.7 treatments compared to the TP5.3 treatment on day 15 (P = 0.01), and when only the differences between treatments were evaluated, there was a trend (P = 0.10) of the control animals presented values higher than TP5.3. There was an effect of day for acetic, butyric and valeric in TP5.3 lambs, which increased over time. Effect of day (increased over time) for propionic acid in all treatments. There was no treatment x day interaction or day effect for the sum of SCFA, pH and MBRT (Supplementary material 3).

There was no effect of treatment on *E. coli* and *Enterobacteria* counts in lamb feces (Table 5). However, lower total coliform counts were found in the feces of TP1.3 lambs when compared to the control. *E. coli* counts between treatment x day (P = 0.05). The TP5.3 animals had a higher count than the TP1.3 animals on day 15. The total coliform count of the TP5.3 was higher than the TP1.3 group on the fifteenth day; however, the animals from the control and TP1.3 treatments had higher counts than the TP2.7 and TP5.3 treatments on day 45 (P = 0.01). Enterobacteriaceae counts did not differ treatment x day and day (P ≥ 0.05). There was an effect of day for *E. coli* and total coliform counts in all treatments, reducing over time (Supplementary material 3).

### 3.3. Experiment 2

The results of growth performance are described in Table 6. Animals that received propolis had greater body weight than animals in the MONENSIN group on days 60 and 75 (interaction; P = 0.05); however, treatment did not affect body weight (P = 0.17). The weight gain of animals that received propolis was higher than those that received MONENSIN (P = 0.05), as was the ADG of animals treated with propolis (P = 0.05). No effect was found between treatments or treatment x day interaction (P ≥ 0.11) for weight gain from day 1–15, DMI, and feed conversion.

**Table 6**
Experiment 2 - Zootechnical performance of lambs fed with red propolis extract mixed with concentrate.

| Variables | Treatments[1] | | SEM | P-Value | |
|---|---|---|---|---|---|
| | MONENSIN | PROPOLIS | | Treat | Treat ×day |
| Body weight (kg) | | | | 0.17 | 0.05 |
| Day 1 | 18.3 | 18.4 | 0.54 | | |
| Initial weight (d15) | 21.0 | 21.4 | 0.69 | | |
| Final weight (d75) | 36.5[b] | 38.1[a] | 0.66 | | |
| Average daily gain (ADG) | 0.240[b] | 0.261[a] | **0.02** | 0.03 | - |
| Dry matter intake (kg/day) | 0.910 | 0.950 | 0.42 | 0.79 | - |
| Feed conversion (kg/kg) | 3.79 | 3.63 | 0.17 | 0.32 | - |

[1]The monensin and propolis treatments represent animals that received 23.45 ppm/kg/DM of monensin and 3.8 mL of animal propolis/day, respectively. [a-b] Within a row means without a common superscript differ (P ≤ 0.05) or tend to differ (P ≤ 0.10).

The results of the hematologic analysis are described in Table 7. Animals fed with propolis had higher counts of lymphocytes than animals of the MONENSIN group on day 15 (P = 0.01). No treatment or treatment x day effects were detected for (P > 0.11) were detected for hematocrit, hemoglobin, erythrocytes, leukocytes, granulocytes, or monocytes. There was an effect of day (P = 0.01), for both treatments (MONENSIN and PROPOLIS), for the variable's total leukocytes, lymphocytes and granulocytes. The sampling day had no effect on the other hematologic variables.

The serum biochemistry results are described in Table 7. Animals fed with propolis showed greater AST activity than the MONENSIN group on days 45 and 75, as well as the treatment effect (P = 0.01). No effects of treatment and treatment x day (P > 0.11) were identified for glucose, triglycerides, cholesterol, total protein, albumin, globulin, and GGT. There was an effect of day (p = 0.03), for both treatments (MONENSIN and PROPOLIS), for AST activity. The sampling day had no effect on the other clinical biochemical variables.

The results of ruminal indicators are presented in Table 8. Animals fed with propolis showed higher pH values on days 45 and 75, with a significant treatment effect (P = 0.01). The PROPOLIS group had a shorter time to MBRT than the MONENSIN animals on days 45 and 75 (P = 0.01). A lower concentration of short-chain fatty acids was found in the rumen fluid of lambs that consumed propolis (Table 8). Treatment or treatment x day interaction did not affect the proportion of acetic, propionic, butyric, isovaleric and valeric acids (P>0.05). The acetic/propionic ratio was higher in the rumen of lambs that consumed propolis compared to monensin. In ruminal fluid, there was an effect of day (p = 0.01) for both treatments (MONENSIN and PROPOLIS), for MBRT time, SCFA concentration and proportion of acetic acid. The sampling day had no effect on the other variables related with ruminal fluid.

The results of the microbiological analysis in feces are described in Table 8. The animals in the PROPOLIS group showed lower *Escherichia coli* counts on days 45 and 75 (P = 0.03), with a significant treatment effect (P = 0.05). The same occurred for the variable total coliforms on days 45 and 75 (P = 0.01), with lower counts in animals in the PROPOLIS group compared to the MONENSIN group. However, there was a significant effect of treatment and a treatment x day interaction for Enterobacteria counts, with lower bacteria counts observed on day 45 (P

**Table 7**
Experiment 2 - Hemogram of lambs fed with red propolis extract mixed with concentrate compared to monensin.

| Variables | Treatments[1] | | SEM | P-value | |
|---|---|---|---|---|---|
| | MONENSIN | PROPOLIS | | Treat | Treat ×day |
| ***Hemogram*** | | | | | |
| Hematocrit (%) | 30.4 | 29.1 | 1.25 | 0.89 | 0.91 |
| Hemoglobin (g/dL) | 11.8 | 11.8 | 0.41 | 0.92 | 0.95 |
| Erythrocytes (x $10^6$µL) | 10.0 | 10.1 | 0.26 | 0.95 | 0.96 |
| Leukocytes (x$10^3$ µL)[2] | 7.04 | 7.24 | 1.01 | 0.82 | 0.32 |
| Lymphocytes (x$10^3$/µL)[2] | | | | 0.68 | **0.01** |
| Day 1 | 10.8 | 11.0 | 0.96 | | |
| Day 15 | 7.43[b] | 8.93[a] | 0.57 | | |
| Day 45 | 4.37 | 4.59 | 0.56 | | |
| Day 75 | 3.13 | 3.03 | 0.55 | | |
| Granulocytes (x$10^3$/µL)[2] | 1.49 | 1.19 | 0.75 | 0.20 | 0.13 |
| Monocytes (x$10^3$/µL) | 0.56 | 0.54 | 0.38 | 0.90 | 0.81 |
| ***Seric biochemistry*** | | | | | |
| Glucose (mg/dL) | 74.7 | 74.2 | 2.85 | 0.94 | 0.92 |
| Triglycerides (mg/dL) | 27.3 | 22.7 | 3.14 | 0.73 | 0.65 |
| Cholesterol (mg/dL) | 65.3 | 65.1 | 2.46 | 0.96 | 0.91 |
| Total protein (g/dL) | 6.11 | 6.06 | 0.26 | 0.93 | 0.92 |
| Albumin (g/dL) | 2.55 | 2.54 | 0.11 | 0.95 | 0.96 |
| Globulin (g/dL) | 3.56 | 3.51 | 0.24 | 0.93 | 0.92 |
| GGT (U/L) | 95.9 | 96.8 | 6.41 | 0.85 | 0.72 |
| AST (U/L)[2] | | | | 0.08 | **0.01** |
| Day 1 | 94.3 | 93.6 | 3.21 | | |
| Day 15 | 90.6 | 92.5 | 3.04 | | |
| Day 45 | 80.3[a] | 108.2[b] | 3.94 | | |
| Day 75 | 93.0[a] | 103.5[b] | 3.82 | | |

[1]The monensin and propolis treatments represent animals that received 23.45 ppm/kg/DM of monensin and 3.8 mL of animal propolis/day, respectively. [2]Aspartate aminotransferase (AST) and gamma-glutamyl transferase (GGT). [2]There was an effect of day ($P < 0.05$). [a-b]Within a row means without a common superscript differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$).
Note: To analyze the treatment effect, results on days 15, 45, and 75, which correspond to the experimental period, were used; the results were presented as an average.

**Table 8**
Experiment 2 - Ruminal indicators and feces microbiology of lambs fed with red propolis extract mixed with concentrate.

| Variables[2] | Treatments[1] | | SEM | P-value | |
|---|---|---|---|---|---|
| | MONENSIN | PROPOLIS | | Treat | Treat × day |
| ***Ruminal liquid*** | | | | | |
| pH ruminal | | | | **0.01** | **0.01** |
| Day 15 | 6.21 | 6.34 | 0.03 | | |
| Day 45 | 6.13[b] | 6.35[a] | 0.03 | | |
| Day 75 | 6.11[b] | 6.38[a] | 0.03 | | |
| MBRT (s)[3] | | | | 0.26 | **0.01** |
| Day 15 | 41.4 | 56.2 | 3.05 | | |
| Day 45 | 67.8[a] | 33.0[b] | 3.65 | | |
| Day 75 | 78.3[a] | 57.8[b] | 3.41 | | |
| Σ SCFA (mmol/L)[3] | | | | **0.05** | **0.01** |
| Day 15 | 121[a] | 98.4[b] | 2.85 | | |
| Day 45 | 104 | 101 | 2.87 | | |
| Day 75 | 119 | 113 | 2.86 | | |
| Acetic acid (%)[3] | 59.7 | 61.3 | 1.97 | 0.56 | 0.23 |
| Propionic acid (%) | 26.3 | 25.4 | 0.95 | 0.89 | 0.61 |
| Butyric acid (%) | 10.6 | 10.2 | 0.64 | 0.93 | 0.86 |
| Isovaleric acid (%) | 1.40 | 1.27 | 0.06 | 0.15 | 0.34 |
| Valeric acid (%) | 1.90 | 1.76 | 0.07 | 0.23 | 0.11 |
| Acetic/propionic | 2.43[b] | 2.57[a] | 0.02 | **0.02** | 0.12 |
| ***Feces microbiology***[2] | | | | | |
| *Escherichia coli* (x$10^4$)[3] | | | | **0.05** | **0.03** |
| Day 15 | 134 | 120 | 5.10 | | |
| Day 45 | 310[a] | 186[b] | 6.16 | | |
| Day 75 | 82.7[a] | 42.6[b] | 3.94 | | |
| Total coliforms (x$10^4$)[3] | | | | **0.02** | **0.01** |
| Day 15 | 235 | 195 | 5.96 | | |
| Day 45 | 610[a] | 357[b] | 8.41 | | |
| Day 75 | 209[a] | 137[b] | 7.36 | | |
| Enterobacteria (x$10^4$)[3] | | | | **0.05** | **0.01** |
| Day 15 | 175 | 159 | 7.28 | | |
| Day 45 | 594[a] | 338[b] | 10.7 | | |
| Day 75 | 129 | 113 | 9.32 | | |

[1]The monensin and propolis treatments represent animals that received 23.45 ppm/kg/DM of monensin and 3.8 mL of animal propolis/day, respectively. [2]MBRT (methylene blue reduction test); SCFA (short-chain fatty acids); Values expressed as log CFU/g of feces. [3]There was an effect of day ($P < 0.05$).a-bWithin a row means without a common superscript differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$).
Note: To analyze the treatment effect, results on days 15, 45, and 75, which correspond to the experimental period, were used; the results were presented as averages.

$= 0.01$) in lambs in the PROPOLIS group. There was an effect of day ($p = 0.01$) for both treatments (MONENSIN and PROPOLIS), regarding the count of *E. coli*, total coliforms and Enterobacteria.

The results of carcass characteristics and the chemical composition of the meat are described in Table 9. There was no treatment effect ($P > 0.11$) for carcass weight, yield, hot carcass pH, DM, fat, protein, and organic matter.

The results of the FA profile in meat are described in Table 9. Meat from animals fed with red propolis showed a lower concentration of palmitic acid, while there was an increase in palmitoleic acid ($P = 0.01$). Furthermore, an effect of the treatment ($P = 0.05$) was detected for the total concentration of monounsaturated fatty acids (MUFA), being higher in animals fed with red propolis when compared to lambs in the MONENSIN group. There was no treatment effect for the other variables related to the FA profile in meat.

## 4. Discussion

Adding red propolis extract to the diet of lambs decreased the oxidative state, improved inflammatory responses and animal growth; these improvements do not always occur in propolis experiments. According to the literature, the chemical composition of propolis depends on the biodiversity in the area where the hives collect their material and their phytogeographic location, and this variation directly affects its pharmacological properties (Afrouzan et al., 2018). Vestitol and medicarpine exist only in the thirteenth type of propolis and have never been mentioned in the other 12 types of Brazilian propolis (Park et al., 2000). Red propolis is rare due to its robust antioxidant, antimicrobial, antiprotozoal and antifungal properties (Freires et al., 2016; Rufatto et al., 2018), as it is produced from resins collected from specific plants that are found in limited geographic regions. Brazilian red propolis is derived primarily from the plant *Dalbergia ecastaphyllum*, which grows in specific coastal areas of the northeastern region of Brazil. Compared to other types of propolis, the production of red propolis is naturally lower due to the limited availability of source plants and the dependence on bee activities in specific environments. It has unique chemical compounds that are not commonly found in other types of

**Table 9**
Experiment 2 - Chemical composition and fatty acid of meat and carcass characteristics of lambs fed with red propolis extract mixed with concentrate.

| Variables | Treatments[1] | | SEM | P-value |
|---|---|---|---|---|
| | MONENSIN | PROPOLIS | | Treat |
| Hot carcass weight (kg) | 19.5 | 18.0 | 0.62 | 0.87 |
| Carcass yield (%) | 47.7 | 47.7 | 0.60 | 0.96 |
| pH hot carcass | 6.61 | 6.60 | 0.04 | 0.97 |
| **Chemical composition (g/kg)** | | | | |
| Dry matter | 27.5 | 27.2 | 0.25 | 0.96 |
| Total lipids | 2.64 | 2.22 | 0.08 | 0.74 |
| Crude protein | 23.3 | 23.5 | 0.55 | 0.95 |
| Organic matter | 1.58 | 1.49 | 0.12 | 0.88 |
| Fatty acid (%) | | | | |
| C8:0 (Caprylic) | 0.048 | 0.046 | 0.002 | 0.87 |
| C10:0 (Capric) | 0.050 | 0.061 | 0.003 | 0.58 |
| C12:0 (Lauric) | 0.046 | 0.060 | 0.002 | 0.44 |
| C14:0 (Mystic) | 1.301 | 1.565 | 0.361 | 0.51 |
| C14:1 (Myristoleic) | 0.068 | 0.089 | 0.025 | 0.19 |
| C15:0 (Pentadecanoic) | 0.229 | 0.285 | 0.012 | 0.65 |
| C16:0 (Palmitic) | 25.00[a] | 22.78[b] | 0.851 | **0.01** |
| C16:1 (Palmitoleic) | 1.626[b] | 2.842[a] | 0.201 | **0.01** |
| C17:0 (Heptadecanoic) | 0.963 | 0.978 | 0.157 | 0.96 |
| C17:1 (cis−10-heptadecenoic) | 0.744 | 0.758 | 0.143 | 0.95 |
| C18:0 (Stearic) | 15.87 | 15.81 | 0.358 | 0.94 |
| C18:1n9t (Elaidic) | 1.646 | 1.705 | 0.092 | 0.89 |
| C18:1n9c (Oleic) | 40.31 | 41.07 | 0.386 | 0.74 |
| C18:2n6c (Linoleic) | 7.921 | 7.758 | 0.654 | 0.94 |
| C20:0 (Arachidic) | 0.119 | 0.122 | 0.040 | 0.91 |
| C18:3n6 (α- linolenic) | 0.104 | 0.108 | 0.015 | 0.97 |
| C18:3n3 (α- linolenic) | 0.249 | 0.250 | 0.139 | 0.99 |
| C21:0 (Henicosanoic) | 0.230 | 0.262 | 0.097 | 0.95 |
| C20:2 (cis−11,14-Eicosadienoic) | 0.094 | 0.085 | 0.008 | 0.88 |
| C22:0 (Behenico) | 0.062 | 0.061 | 0.006 | 0.96 |
| C20:3n6 (cis−8,11,14-Eicosatrienoic) | 0.215 | 0.214 | 0.023 | 0.98 |
| C20:4n6 (Arachidonic) | 2.913 | 2.884 | 0.113 | 0.90 |
| C20:5n3 (cis−5,8,11,14,17-Eicosapentaenoic) | 0.075 | 0.079 | 0.011 | 0.92 |
| C24:1n9 (nervous) | 0.041 | 0.038 | 0.008 | 0.87 |
| C22:6n3 (cis−4,7,10,13,16,19-docosahexaenoic) | 0.057 | 0.077 | 0.011 | 0.65 |
| SFA | 18.92 | 19.25 | 0.241 | 0.81 |
| MUFA | 44.44[b] | 46.50[a] | 0.468 | **0.05** |
| PUFA | 11.62 | 11.45 | 0.197 | 0.96 |

[1]The monensin and propolis treatments represent animals that received 23.45 ppm/kg/DM of monensin and 3.8 mL of animal propolis/day, respectively. [2]SFA (saturated fatty acids); MUFA (monounsaturated fatty acids); PUFA (polyunsaturated fatty acids).
[a-b] Within a line, means without a common superscript differ ($P \leq 0.05$).

propolis. These compounds include isoflavones, for example. However, in the propolis of this study, no rare compounds were found to classify it as the thirteenth type. This finding occurs because the propolis of each study originates from different places, and their respective compositions are also different. Therefore, the values of phenols and flavonoids are unique to this study and are challenging to compare with the literature. Nevertheless, it is possible that red propolis directly and indirectly influences our results, as discussed below.

TP2.7 animals have more ADG. However, the lambs in the PROPOLIS treatment in experiment 2 achieved greater body weight due to greater weight gain and daily gain during the experimental period. Suckling lambs that were supplemented with green propolis at a dose of 150 μL/kg of body weight showed significant weight gain (Cécere et al., 2021), while Ítavo et al. (2011) found a more significant daily gain in confined lambs fed green propolis at a dose of 15 mL/day (67.0 mg of flavonoid). Propolis has high bioactivity and synergism between its compounds; for this reason, it is called a feed additive. Like monensin, propolis acts against gram-positive bacteria, which benefits rumen fermentation in terms of nutrient utilization, improving animal performance (Zawadzki et al., 2011).

The leukogram must be interpreted with caution, as an increase in cells may indicate an increase in the inflammatory response (negative effect on animal productivity) or stimulation of the immune system (positive effect for the animal). An increase in the inflammatory response in animals can lead to a series of adverse effects that negatively impact productivity, such as reduced growth, as the energy that animals would use for growth and production can be diverted to combat inflammation, and inflammation can affect digestion and nutrient absorption, resulting in lower feed efficiency; a reduction in these defense cells may indicate a negative interference in the immune response or simply a desirable anti-inflammatory effect due to the consumption of the additive. In the pilot study, there was a lower number of leukocytes in animals fed propolis extract, although the value was within the normal range for the species (González and Silva, 2022), suggesting a beneficial effect on lamb performance. Morsy et al. (2016) found a higher number of leukocytes in sheep fed red propolis extract; Slazon et al. (2019) found no difference in the leukogram of calves.

Propolis acts as an immunostimulant due to the presence of its flavonoids (Nassar et al., 2012) and an anti-inflammatory action resulting from compounds present in propolis (Ramos and Miranda, 2007). The immunomodulatory effect is probably due to the components of propolis that increase and inhibit biomarkers of the immune system. These contradictory effects described in the literature are probably due to the complex chemical variations, the presence in different geographic regions and the forms of extraction (Berretta et al., 2017). The contradictory effects refer to the different ways in which propolis may influence the immune system, as described in the scientific literature. In other words, some studies report that propolis may increase certain biomarkers of the immune system, while others indicate that it may inhibit these same biomarkers. These contradictory observations suggest that the effects of propolis on the immune system are not consistent.

The lower lymphocyte count is responsible for the lower number of leukocytes in lambs that fed red propolis extract. This decrease did not interfere with the concentration of globulins; however, it affected the levels of acute phase proteins, that is, levels of ceruloplasmin, and C-reactive protein were in lower concentrations in animals fed with propolis, suggesting an anti-inflammatory effect. Flavonoids are potent anti-inflammatories, which explains our results. The elevation of these proteins (ceruloplasmin, and C-reactive protein) may suggest an overload response due to tissue injuries and local infections (Gabay and Kushner, 1999), leading to a greater ATP consumption by the animal organisms, reflecting less energy to produce muscle mass and provide weight gain for the animals. Therefore, indirectly, due to the anti-inflammatory action, the ingestion of propolis by the lambs favored their growth. According to the literature, flavonoids can also regulate arachidonic acid proteins (Hou et al., 2007), decrease reactive oxygen species with consequent inhibition of the inflammatory pathway (Salzano et al., 2014), inhibit the path of NF-kB through activation of PPAR-gamma (Martinez-Micaelo et al., 2015), and regulate mitogen-activated protein kinases (Wadsworth et al., 2001; Cho et al., 2003). Cécere et al. (2021) evaluated acute phase proteins such as ceruloplasmin in lambs fed green propolis and found no differences, even at a higher dose (250 μl/kg body weight).

The reduction of TBARS levels capable of reacting to thiobarbituric acid and the induction of activation of antioxidant enzymes are associated with compounds found in propolis (Jasprica et al., 2007). In the present study, there were flavonoids and phenols, antioxidant agents capable of eliminating free radicals and protecting the action of lipid peroxidation (Paulino et al., 2018; Yousef and Salama, 2009). As a result, we can attribute an indirect hepatoprotective effect, as GST reduced its activity, a critical enzyme in liver detoxification; this effect is probably related to the reduction of oxidations and, consequently, free radicals.

There was an unexpected result because the animals that consumed propolis in the pilot study (TP2.7) had more weight gain and the lowest

levels of SCFAs in the ruminal environment. At the same time, in experiment 2, we observed no difference in levels at the end of the experiment. Morsy et al. (2021) found an increase in acetate, propionate, valerate, and total SCFA when red propolis was fed to sheep. We need to understand better how the flavonoids present in red propolis (vestitol, medicarpin, isoliquiritigenin, and formononetin) can affect the profile of SCFAs, which we will do in a future study investigating the ruminal microbiota.

The microorganisms in the rumen are indispensable for the efficient digestion of the nutrients present in the diet (Vázquez-Anón and Jenkins, 2007; Freires et al., 2016); however, in this study, an antimicrobial effect may have occurred in the rumen and feces, consequently interfering with SCFA synthesis. We hypothesize that ingesting red propolis extract by lambs would increase ruminal nutrient degradation and produce individual proportions and total SCFA while mitigating methane formation based on information from other researchers (Morsy et al., 2015; Soltan and Patra, 2020). We attribute the lowest levels of SCFA to the extract not having vestitol, medicarpin, isoliquiritigenin, or formononetin, present in the study by Morsy et al. (2021); according to the authors, these compounds helped increase SCFA production. However, the decrease in SCFA concentrations did not interfere with the performance results, which we believe is related to the indirect effects, such as anti-inflammatory and antioxidant action, which improve the lambs' health.

Lower counts of total coliforms in the feces of TP1.3 lambs had already been described in another study by our research group when we used green propolis at a dose of 150 μL/kg of body weight (Cécere et al., 2021). In experiment 2, we obtained a decrease in the total coliforms, *E. coli,* and Enterobacteria count for animals fed with red propolis extract at a 3.8 mL animal/day dose. We attribute this effect to flavonoids and terpenes with antibacterial characteristics. According to researchers (Krol et al. 1993), these agents present synergism and enhance the antibacterial mechanism when combined. In vitro, 30 % green propolis has been shown to inhibit cell division in gram-negative bacteria, including *E. coli*, and to cause bacteriolysis (Krol et al., 1993). Notably, the extract in the present study had a concentration of 10 %, suggesting a more significant antibacterial action than other chemical compositions found in the literature at lower doses but efficiently reducing the count of gram-negative bacteria.

The results obtained for fatty acids in meat show that red propolis extract influenced the acid profile. Propolis increased the concentration of palmitoleic acid, a monounsaturated acid. Palmitic acid, a saturated acid, was reduced. This finding corroborates Aguiar et al. (2014), where the authors also found an increase in the concentration of palmitoleic acid when feeding propolis to confined cattle. Furthermore, Ítavo et al. (2019), when supplementing lambs with brown propolis, observed a reduction in the levels of saturated fatty acids and an increase in monounsaturated fatty acids (oleic acid (C18:1n-9) and polyunsaturated fatty acids (linoleic acid, C18:2n−6). Although we did not find an increase in other monounsaturated fatty acids alone in this study, we did observe an increase in their total. Therefore, as serum cholesterol is affected by the composition of fatty acids and monounsaturated fatty acids play an essential role in reducing blood cholesterol (Madruga et al., 2005), it can be stated that the addition of red propolis improved the profile of fatty acids in lamb meat, which is essential from the consumer's point of view. Aguiar et al. (2014) evaluated the effect of propolis on the FA profile of the milk of mid-lactation dairy cows. They observed that the FA profile of the milk was altered by the addition of propolis, which shows that it plays an essential role in microorganisms. The rumen is involved in the biohydrogenation process. However, in the literature, it is unclear which compounds in red propolis can modulate these effects on meat quality and FA profile, making it difficult to interpret the results; therefore, more studies are needed. However, in another recent study, researchers found that the red propolis extract improved the lipid profile of lamb meat, i.e., the treatment with propolis had a more significant antioxidant effect and lower content of saturated fatty acids in meat (Silva et al., 2024). Furthermore, an increase in the content of monounsaturated acids and, consequently, an improvement in nutritional quality, atherogenicity, and thrombogenicity indexes of desirable fatty acids was verified (Silva et al., 2024).

The "n" sample was one of the limitations of our pilot study, especially for the zootechnical performance evaluation; however, we highlight that performance analysis is only complementary data, and the main objective of the study was to understand the effects of propolis on the antioxidant response, inflammation, and the SCFA profile in the rumen. However, experiment 2 was carried out to complement these performance data and add more information about the action of red propolis in confined lambs.

## 5. Conclusion

The addition of red propolis extract to the concentrate provided a positive oxidative balance, which means there is an excess of antioxidants compared to free radicals. In terms of oxidative balance, this may indicate a condition in which the organism is well protected against oxidative stress, potentially promoting cellular health and preventing related diseases. The feed additive had anti-inflammatory and antimicrobial action, a diet containing propolis reduced SCFAs in the rumen. In addition, propolis extract at the tested dose of 3.8 mL of propolis extract/day was more efficient in the growth performance of lambs compared to the control group without propolis.

## CRediT authorship contribution statement

**Charles M. Giacomelli:** Methodology, Investigation, Formal analysis. **Luisa Nora:** Writing – original draft, Methodology, Investigation. **Fernanda Picoli:** Writing – original draft, Methodology, Investigation. **Aleksandro S. da Silva:** Writing – review & editing, Validation, Supervision, Software, Resources, Project administration, Investigation, Formal analysis, Conceptualization. **Luiz Eduardo L. Silva:** Writing – original draft, Methodology, Investigation, Formal analysis. **Priscila M. Copetti:** Validation, Methodology, Formal analysis, Data curation. **Bianca F. Bissacotti:** Validation, Methodology, Formal analysis, Data curation. **Bruna Klein:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Bruno G.O. Cécere:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Denise N. Araujo:** Writing – original draft, Supervision, Resources, Project administration, Conceptualization. **Vitor L. Molosse:** Writing – original draft, Methodology, Formal analysis, Data curation. **Aniela P. Kempka:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Guilherme L. Deolindo:** Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Roger Wagner:** Writing – original draft, Supervision, Resources, Formal analysis.

## Declaration of Competing Interest

The authors declare no conflict of interest.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.smallrumres.2024.107379.

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# Performance, Metabolism, and Economic Implications of Replacing Soybean Meal with Dried Distillers Grains with Solubles in Feedlot Cattle Diets

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## Abstract

The growing demand for biofuels, especially ethanol produced from corn, has driven the production of co-products such as dried distillers grains with solubles (DDGS). With a high protein content (around 30%), fiber, and minerals, DDGS presents an economical alternative for animal nutrition, replacing traditional sources like soybean meal while maintaining productive performance and reducing costs. This study evaluated the total replacement of soybean meal with DDGS in the diet of confined Holstein cattle, focusing on weight gain, feed intake, digestibility, feed efficiency, animal health, meat quality, and economic viability. The 24 animals received diets with 80% concentrate, containing either DDGS or soybean meal, and no significant differences were observed in terms of body weight ($p = 0.92$), feed intake ($p = 0.98$), or feed efficiency ($p = 0.97$) between the two treatments. The average daily gain was 1.25 and 1.28 kg for cattle in the DDGS and soybean meal groups, respectively ($p = 0.92$). Regarding metabolic and digestive parameters, no relevant changes were found in blood levels, except for higher serum cholesterol ($p = 0.03$) levels in animals fed DDGS. The digestibility of neutral detergent fiber (NDF) ($p = 0.03$) and acid detergent fiber (ADF) ($p = 0.05$) was lower in the DDGS group, while the digestibility of ether extract was higher ($p = 0.02$). Rumen fluid analysis revealed an increase in the production of short-chain fatty acids ($p = 0.01$), such as acetic and butyric acids ($p = 0.01$), in the DDG-fed animals. In terms of meat quality, animals fed DDGS produced meat with lower levels of saturated fatty acids (SFA) ($p = 0.05$) and higher levels of unsaturated fatty acids (UFA) ($p = 0.02$), especially oleic acid ($p = 0.05$). This resulted in a healthier lipid profile, with a higher UFA/SFA ratio ($p = 0.01$). In terms of economic viability, DDGS-based diets were 10.5% cheaper, reducing the cost of production per animal by 7.67%. Profitability increased by 110% with DDGS compared to soybean meal, despite the high transportation costs. Therefore, replacing soybean meal with DDGS is an efficient and

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economical alternative for feeding confined cattle, maintaining zootechnical performance, increasing meat lipid content and improving fatty acid profile, and promoting higher profitability. This alternative is particularly advantageous in regions with easy access to the product.

**Keywords:** DDGS; meat quality; digestibility; volatile fatty acids

## 1. Introduction

The growing global demand for biofuels and the considerable increase in industries specialized in producing ethanol from cereals, especially corn, produce renewable compounds that seek to reduce environmental damage compared to the production and use of fossil fuels [1]. Corn is one of the most produced cereals in the world, especially in countries such as the United States and Brazil, where the production of biofuels from this cereal is increasing due to the excellent availability of the raw material. To produce ethanol, corn is subjected to fermentation and high temperatures, where sugars present in the grains, including starch, are used by yeast, transforming glucose into alcohol, resulting in its production [2].

After drying the residue, we have the dried distiller's grain (DDG), where proteins, fibers, and minerals are retained [1]. DDG is the solid part obtained in this process, to which vinasse or syrup, rich in amino acids and lipids, also received in the fermentation process, can be added, giving rise to the DDGS, dried distillers grains with solubles. This coproduct has proteins, fats, fibers, and minerals with great potential in animal nutrition, providing carbon and nitrogen for microbial fermentation [3]. It is considered a protein food used in the diet of both monogastric and ruminant animals, presenting approximately 30% crude protein [4]. In animal nutrition, protein components and non-voluminous energy increase feed costs when added to diets [5]. The search for economically viable alternatives, reducing costs, such as using coproducts, which allow maintaining performance and productivity, such as DDGS, is constantly growing.

The protein source typically used in ruminant feed is soybean meal, which has good levels of rumen-degradable protein and provides essential amino acids for the animal, such as lysine [6]. However, a limitation on the use of this product is its high demand, which increases its market value and production costs. DDGS appear as an interesting source of protein, containing amino acids in its composition, especially methionine, allowing its inclusion in animal feed, reducing costs, and maintaining productive performance [7]. The search for levels of inclusion of this coproduct in ruminant feed is the focus of many studies, aiming at safe levels at which DDGS can be included in the diet without harming performance or causing problems in the animal or the products derived from it due to its lower starch concentration. In addition, this research aims to verify whether DDGS is a viable feed in the diet of male Holstein calves in feedlots, animals that are usually discarded at birth due to high production costs and low carcass yield, which makes their breeding unfeasible from an economic point of view. Thus, it is important to know whether the use of co-products can be effective for performance, and allow a profitable activity that farmers seek to consider this breed as a market niche.

As a hypothesis of our study, we expected that the replacement of soybean meal by DDGS would maintain the growth performance of animals without causing significant changes in ruminal parameters and their products, despite the differences in the composition between the feeds, promoting economic gain due to the lower cost of the diet. Among the implications of this research with DDGS, it is important to highlight the focus on envi-

ronmental sustainability and diversification of feed resources, providing more scientific support for the use of industrial waste to feed ruminants.

As already mentioned, there are many justifications for this research, from reducing production costs while maintaining productivity, a lower-cost feed source for Holstein cattle that are discarded at birth on many dairy farms, but mainly for the valorization of Bioresources from agri-food waste for sustainable cattle feeding, as is the case with DDGS. Therefore, the objective of the study was to evaluate whether the total replacement of soybean meal by DDGS in the diet of Holstein steers in the finishing phase under confinement conditions is an advantageous option considering weight gain, feed intake, dry matter digestibility, feed efficiency, animal health, meat quality, and economic viability.

## 2. Materials and Methods

### 2.1. DDGS and Soybean Meal

The DDGS used in the experiment was acquired from Cooperativa Cooper A1 (Palmitos, Santa Catarina, Brazil), presenting the following composition data, % (Dry matter (DM): 90.82; total digestible nutrients (TDN): 75.70; crude protein (CP): 34.71; neutral detergent fiber (NDF): 33.48; Starch: 7.36; ether extract (EE): 7.28; ash: 9.37), where the proportion of inclusion of the same in the concentrate was 23.75%. The soybean meal used was produced and supplied by Cooperativa Agroindustrial Alfa (Cooperalfa, Chpaecó, Santa Catarina, Brazil), with the following composition, % (DM: 93.20; TDN: 77.33; CP: 48.13; NDF: 14.32; EE: 2.33; ash: 7.29) corresponding to 12.50% inclusion in the concentrate.

### 2.2. Animals and Installations

Twenty-four intact male Holstein calves, 10 months old, weighing 345.6 $\pm$ 3.84 kg, were used in this study. The animals were housed in a barn with individual stalls measuring 15 $m^2$, partially covered and equipped with feeders and drinkers. The study lasted 90 days, with the first 21 days spent adapting to the facility and diet.

### 2.3. Experimental Design and Diets

The cattle were divided into two groups in a completely randomized design with 12 replicates per group. The diet provided (Table 1), formulated based on the nutritional requirements, provided 2.4% DM in relation to their live weight, in a concentrate/forage ratio (80:20). It was offered three times a day, at 8:00 a.m., 10:15 a.m., and 5:00 p.m., weighed and supplied individually directly into the feeder. Differences in the diet consisted of protein sources, soybean meal, and DDGS. The diets formulated for both groups were isoenergetic and isonitrogenous.

**Table 1.** Chemical composition and fatty acid profile in Total Mixed Ration (TMR).

| Variables | TMR: DDGS | TMR: Soybean Meal |
|---|---|---|
| Dry matter [1], % | 52.0 | 52.1 |
| Mineral matter, % | 6.15 | 5.79 |
| Crude protein, % | 14.2 | 13.9 |
| NDF, % | 22.4 | 24.0 |
| ADF, % | 11.2 | 14.3 |
| Ash, % | 3.53 | 3.29 |
| Fatty acid, % | | |
| C8:0 (Caprylic) | 0.02 | 0.03 |
| C12:0 (Lauric) | 0.07 | 0.05 |
| C14:0 (Myristic) | 0.20 | 0.18 |

**Table 1.** *Cont.*

| Variables | TMR: DDGS | TMR: Soybean Meal |
|---|---|---|
| C15:0 (Pentadecanoic) | 0.07 | 0.10 |
| C16:0 (Palmitic) | 19.2 | 21.4 |
| C16:1 (Palmitoleic) | 0.21 | 0.23 |
| C17:0 (Heptadecanoic) | 0.14 | 0.17 |
| C17:1 (cis-10-Heptadecenoic) | 0.05 | 0.05 |
| C18:0 (Stearic) | 3.04 | 3.46 |
| C18:1n9t (Elaidic) | 0.09 | 0.11 |
| C18:1n9c (Oleic) | 28.8 | 26.5 |
| C18:2n6c (Linoleic) | 43.7 | 42.5 |
| C20:0 (Arachidic) | 0.52 | 0.50 |
| C20:1n9 (cis-11-Eicosenoic) | 0.22 | 0.28 |
| C18:3n3 (a-Linolenic) | 2.54 | 3.28 |
| C20:2 (cis-11,14-Eicosadienoic) | 0.12 | 0.10 |
| C22:0 (Behenic) | 0.34 | 0.38 |
| C24:0 (Lignoceric) | 0.46 | 0.48 |
| ∑ Saturated fatty acids (SFA), % | 24.1 | 26.8 |
| ∑ Unsaturated fatty acids (UFA), % | 75.8 | 73.1 |
| ∑ Monounsaturated fatty acids (MUFA), % | 29.4 | 27.2 |
| ∑ Polyunsaturated fatty acids (PUFA), % | 46.3 | 45.9 |
| UFA/SFA | 3.16 | 2.73 |

The diet (TMR) of the DDGS group was formulated based on corn silage (30.0%), ground corn (40.1%) soybean hulls (3.3%), DDGS (23.6%), and mineral core (3.0%). The diet (TMR) of the soybean meal group was based on corn silage (30.0%), ground corn (41.2%), soybean hulls (12.4%), soybean meal (13.4%), and mineral core (3.0%). The mineral supplement used had the following micro-ingredients and their guaranteed levels: calcium (g/kg) (min 231.4); phosphorus (g/kg) (min 40.0); sulfur (g/kg) (min 40.0); magnesium (g/kg) (min 25.0); potassium (g/kg) (min 10.0); sodium (g/kg) (min 70.0); cobalt (mg/kg) (min 15.0); copper (mg/kg) (min 750.0); chromium (mg/kg) (min 20.0); iron (mg/kg) (min 700.0). [1]: Dry matter content (DM%), crude protein (CP%), neutral detergent fiber (NDF%), acid detergent fiber (ADF%), and ether extract (EE).

### *2.4. Data and Sample Collection*

At all times of data and sample collection, the cattle had been without food for approximately 14 h, but had free access to water. The collections occurred in the morning period between 07:00 and 08:30 h, considering the same feeding order of the animals as on the previous day. This practice was used to standardize the animals and thus have less fluctuation in the parameters of body weight, blood, and ruminal fluid due to feeding.

Weight was measured on days 1, 21, 55, and 90 of the experiment, using a digital scale before the morning feeding to monitor performance. From this data, weight gain and average daily weight gain were calculated. Based on the assessment of consumption, feed efficiency was measured.

Blood collections were performed on the same days as the weighing, with 8 mL collected per animal from the coccygeal vein and divided into two tubes: a vacuolated tube with anticoagulant (EDTA) for blood count analysis and a vacuolated tube without anticoagulant for subsequent centrifugation and serum collection for serum biochemistry analysis. The tubes were refrigerated at 10 °C in an isothermal box until they arrived at the laboratory. To separate the serum, the tubes were centrifuged without anticoagulant (7500 RPM for 10 min). The serum was transferred to microtubes, labeled, and stored at −20 °C until analysis.

The ruminal fluid collection was performed on days 55, and 90 of the experiment, with the aid of an esophageal probe and vacuum pump, instantaneous pH measurement with the help of a pH meter (Testo 0563 2051, Campinas, SP, Brazil) and freezing of samples at −20 °C for analysis of volatile fatty acid profile. Feces, diet, and respective leftovers for

analysis of apparent digestibility were collected once a day for three consecutive days at different times, on days 78, 79, and 80 of the experiment.

After 90 days of experimentation, the animals were slaughtered at a local slaughterhouse, following the procedures described in the legislation, with a state inspection system. After fasting, with access only to water, the animals were stunned with a pneumatic pistol, followed by bleeding and skinning. A portion of the longissimus dorsi muscle was removed and stored under refrigeration (6 °C) in non-vacuum plastic bags for 24 h to transform the muscle into meat (rigor mortis) for further analysis. After this process, the samples were refrigerated at −20 °C until analysis.

### *2.5. Zootechnical Performance*

From the measurement of body weight (BW), it was possible to calculate the other variables. Weight gain (WG) was calculated from the data from the last weighing by subtracting the weight from the previous weighing (final weight−initial weight); daily weight gain (ADG) was calculated from the weight gain divided by the period in days (WG/time (days)); feed intake (dry matter intake—DMI) was calculated from the weighing of the amount of feed provided and subtracting its respective leftovers later. The leftovers were weighed daily in the morning individually. To calculate feed efficiency, the equation was used: feed efficiency = ADG/DMI.

### *2.6. Laboratory Analysis*

#### 2.6.1. Chemical Composition of Diet and Feces

Samples of the feed provided and their respective leftovers were collected and subsequently dried in a forced ventilation oven for 72 h at a temperature of 55 °C, after which they were ground in a knife mill to a size of 1 mm and submitted to analyses to determine the dry matter content (DM%), crude protein (CP%), neutral detergent fiber (NDF%), acid detergent fiber (ADF%) ether extract (EE), and mineral matter (MM%) according to the methodology of literature [8–10], as described by Brunetto et al. [11].

#### 2.6.2. Hemogram

Blood with an anticoagulant was used to evaluate the total blood count. An automatic blood cell counter (EQUIP VET 3000®, Equip, Itatiba, SP, Brazil) quantified the number of leukocytes, platelets, and erythrocytes, hemoglobin concentrations, hematocrit percentage, and the leukocyte differential. The results were extracted from the equipment and then tabulated for analysis.

#### 2.6.3. Metabolic Biochemistry

Serum levels of total proteins, albumin, globulins, glucose, amylase, gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST), cholesterol, triglycerides, fructosamine, urea, calcium, phosphorus, magnesium, and iron were measured using commercial kits (Analisa®, Gold Analisa Diagnóstica Ltd.a, Belo Horizonte, MG, Brazil) following the manufacturer's recommendations, and using automatic equipment (Zybio EXC 200®, Equip, Itatiba, SP, Brazil).

#### 2.6.4. pH and Volatile Fatty Acids in Ruminal Fluid

The ruminal fluid underwent a slow thawing process at 5 °C, and then the sample was homogenized manually. A volume of 1 mL of the supernatant from the ruminal fluid samples was placed in polypropylene microtubes and then centrifuged for 5 min (12,300× *g*). An aliquot of 250 µL of the supernatant was transferred to a new microtube containing formic acid (250 µL), following the same protocol recently published [11]. Fatty acid quantification was performed using a gas chromatograph equipped with a flame

ionization detector (GC-FID; Varian Star 3400, Palo Alto, CA, USA) and an autosampler (Varian 8200CX, Palo Alto, CA, USA) in split mode (1:10) up to 250 °C. Acetic, propionic, butyric, valeric, and isovaleric acids were separated by a CP-Wax 52CB capillary column (50 m × 0.32 mm; stationary phase thickness of 0.20 µm), according to standard methodology [11]. Method validation was performed, and the standardization results were presented in Table S1. The results were expressed as 100 mol/L of each short-chain fatty acid (SCFA) in the ruminal fluid.

#### 2.6.5. Digestibility Coefficient

For the analysis of apparent digestibility, individual fecal samples were collected once a day, for a period of three consecutive days, at different times each day (08:00 h, 13:00 h, and 18:00 h). To measure daily fecal excretion, the internal indicator indigestible neutral detergent fiber (NDF) was used, estimated from the feed provided, daily leftovers, and feces, using the in situ digestibility technique [12], adapted for an incubation period of 240 h [13]. Then, the percentage of DM and NDF was measured [9]. The final residue of the NDF analysis was incubated in fistulated animals. With these data, fecal excretion was estimated by the difference between the ingested NDF and the iNDF excreted in feces (kg DM/day). Subsequently, the apparent digestibility coefficient (ADC) of DM was determined by the difference between the amount consumed and that obtained in feces; the same methodology was used to determine the ADC of other nutrients.

### *2.7. Meat Analysis*

We used a portion of the collected sample to analyze the meat's composition. Meat was processed, and the subcutaneous fat and connective tissue fragments were removed. The sample was then placed in a device, near-infrared reflection spectrometer, model Spectra Star 2600 XT series of near-infrared analyzers (Unity Scientific®, Jakarta, Indonesia), where data on the percentage of DM, fat, and protein were obtained.

We used the Bligh and Dyer method [10] for lipid extraction, with some modifications previously published by Brunetto et al. [11]; then, fatty acid methylation was performed by the transesterification method [14]. The extracted lipids were mixed with 1 mL of 0.4 M KOH methanolic solution, and the test tubes were vortexed for 1 min. Then, the sample aliquots were kept in a water bath for 10 min at boiling point, followed by cooling to room temperature, following a methodology previously published by the research group [11]. Using a TRACE 1310 gas chromatograph with a flame ionization detector, fatty acid methyl esters (FAME) were measured. An aliquot of 1 µL of the prepared sample was injected into a splitless injector operated in 1:10 split mode at 250 °C, with hydrogen used as carrier gas [11]. The separation of FAMEs was performed with an RT 2560 chromatographic column (film thickness of 100 m × 0.25 mm × 0.20 µm, Restek, Bellefonte, PA, USA), following standard analysis methodology [11]. The FAMEs were identified based on the experimental retention time, when compared to the authentic standard (FAME Mix-37, Sigma Aldrich, St. Louis, MO, USA). Results are shown as a percentage of each FA identified in the lipid fraction, according to Visentainer and Franco [15].

### *2.8. Cost Analysis*

The cost analysis was calculated using dry matter intake (DMI) data [16]. Knowing the cost/kg of DM of the diet consumed, it was possible to calculate the total feeding costs during the experimental period, adding a percentage of 20% to this value, such as extra expenses related to facilities, electricity, medicines, and labor. With the commercialization of the carcasses, a value of US$ 3.13 per kg was collected; thus, we discounted the total production cost, obtaining the profit (US$/animal).

### *2.9. Statistical Analysis*

All data were analyzed using the "MIXED procedure" of SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4), with Satterthwaite approximation to determine degrees of freedom for the fixed effects test. Growth performance (except for BW), meat, and production costs data were tested for treatment fixed effects using animal (treatment) as a random effect. BW, blood count, biochemistry, and serum ruminal biomarker data were analyzed as repeated measures and tested for treatment, day, and treatment × day fixed effects using animal (treatment) as a random effect. The d1 results were included as an independent covariate because they reflect different results prior to the start of the experiment, without any influence from DDGS, indicating an individual effect of each animal. Day 1 results were removed from the data set to generate the treatment mean but were retained as a covariate. In order to construct the first-order autoregressive covariance structure, we used the Akaike information criterion. The means were separated using the PDIFF method (*t*-test), and all results were reported as LSMEANS followed by SEM. Significance was defined when $p \leq 0.05$, and trend when $p > 0.05$ and $\leq 0.10$.

## 3. Results

### *3.1. Performance and Clinical Changes*

During the experimental period, there were a few days in which some animals (between 4 to 7 days) presented leftover feed, the vast majority without any other clinical changes. However, during the study, five cattle reduced their consumption and presented altered fecal consistency (diarrhea) (two in the DDGS group and three in the soybean meal group); it was necessary to intervene with oral bicarbonate and injectable dipyrone for 3 days at 24-h intervals. Within 48 h after the drug intervention, the animals resumed consuming all the feed provided.

Table 2 presents the results for body weight, daily weight gain, daily feed intake, and feed efficiency; all of these variables had no effect of treatment and/or treatment × day interaction ($p > 0.05$). Only a day effect was observed for body weight in both groups, which is expected in animals in the growth and fattening phase.

**Table 2.** Body weight, weight gain, daily weight gain, feed intake, and feed efficiency of cattle fed DDGS replacing soybean meal under confinement conditions.

| Variables | DDGS | Soybean Meal | SEM | P-Treat [1] | P-Day [1] | P-Treat × Day [1] |
|---|---|---|---|---|---|---|
| Body Weight. kg | | | | 0.92 | 0.01 | 0.88 |
| d1 | 345.9 | 345.3 | 3.84 | | | |
| d21 | 374.3 | 371.3 | 3.56 | | | |
| d90 | 458.9 | 460.9 | 3.51 | | | |
| Average daily gain (ADG). kg/day | | | | | | |
| d1-90 | 1.25 | 1.28 | 0.05 | 0.92 | - | - |
| d21-90 | 1.22 | 1.29 | 0.04 | 0.86 | - | - |
| Dry matter intake (DMI). kg/day | | | | | | |
| d1-90 | 8.77 | 8.80 | 0.02 | 0.98 | - | - |
| d21-90 | 8.83 | 8.87 | 0.03 | 0.97 | - | - |
| Feed efficiency [2]. kg/kg | | | | | | |
| d1-90 | 0.142 | 0.145 | 0.01 | 0.97 | - | - |
| d21-90 | 0.138 | 0.145 | 0.02 | 0.81 | - | - |

[1]: no statistical difference between groups and interaction treatment vs. day ($p > 0.05$). [2]: Feed efficiency = ADG/DMI.

### 3.2. Hematology and Serum Biochemistry

Hematology and biochemistry results are presented in Table 3. There was no treatment effect or treatment × day interaction for all hematologic variables (erythrocytes, hematocrit, hemoglobin, platelets, total leukocytes, granulocytes, lymphocytes, and monocytes). Among the biochemical variables, there was only a treatment effect for cholesterol levels, which was higher in the cattle serum that consumed DDGS ($p = 0.03$). There was no treatment effect or treatment × day interaction for albumin, total protein, globulin, triglycerides, fructosamine, urea, calcium, phosphorus, magnesium, and iron levels, as well as amylase, GGT, and AST activity ($p > 0.05$).

**Table 3.** Serum hematological and biochemical biomarkers (days 1, 21, 55, and 90) of cattle fed DDGS as a substitute for soybean meal under confinement conditions.

| Variables | DDGS | Soybean Meal | SEM | P-Treat | P-Treat × Day |
|---|---|---|---|---|---|
| Hemogram | | | | | |
| Total leukocyte ($\times 10^3/\mu L$) | 6.90 | 7.71 | 0.34 | 0.51 | 0.28 |
| Lymphocyte ($\times 10^3/\mu L$) | 4.06 | 4.62 | 0.23 | 0.64 | 0.37 |
| Granulocytes ($\times 10^3/\mu L$) | 1.79 | 1.96 | 0.12 | 0.82 | 0.70 |
| Monocyte ($\times 10^3/\mu L$) | 1.05 | 1.12 | 0.06 | 0.75 | 0.86 |
| Erythrocytes ($\times 10^6/\mu L$) | 7.60 | 7.33 | 0.07 | 0.92 | 0.88 |
| Hemoglobin (mg/dL) | 12.1 | 11.9 | 0.13 | 0.94 | 0.93 |
| Hematocrit (%) | 32.9 | 32.7 | 0.26 | 0.91 | 0.96 |
| Platelets ($\times 10^3$ µL) | 294 | 216 | 22.6 | 0.11 | 0.16 |
| Serum biochemistry | | | | | |
| Albumin (g/dL) | 3.01 | 3.05 | 0.02 | 0.96 | 0.98 |
| Total protein (g/dL) | 7.41 | 7.51 | 0.06 | 0.89 | 0.91 |
| Globulin (g/dL) | 4.40 | 4.46 | 0.07 | 0.93 | 0.96 |
| Amylase (U/L) | 124 | 122 | 6.42 | 0.88 | 0.91 |
| GGT (U/L) | 28.3 | 29.0 | 0.78 | 0.94 | 0.87 |
| AST (U/L) | 123 | 121 | 4.57 | 0.92 | 0.83 |
| Cholesterol (mg/dL) | 93.0 [a] | 84.7 [b] | 2.37 | 0.03 | 0.18 |
| Triglycerides (mg/dL) | 14.8 | 15.5 | 0.37 | 0.84 | 0.89 |
| Fructosamine (mg/dL) | 2.80 | 2.77 | 0.02 | 0.95 | 0.97 |
| Urea (mg/dL) | 16.9 | 20.4 | 1.02 | 0.22 | 0.11 |
| Calcium (mcg/dL) | 8.87 | 8.83 | 0.06 | 0.96 | 0.97 |
| Phosphorus (mcg/dL) | 9.69 | 9.98 | 0.11 | 0.95 | 0.92 |
| Magnesium (mcg/dL) | 2.64 | 2.82 | 0.05 | 0.76 | 0.65 |
| Iron (mcg/dL) | 207 | 215 | 4.64 | 0.81 | 0.55 |

Note: When $p \leq 0.05$, there was a treatment effect, illustrated by different letters on the same line; there was no treatment × day interaction for serum biomarkers of biochemistry and hematology.

### 3.3. Short-Chain Fatty Acids in Ruminal Fluid

Table 4 presents the results of pH and SCFA in the ruminal fluid. There was no effect of treatment or interaction for pH ($p > 0.05$). There was a treatment × day interaction for levels of total SCFA and butyric acids, which were higher in cattle that consumed DDGS than the other group on day 55. Treatment effect and treatment × day interaction for levels of acetic acid in the rumen, being higher on both collection days (d55 and d90) in cattle that consumed DDGS. No treatment effect or interaction existed for propionic, isovaleric, and valeric acid levels.

**Table 4.** Fatty acid profile in ruminal fluid of cattle fed DDGS as a replacement for soybean meal under confinement conditions.

| Variables | DDGS | Soybean Meal | SEM | P-Treat | P-Treat × Day |
|---|---|---|---|---|---|
| pH | 6.68 | 6.64 | 0.04 | 0.92 | 0.73 |
| Total SCFA (mmol $L^{-1}$) | | | | 0.26 | 0.01 |
| d55 | 81.3 [a] | 67.4 [b] | 1.52 | | |
| d90 | 71.6 | 69.5 | 1.32 | | |
| Acetic acid (mmol $L^{-1}$) | | | | 0.05 | 0.01 |
| d55 | 58.7 [a] | 48.0 [b] | 1.24 | | |
| d90 | 46.5 [a] | 42.6 [b] | 1.03 | | |
| Butyric acid (mmol $L^{-1}$) | | | | 0.24 | 0.01 |
| d55 | 9.25 [a] | 6.39 [b] | 0.85 | | |
| d90 | 8.04 | 9.41 | 0.97 | | |
| Propionic acid (mmol $L^{-1}$) | 12.2 | 12.2 | 0.51 | 0.92 | 0.95 |
| Isovaleric acid (mmol $L^{-1}$) | 1.84 | 1.76 | 0.22 | 0.75 | 0.84 |
| Valeric acid (mmol $L^{-1}$) | 1.14 | 1.19 | 0.08 | 0.96 | 0.92 |

Note: When $p \leq 0.05$, there was a treatment effect or treatment × day interaction, illustrated by different letters on the same line.

### *3.4. Digestibility*

Table 5 presents the apparent digestibility coefficient (ADC) of nutrients. Cattle that consumed DDGS had lower ADCs of NDF and ADF than those fed soybean meal; conversely, the ADC of ether extract was higher in cattle fed DDGS than in the other group. No effect of treatment was observed for the ADC of DM, organic matter, and crude protein.

**Table 5.** Apparent digestibility coefficient (ADC) in confined cattle fed with DDGS as a replacement for soybean meal.

| Variable | DDGS | Soybean Meal | SEM | *p*-Value |
|---|---|---|---|---|
| ADC—Dry material | 0.68 | 0.70 | 0.01 | 0.62 |
| ADC—Organic material | 0.71 | 0.73 | 0.01 | 0.56 |
| ADC—Crude protein | 0.64 | 0.71 | 0.03 | 0.25 |
| ADC—Neutral detergent fiber | 0.38 [b] | 0.50 [a] | 0.02 | 0.03 |
| ADC—Acid detergent fiber | 0.44 [b] | 0.52 [a] | 0.02 | 0.05 |
| ADC—Ether extract | 0.70 [a] | 0.63 [b] | 0.01 | 0.02 |

Note: When $p \leq 0.05$, there was a treatment effect or treatment × day interaction, illustrated by different letters on the same line.

### *3.5. Total Lipids and Fatty Acid Profile in Meat*

Table 6 presents the meat results. A higher percentage of fat was observed in the meat of cattle that consumed DDGS. In the meat of cattle that consumed DDGS, a lower rate of myristoleic, palmitic, stearic, and linolenic acids was observed compared to animals that consumed soybean meal, as well as a higher percentage of oleic acid in the DDGS group. The sum of SFA was lower in animals that consumed DDGS than in the other group, and these animals had a higher sum of UFA in the meat. PUFA was higher in the DDGS group meat than in the control (soybean meal). A higher UFA/SFA ratio was also observed in cattle that consumed DDGS than in those that consumed soybean meal.

**Table 6.** Total lipids and fatty acid profile in meat from cattle fed DDGS as a replacement for soybean meal under confinement conditions.

| Variables | DDGS | Soybean Meal | SEM | *p*-Value |
|---|---|---|---|---|
| Total lipids % | 2.44 | 2.08 | 0.07 | 0.03 |
| C8:0 (Caprylic) % | 0.01 | 0.02 | 0.01 | 0.95 |
| C12:0 (Lauric) % | 0.04 | 0.06 | 0.02 | 0.51 |
| C14:0 (Myristic) % | 1.55 | 1.65 | 0.31 | 0.34 |
| C14:1 (Myristoleic) % | 0.32 | 0.53 | 0.31 | 0.05 |
| C15:0 (Pentadecanoic) % | 0.23 | 0.35 | 0.22 | 0.41 |
| C16:0 (Palmitic) % | 25.06 | 26.85 | 0.34 | 0.05 |
| C16:1 (Palmitoleic) % | 3.16 | 3.14 | 0.58 | 0.95 |
| C17:0 (Heptadecanoic) % | 0.38 | 0.42 | 0.07 | 0.86 |
| C18:0 (Stearic) % | 12.97 | 14.09 | 0.27 | 0.02 |
| C18:1n9t (Elaidic) % | 1.63 | 1.65 | 0.12 | 0.96 |
| C18:1n9c (Oleic) % | 36.58 | 34.97 | 0.42 | 0.05 |
| C18:2n6c (Linoleic) % | 12.61 | 11.09 | 0.19 | 0.11 |
| C20:0 (Arachidic) % | 0.10 | 0.09 | 0.01 | 0.95 |
| C18:3n6 (γ-Linolenic) % | 0.07 | 0.07 | 0.01 | 0.98 |
| C20:1n9 (cis-11-Eicosenoic) % | 0.16 | 0.18 | 0.02 | 0.95 |
| C18:3n3 (Linolenic) % | 0.24 | 0.34 | 0.03 | 0.02 |
| C21:0 (Henicosanoic) % | 0.26 | 0.26 | 0.08 | 0.92 |
| C20:2 (cis-11.14-Eicosadienoic) % | 0.14 | 0.14 | 0.03 | 0.95 |
| C22:0 (Behenic) % | 0.04 | 0.04 | 0.02 | 0.98 |
| C20:3n6 (cis-8.11.14-Eicosatrienoic) % | 0.70 | 0.64 | 0.03 | 0.78 |
| C20:4n6 (Arachidonic) % | 3.52 | 3.17 | 0.15 | 0.91 |
| C22:2 (cis-13.16-Docosadienoic) % | 0.02 | 0.03 | 0.70 | 0.98 |
| C24:0 (Lignoceric) % | 0.05 | 0.04 | 0.01 | 0.97 |
| C20:5n3 (cis-5.8.11.14.17-Eicosapentaenoic) % | 0.11 | 0.12 | 0.01 | 0.98 |
| C24:1n9 (Nervonic) % | 0.02 | 0.02 | 0.03 | 0.99 |
| C22:6n3 (cis-4.7.10.13.16.19-Docosahexaenoic) % | 0.04 | 0.04 | 0.00 | 0.99 |
| Other variables | | | | |
| ∑ Saturated fatty acids (SFA) % | 40.69 | 43.87 | 0.71 | 0.05 |
| ∑ Unsaturated fatty acids (UFA) % | 59.31 | 56.13 | 0.65 | 0.02 |
| ∑ Monounsaturated fatty acids (MUFA) % | 41.87 | 40.49 | 0.48 | 0.25 |
| ∑ Polyunsaturated fatty acids (PUFA) % | 17.44 | 15.64 | 0.32 | 0.01 |
| UFA/SFA | 1.46 | 1.28 | 0.06 | 0.01 |

### *3.6. Economic Viability*

Table 7 presents the economic viability results. The addition of DDGS reduced the cost of the diet by 10.5%. Considering the intake, the cost of producing each steer fed with DDGS was 7.67% lower than that of soybean meal. This situation resulted in a profit of 110% when DDGS were used in the cattle diet instead of soybean meal.

**Table 7.** Production costs, revenues, and profits from the confinement of cattle fed DDGS as a replacement for soybean meal.

| Variables | DDGS | Soybean Meal | SEM | *p*-Value | Difference (%) [2] |
|---|---|---|---|---|---|
| Diet costs (US$ animal/day) | 2.37 | 2.65 | - | - | 10.57 ↓ |
| Total production cost considering consumption (US$/animal) [1] | 680 | 698 | 3.78 | 0.31 | 7.67 ↓ |

**Table 7.** *Cont.*

| Variables | DDGS | Soybean Meal | SEM | *p*-Value | Difference (%) [2] |
|---|---|---|---|---|---|
| Carcass weight, kg | 228.1 | 228.1 | 6.32 | 0.97 | 0.02 ↑ |
| Amount paid per carcass (US$ 3.13 per kg) | 714 | 714 | 3.27 | 0.92 | 0.02 ↑ |
| Profit (US$/animal) | 34.44 | 16.34 | 2.52 | 0.02 | 110.7 ↑ |

[1]: value refers to the costs of cattle (US$ 6.50 kg BW), feed and 20% added to feed for other expenses. According to the literature [16], these expenses would be related to electricity, installation wear and tear, medications/vaccines and daily labor. [2]: The difference between the groups for each of the variables was presented as a percentage, with the DDGS group always being compared to the control (soybean meal).

## 4. Discussion

The use of unconventional feeds or coproducts in animal feed, such as DDGS, has gained prominence due to its nutritional composition, which includes good protein and energy levels, high fiber percentage, and lower cost, encouraging its use. Including DDGS in the diet of confined cattle has proven to be an effective alternative for maintaining animal performance when used as a substitute for soybean meal in the diet, focusing on protein replacement. As shown in Table 2, the replacement did not change the parameters of weight gain, DM intake, and feed efficiency in isoenergetic and isoprotein diets. This high replacement power is due to the high protein content present in DDGS (above 30%), its relevant fiber fraction (approximately 33%), and the considerable amount of lipids originating from soluble fats [17], essential components for animals in growth and finishing conditions. However, it is important to highlight that the composition of DDGS may vary depending on the raw material used in its production, impacting its effects on animal metabolism and formulation [18], which may result in non-compliance with nutritional requirements according to the animal category.

In addition to the crude protein content, the fiber percentage is the main difference between soybean meal and DDGS, as soybean meal has lower levels of NDF compared to DDGS [19]. This difference can influence the precision profile in the rumen, promoting more stable productivity and a lower risk of metabolic problems in animals fed DDGS. As shown, the use of DDGS was proven in changes in the profile of fatty acids produced during ruminal fermentation, with an increase in the concentrations of short-chain fatty acids (SCFA). According to Shen et al. [19], changes in the ruminal microbiota, such as changes in the population of protozoa and bacteria, can alter the concentrations of SCFA caused by different feed compositions when this substitution is performed. This increase was attributed to the higher concentrations of acetic and butyric acid in the ruminal fluid of animals that consumed DDGS. The higher amount of fiber in DDGS stimulates the patent of bacteria specialized in fiber fermentation [20], resulting in a more significant production of acetate, a two-carbon SCFA, and an essential energy source for ruminants associated with the formation of fatty acids. Knowing the ruminal microbiota may be a possibility to understand the mechanism involved in the increase in volatile fatty acids, such as acetic and propionic.

Regarding the digestibility coefficient of the fibrous portion, a reduction was observed in the group fed DDGS. This is due, in part, to the lower availability of protein at the ruminal level for the proliferation of microorganisms and aid in the digestion and fermentation of this fiber when compared to soybean meal. The reduced digestibility of fiber is related to the fact that this process occurs predominantly in the rumen, where the interaction between microorganisms, nutrients, especially protein, and environmental conditions determines the efficiency of fibrous fermentation. DDGS has a high concentration of UFA in the ether extract (EE), which can be associated with increased serum cholesterol levels in animals. This effect is explained by the more significant digestibility of EE in animals that received

DDGS, indicating more substantial absorption and availability of these compounds for the tissues. This more considerable digestibility contributes to a higher percentage of intramuscular fat, increasing the fat content in the meat of animals treated with DDGS [21].

The inclusion of DDGS also influences the fatty acid profile of meat. Due to the high concentration of UFA in the coproduct, including oleic acid and linoleic acid, there is a modulation in the processes of lipolysis, biohydrogenation, and ruminal synthesis [22], resulting in a greater flow of unsaturated compounds to the intestine, where they are absorbed. This process alters the lipid profile of meat, favoring a composition with a higher proportion of UFA, such as oleic acid, which can bring nutritional and sensory benefits to the final product. This interference in the biohydrogenation process causes the conversion of PUFA into saturated fatty acids (SFA) to be reduced, resulting in a lower concentration of palmitic (C16:0) and stearic (C18:0) acid and causing changes in the ratio of unsaturated/saturated fatty acids [22]. Dietary sulfur levels can promote changes in the fatty acid profile of meat from animals receiving DDGS, as this mineral is present in good quantities in this residue. This can promote changes in the populations of microorganisms present in the ruminal environment and alter fatty acid biohydrogenation processes [23].

Regardless of the protein source used in the diet, the blood count, serum biochemistry, and metabolic parameters were similar, except for total cholesterol levels, which were higher in cattle that consumed DDGS. This effect on serum lipids is probably related to the more substantial digestibility of the EE, which allowed greater availability of fat to be absorbed by the intestine, resulting in this higher cholesterol concentration. Still, it is worth noting that the values were within the normal range for adult cattle in both groups [24].

The cost of producing confined cattle is high in Brazil, mainly due to the cost of concentrate, which has ingredients that suffer from price fluctuations throughout the year due to the effects of the harvest (law of supply and demand) but are also impacted by weather conditions, which have resulted in extreme conditions in recent years, from a brief drought to severe dry seasons or floods. This situation has significantly influenced the use of industry coproducts in animal feed, emphasizing DDG and DDGS. The diet that replaced soybean meal with DGGS had a lower daily animal cost and, as it did not affect performance, resulted in a higher profit per animal. It is essential to clarify that the cost of DDGS used in this experiment was high due to the costs of the product and the freight of over 900 km. When we consider the cost of this coproduct in regions where it is available in the industry, the value of DDGS can be up to 30% lower during the harvest period compared to the cost in our research.

The diet was formulated for the cattle to gain 1.5 kg per day, but this did not happen, with the maximum average daily gain in this experiment being 1.28 kg and 1.25 kg (soybean meal and DDGS, respectively). This is due to the diet used in the study, composed of 80% concentrate and 20% corn silage, which presents positive results for beef cattle. However, when used in cattle of breeds suitable for milk production, such as the Holstein, the animals did not respond in weight gain due to a condition of subclinical and/or clinical acidosis (three animals: two in the DDGS group and one in the soybean meal group), similar to the ADG of 1.32 kg detected in a diet with a greater inclusion of concentrate (70%) and (30%) silage [25]. However, it is much lower than what occurred in colder diets, that is, with a lower proportion of concentrate or fast-fermenting carbohydrates in the diet, with (55%) concentrate and (45%) silage, where the ADG was 1.62 kg [26]. This is the explanation for the low profitability of the confinement system here; the animals did not gain the projected body weight, reducing feed efficiency. In addition to these studies by our research group, others not yet published show that Holstein cattle respond positively to ADG formulated in cold diets, with a maximum of 65% concentrate.

According to the literature, there are benefits to cattle performance when DDGS is used in the diet; however, it can result in operational difficulties [27]. As practical implications of these results with DDGS, we highlight the lower production cost, which allows for greater profit; in addition, this study shows that DDGS can be used as a substitute for a higher-cost protein source, providing an alternative byproduct as feed for confinement. Having a sustainable destination for the residue is valuable, especially because the number of ethanol-producing plants is growing in Brazil, which prevents the residue from being disposed of inappropriately, leading to environmental contamination.

## 5. Conclusions

Replacing soybean meal with DDGS in the diet of Holstein cattle in confinement is an advantageous option because the animals maintain similar weight gain and feed efficiency between treatments without causing any harm to animal health and metabolism. In addition, the use of DDGS was able to improve the digestibility of EE, increase serum cholesterol levels, and modulate the fatty acid profile in the meat, with emphasis on a lower amount of SFA and a higher amount of UFA, resulting in meat that is more desirable to the consumer from a health point of view. Furthermore, the diet with DDGS has a lower cost, and consequently results in greater profitability in the production system, providing economic and environmental sustainability.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11070363/s1, Table S1. Standardization of the quantification analysis of short-chain fatty acids in the ruminal fluid of cattle.

**Author Contributions:** A.L.R.B. and A.S.d.S. contributed to the research design and implementation to analyze the results. R.W. and G.V.K. helped elaborate on the project's execution and financing. A.L.R.B., G.L.D., A.L.d.F.d.S., L.N., M.G.d.V., R.S.d.J. and A.S.d.S. participated in the execution of the experiment and collection of samples and data. B.K., and L.E.L.e.S. did the laboratory analysis. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** Data remained with the authors but may be made available upon request.

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**Conflicts of Interest:** The authors declare no conflicts of interest.

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REGULAR ARTICLES

# Inclusion of yeast and saccharides based-product to replace monensin in the diet of confined steers: performance, rumen environment, metabolism, animal health, and meat quality

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## Abstract

The present study aimed to evaluate a blend of yeast and saccharides based-product fed via diet to feedlot cattle on performance, health, meat quality, and rumen metabolism when compared to monensin. Twenty-four non-castrated crossbred male steers (Charolais x Nellore) were divided into control ($n=12$), receiving the basal diet with monensin (215 mg/animal/day) and treatment ($n=12$) receiving the basal diet with the blend of yeast and saccharides based-product (17.2 g/animal/day). Animals were weighed on days 1, 20, and 113, and on days 20, 70, and 113, blood and rumen fluid were collected, which was also used to determine the genera of bacteria present in the rumen and to quantify protozoa. Total feces were collected to determine apparent total tract digestibility from days 108 to 112 of the experiment. There was no treatment effect for weight gain, feed intake, or feed efficiency. There was a gain in the loin eye area in the control group ($P=0.05$) and greater subcutaneous fat thickness in the loin and rump cap control group ($P=0.01$). In the ruminal fluid, there was higher protozoa count in the treatment group on day 113 ($P=0.03$) and a higher proportion of propionic acid in the treatment group on day 70 ($P=0.03$). Total short-chain fatty acids were lower in steers in the treatment group. There was no treatment effect on the ruminal microbiota. There were lower counts of lymphocytes and granulocytes in the blood of steer in the treatment group ($P<0,05$). A significant increase in the concentrations of immunoglobin A (IgA) and others heavy-chain immunoglobulins (IgM, IgG, IgM and IgE) were observed in the treatment group. There was an effect of treatment on glutathione S-transferase (GST) activity on days 70 and 113 ($P=0.02$). Likewise, high GST activity in the liver was identified in the treatment group ($P=0.01$). For fatty acid profile in meat, oleic acid presented higher proportion in the treatment group ($P=0.04$), in contrast to docis-5,8,11,14,17-eicosapentaenoic acid, which was lower in the same group ($P=0.05$). It is concluded that the blend of yeast and saccharides based-product played a role similar to monensin's in productive performance, demonstrating a potential to substitute monensin. Furthermore, the inclusion of yeast and saccharides in the diet stimulated the humoral immune response and antioxidant action without affecting meat quality.

**Keywords** Beef cattle · Oligosaccharides · Prebiotics · Monensin · Meat quality · Growth performance

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## Introduction

Monensin is a widely used feed additive in feedlot in beef cattle diets to improve feed efficiency (Duffield et al. 2012), as it can reduce dry matter intake (DMI) by up to 3% (NASEM 2016) without affecting weight gain. Monensin is an ionophore commonly used in beef cattle as a performance improver, modulating the ruminal microbiota and decreasing feed intake without affecting weight gain (McCann et al. 2017; Yang et al. 2023); it improves energy efficiency by increasing propionate production and reducing methane emissions (Neumann et al. 2018). However, monensin has been banned as an animal growth promoter in some countries, including the European Union, since 2006 to prevent antimicrobial resistance (Manero et al. 2006). Given this scenario, there is a search for new feed additives that stimulate animal growth to reduce or eliminate risks to human health. As a result, the space in the market for alternative feed additives to chemicals has increased, aiming to maintain or enhance the efficiency of animals when replacing monensin (Russell and Houlihan, 2033).

Due to the demand for meat and the demands on its production, new alternatives are sought to maintain or improve the feed efficiency of their herds in the absence of conventional chemical improvers when using feeds with a high grain content (Nagaraja and Lechtenberg 2007; de Souza et al. 2018). Yeast and saccharides favor microbial growth, improving digestion of nutrients and production of short chain fatty acid (SCFA). (Pimentel et al. 2022). Yeast in prebiotic form in the diet of weaned calves improved growth performance, ruminal fermentation, immune response, and nutrient digestibility (Jain et al. 2011). Yeast and saccharides can be an option to improve animal performance. Recently, Cagliari et al. (2023) in a in vitro study using the same product used in this experiment to observe the improvement in feed digestibility and a reduction in methane production, showing a potential to be a substitute for monensin.

Prebiotics, such as yeast and saccharides are feed substances that modulate metabolic processes, favoring the growth of beneficial bacteria. These bacteria can generate metabolites, such as branched-chain and SCFA, which benefit the host animal (Markowiak and Ślizewska 2018). Oligosaccharides are also classified as prebiotics, and in ruminant nutrition they generally modulate rumen microbiota and animal health (Moallem et al. 2009; Gaggìa et al. 2010; Ferraretto et al. 2012; Uyeno et al. 2015). Oligosaccharides, including fructooligosaccharides (FOS), mannan-oligosaccharides (MOS), galactooligosaccharides (GOS), and non-starch polysaccharides, constitute the main prebiotics used (Jahan et al. 2022). However, the most widely available are MOS and FOS (Ghosh and Mehla 2012). Mannan-oligosaccharides are considered an effective alternative to antibiotics used as growth promoters. These non-digestible carbohydrates are extracted from the yeast wall of *Saccharomyces cerevisiae*. Even though it is an oligosaccharide, its mode of action differs from others, acting through competitive bonds with gram-positive bacteria (Halas and Nochta 2012; Singh et al. 2017). On the other hand, FOS are synthesized by microorganisms in internal cells, participating in the biotransformation of sucrose, which requires strains capable of tolerating high osmotic pressure (Mano et al. 2018). Like MOS, these carbohydrate components of cellular arrest are extracted from the yeast *S. cerevisiae*. They can positively influence leukocyte phagocytic, cytotoxic, and antimicrobial activities (Singh et al. 2017). The study by Chang et al. (2022) reveals that administering GOS to suckling calves resulted in superior feed efficiency in the short term, with rumen parameters indicating higher concentrations of propionate and SCFA. Therefore, the present study evaluated whether blend of yeast and saccharides based-product supplied via diet to cattle in feedlot would improve animal performance, health, carcass conformation, and meat quality in cattle that consumed monensin as a performance enhancer.

## Materials and methods

### Test product

The test product is a commercial prebiotic (YES GOLF®; Campinas, SP, Brazil), composed of *Saccharomyces cerevisiae* fragment (min. 388 g/kg), and beta-glucans (min. 150 g/kg), fructo-oligosaccharides (min. 120 g/kg), galactooligosaccharides (min. 72 g/kg), glucomannan (min. 210 g/kg) and mannan oligosaccharides (min. 60 g/kg).

### Animals and facilities

The experiment was performed at the Experimental Farm of the Centro de Educação Superior do Oeste of the State University of Santa Catarina (FECEO, Guatambu—SC, Brazil). We used 24 crossbred (Charolais x Nelore) steers with an average weight of $270 \pm 11.2$ kg and an age of $9 \pm 0.75$ months.

The animals were housed in a feedlot with individual shelters of 9 $m^2$ (concrete floor), equipped with automatic drinkers and feeders. The feeding area has a roof where the animals can shelter from the weather.

## Experimental design and diet

A completely randomized design with two treatments and 12 replications was used. The animals were divided into control ($n = 12$), receiving a basal diet with monensin (215 mg/animal/day), and treatment ($n = 12$), receiving the basal diet with the blend of yeast and saccharides (17.2 g/animal/day). The dose was defined in a previous in vitro study (Cagliari et al. 2023).

The experimental period lasted 113 days, with 20 days of adapting to the diet and 93 days of consuming the final diet. The animals received individualized feed, adjusted according to body weight (BW), with constant measurement of the surplus, varying from 3 to 5% of the amount offered to the animal. This optimized dry matter intake (DMI) to achieve the desired performance. The diet was formulated using the BR-CORTE software, the diet comprised a concentrate with a 70:30 ratio between concentrate and roughage, calculated based on body weight and expected average daily gain (ADG) of 1.5 kg (2016). The diet is described in Table 1, and it was provided daily and divided into two equal meals (8 AM and 4 PM). Before supplying the first meal in the morning, the leftovers from each feeder were weighed to calculate the individual DMI.

**Table 1** Composition of the ingredients and total diet for cattle

| Variables | Concentrate (g/kg of DM) | Corn silage (g/kg of DM) |
|---|---|---|
| Dry matter | 892 | 368 |
| Crude protein | 197 | 63.8 |
| Ash | 67.4 | 254 |
| Ethereal extract | 36.7 | 15.8 |
| NDF | 375 | 487 |
| ADF | 167 | 265 |
| | TMR Control (g/kg of DM) | TMR treatment (g/kg of DM) |
| Dry matter | 583 | 585 |
| Crude protein | 171 | 170 |
| Ash | 64.0 | 66.4 |
| Ether extract | 32.8 | 32.8 |
| NDF | 360 | 359 |
| ADF | 196 | 195 |

The concentrate was formulated based on corn (30%), soy bran (12.9%), soy hulls (32.9%), wheat bran (18.6%), urea (1.43%), and core (4.3%). NDF: neutral detergent fiber. ADF: acid detergent fiber

## Performance

The steers were weighed individually using a digital scale (M.A Troncos e Balanças, Uberlandia, Brazil) four times (days 1, 20, 70, and 113). On days 1, 20, and 113, fasting animals were weighed. On day 70, animals were also weighed, but after feeding, this weight information was used only to monitor the animals' weight gain and diet adjustment (data not shown). Weight gain (WG = BW final – starting BW) and ADG were calculated with the BW data. With the BW and DMI data, the feed efficiency of each experimental unit was calculated (ADG/DMI – kg/kg).

## Ultrasound

On days 20 and 113, the loin eye area (LEA/cm$^2$), subcutaneous fat thickness on the sirloin steak (SFT/mm), and subcutaneous fat thickness at the rump cap (FTRC/mm) were also measured by ultrasonography, with the Exago IMVimaging® equipment using a 17.2 cm linear probe at 3.5 MHz.

## Blood sample collection

Blood was collected through the coccygeal vein using needles and vacuolated tubes, one tube without anticoagulant and the other with anticoagulant (EDTA). Immediately after the collections, the tubes were refrigerated at approximately 10 ºC in an isothermal box until they arrived at the laboratory. The tube with EDTA was used for hematologic analyses, and the tube without anticoagulant was centrifuged (1620 g for 10 min) to obtain serum, which was transferred to microtubes, labeled, and stored at –20 °C until measurements of serum biochemistries proteinograms, and antioxidant and oxidant levels.

### Hemogram and serum biochemistries

Hematologic analyses were performed with the blood collected in tubes with anticoagulant using automatic equipment (EquipVet 3000). We recorded the total number of leukocytes and the differential (lymphocytes, granulocytes, and monocytes), total erythrocyte count, hemoglobin concentration, and hematocrit.

According to manufacturers' instructions, total proteins, glucose, albumin, cholesterol, and urea were measured using a semi-automatic analyzer (Bio-2000, BioPlus®) and Analisa® commercial kits. Globulins were calculated as total proteins – albumin.

### Oxidative status

Glutathione S-transferase activity was measured based on Habig et al. (1974) and expressed as U GST/mg protein.

Lipid peroxidation was determined as thiobarbituric acid reactive substances (TBARS) levels according to Jentzsch et al. (1996) for serum samples; the results were expressed as nM malondialdehyde (MDA)/ml. According to Ellman (1959), total thiols were measured, and the results were expressed as µmol SH/mg protein.

#### Proteinogram

For protein fractionation of blood serum, sodium sulfate–polyacrylamide gel electrophoresis was performed according to Fagliari et al. (1998) and adapted by Tomasi et al. (2018) using mini-gels (10 × 10 cm). The gels were stained with Coomassie Blue and photographed to identify and quantify protein fractions using Labimage1D software (Loccus Biotechnology). A standard containing fractions with molecular weight between 10 and 250 KD (Kaleidoscope—BIO-RAD) was used as a reference.

### Ruminal fluid analysis

On days 20, 70, and 113, ruminal fluid was collected 4 h after feeding using an esophageal probe suitable for cattle and a vacuum pump coupled to the probe to facilitate fluid extraction.

#### Bacterial activity, pH, and protozoan count

Immediately after collecting ruminal fluid, the pH was measured using a portable pH meter – Testo 205®. The bacterial activity was measured using the methylene blue reduction test (MBRT), according to Dirksen (1993). Using 10 mL of ruminal fluid, protozoa were quantified according to Dehority (1993).

#### Profile of volatile fatty acids in the rumen

Ruminal fluid samples were thawed at 5 °C and manually shaken for homogenization. Aliquots of 1 mL of the supernatant of ruminal fluid samples were collected in polypropylene microtubes (2 mL) and centrifuged for 5 min (12,300 × *g*). Then, 250 µL of the supernatant was transferred to a new microtube containing 250 µL of formic acid. The mixture was manually shaken and centrifuged for 3 min. After centrifugation, 250 µL of the supernatant of the mixture was collected in another polypropylene tube previously containing 500 µL of 3-octanol solution (665 µg $mL^{-1}$ in methanol), used as an internal standard and was homogenized and centrifuged again; 600 µL of the sample was inserted into an injection vial and injected into a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3400, Palo Alto, USA) and an autosampler (Varian 8200CX, Palo Alto, USA); 1 µL of the extract was injected in split mode at 1:10. The carrier gas used was hydrogen at a constant pressure of 20 psi. The analytes (acetic, propionic, butyric, valeric, and isovaleric acids) were separated on a CP-Wax 52CB capillary column (60 m × 0.25 mm; 0.25 µm stationary phase thickness). The initial column temperature was set at 80 °C for 1 min and increased to 120 °C at a rate of 8 °C $min^{-1}$, then to 230 °C for 20 °C $min^{-1}$, where it remained for 1 min. The injector and detector temperatures were set to 250 °C. Method validation was as follows: selectivity, linearity, linear range, repeatability, precision, limit of detection (LOD), and limit of quantification (LOQ) for acetic, propionic, butyric, and isovaleric acids. Linearity was assessed by calculating a regression equation using the least squares method. LOD and LOQ values were obtained by sequential dilutions to signal-to-noise ratios of 3:1 and 6:1, respectively. Precision was analyzed by analyzing the repeatability of six replicated samples. Accuracy was determined by recovering known amounts of standard substances added to a diluted sample (Supplementary material 1). The valeric acid was expressed as the equivalent of isovaleric acid. The results were expressed in mol 100 $mol^{-1}$ of each SCFA in the ruminal fluid.

#### Ruminal microbiota

On days 20, 70, and 113, ruminal fluid was collected and stored in 3 M™ Quick Swabs for qualitative and quantitative detection of microorganisms using metagenomics by sequencing the 16S rRNA gene, performed by the laboratory BPI—Biotechnology Research and Innovation®, Botucatu, São Paulo, Brazil. One swab of each total diet per treatment was also made to identify the bacteria in the feed consumed.

Total DNA was extracted from 200 mg (wet weight) of samples with the ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research, USA). Primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) were selected to amplify the V3–V4 region of bacterial 16S rRNA gene by the polymerase chain reaction (Hjelmsø et al. 2014).

Libraries were quantified by qualitative polymerase chain reaction using the Kapa Library Quantification Kit (Illumina, San Diego, CA, US) following the manufacturer's recommendations. Samples were normalized to a final concentration of 2 nM and sequenced with an Illumina MiSeq for 250 cycles from each end.

### Apparent total tract digestibility

During the final five days of the experiment, feces were collected and weighed and approximately 150 g were frozen for later analysis. The samples were thawed, mixed, and taken from a pool of 400 to determine dry matter, and then the chemical composition was determined. Samples of the diets

and leftovers were also pooled to determine the chemical composition of: ash, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and ether extract (EE).

The samples were pre-dried in a forced ventilation oven at 54 °C for 72 h and ground to facilitate further analyses. The samples were placed in another forced ventilation study at 105 °C for 24 h to measure and weigh the dry matter. Crude protein quantification was performed following digestion, distillation, and titration using the Kjeldahl method (AOAC 2002; Method 2001.11). To obtain the ash, the samples were placed in a muffle furnace at 600 ºC for six hours, which extracted all the organic matter from the sample, leaving only the ashes, which were weighed to obtain the percentage of ash. The quantification of NDF and ADF was performed according to Van Soest (1963), Van Soest and Wine (1967), and Silva and Queiroz (2002). According to the manufacturer's instructions, an automatic fat and lipid extractor (SER 158/6 VELP®) was used to determine the quantification of the ether extract.

The concentration of nutrients in the diet compared to what was defecated was calculated to calculate the digestibility of each nutrient of the diet (Titgemeyer 1997).

## Meat analyses

At the end of the experiment (day 114), the animals were slaughtered in a commercial cattle slaughterhouse in São Miguel, Santa Catarina, Brazil. Immediately after slaughter, the pH of the hot carcass was measured, and samples of the liver and longissimus dorsi muscle were collected. After 24 h, the pH was measured again to obtain cold carcass values. We used a portable pH meter for the analyses (Testo 205®).

### Fatty acids in meat

The extraction was performed according to Bligh and Dyer (1959) with some modifications: 1.5 g of bovine muscle samples, 0.5 mL of water, 5 mL of methanol, and 2.5 mL of chloroform were added into a 15-mL polypropylene tube, and mechanical shaking was performed for 60 min. We added 2.5 mL of chloroform and $Na_2SO_4$ 1.5% solution to promote a biphasic system. This mixture was shaken for 2 min and centrifuged for 15 min at 2000 rpm. Lipids obtained from the chloroform phase were subjected to fatty acid analysis.

The FA methylation was performed using a transesterification method proposed by Hartman and Lago (1973). We added 1 mL of 0.4 M KOH methanolic solution to the extracted lipids in a test tube and shook them in a vortex for 1 min. Samples were kept in a water bath for 10 min at the boiling point. Samples were then cooled to room temperature, and 3 mL of 1 M $H_2SO_4$ solution was added, shaken in a vortex, and maintained in a water bath for 10 min. After cooling, we added 2 mL of hexane and centrifuged at 2000 rpm for 10 min. Finally, hexane with the fatty acid methyl esters (FAME) was subjected to chromatography.

For the FAME determination, we used a gas chromatograph model TRACE 1310 equipped with a flame ionization detector (Thermo Scientific). One microliter of samples was injected in a split/splitless injector, operated in 1:20 ratio split mode at 250 °C. Hydrogen was used as carrier gas at a constant flow rate of 1.5 mL/min. FAME separation was performed using an RT 2560 (100 m × 0.25 mm × 0.20 µm of thickness film, Restek, USA) chromatography column. The initial oven temperature was set to 100 °C for 5 min and increased to 180 °C at 8 °C/minute. The temperature was increased to 210 °C at 4 °C/min, and finally to 240 °C, increasing by 20 °C/min, and maintained for 20 min at isothermal conditions. The detector temperature was kept constant at 250 °C. The FAME compounds were identified by comparison with experimental retention times of standards (FAME Mix-37, Sigma Aldrich, St. Louis, MO). The results were expressed as a percentage of each fatty acid identified in the lipid fraction, considering the chain size equivalent factor of FAME for FID and conversion factor of ester to the respective acid, according to Visentainer and Franco (2006). The dietary fatty acid profile is displayed in Supplementary Material 2.

### Oxidative stability

Liver and meat samples were thawed, and a fragment was crushed in saline solution at 1v/9v. Then, the homogenate was centrifuged (7000 *g* for 10 min), and the supernatants were collected and used for oxidative status analyses. GST activity was measured according to Habig et al. (1974). Lipid peroxidation was determined as TBARS levels according to Ohkawa et al. (1979). Total thiols were measured according to Ellman's methodology (1959).

### Statistical analyses

All data were analyzed using the 'MIXED procedure' of SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4), with the Satterthwaite approximation to determine the denominator degrees of freedom for the test of fixed effects. The BW gain, feed intake, and feed efficiency were tested for the fixed effect of treatment using animal (treatment) as a random effect. The data of BW and all blood results were analyzed as repeated measures and were tested for fixed effects of treatment, day, and treatment × day, using animal (treatment) as the random effect. The d 1 results were included as an independent covariate.

To generate the average per treatment, the d 1 results were removed from the data set but were kept as a covariate. The first-order autoregressive covariance structure was selected according to the lowest Akaike information criterion. Means were separated using the PDIFF method, and all results were reported as LSMEANS followed by the standard error of the mean. Significance was defined when $P \leq 0.05$, and tendency when $P > 0.05$ and $\leq 0.10$.

The sequence data were processed using Mothur v.1.39.5 (Schloss et al. 2009), in line with the Mothur MiSeq SOP (Kozich et al. 2013). Taxonomy was assigned by querying the representative sequence of each oligotype against the Silva database (release 132) (Quast et al. 2013). Closed-reference clustered operational taxonomic unit data were exported for analysis with Phyloseq v1.41 (McMurdie and Holmes 2013) in R 4.3.1 (R Core Team, 2023). The inverse Simpson diversity index obtained alpha diversity values for bacterial communities. The inverse Simpson calculator is preferred over other alpha diversity measures because it indicates the richness of a community with a uniform evenness with the same diversity level and thus allows biological interpretation (Morris et al. 2014). The tax glom function of Phyloseq was used to aggregate abundance data by genera to generate relative abundance plots of the ten most abundant genera across all samples, plotted by averaging abundances by study and animal. Relative abundance of well-known potentially pathogenic *Enterobacter, Enterococcus, Clostridium*, *Escherichia-Shigella, Bacteroides*, and *Klebsiella* (Songer and Anderson 2006) was calculated. All figures were generated using the ggplot2 package v3.2.1 (Wickham 2016).

## Results

### Animal performance

There was no treatment effect or treatment × day interaction for BW or DMI and no treatment effect for feed efficiency (Table 2).

Loin eye area did not differ between treatments, but there was a gain in loin eye area from day 20 to day 113 in the control group ($P = 0.25$; Table 2). Subcutaneous fat thickness on the sirloin steak and subcutaneous fat thickness at the rump cap on day 113 were higher in animals in the control group ($P = 0.01$), as was the gain in fat thickness between the period from day 20 to day 113 ($P = 0.01$).

The NDF apparent digestibility coefficient was higher in control animals ($P = 0.01$) compared to treatment (Table 2). Likewise, the ADF digestibility coefficient was higher in control animals ($P = 0.08$). The digestibility coefficient of dry matter and other nutrients (mineral matter, protein, and ether extract) did not differ between treatments (Table 2).

**Table 2** Growth performance, loin eye area (LEA), longissimus subcutaneous fat thickness (SFT) and biceps femoris fat thickness (FTRC), and nutrient digestibility of cattle consuming monensin and prebiotics

| Items | Treatments[1] | | SEM | *P* – values | |
|---|---|---|---|---|---|
| | Control | Treatment | | Treat | Treat × Day |
| Body weight (kg) | | | | 0.65 | 0.41 |
| d 1 | 273 | 281 | 4.06 | | |
| d 20 | 278 | 286 | 4.02 | | |
| d 113 | 433 | 433 | 4.01 | | |
| Weight gain (kg) | | | | | |
| d 20 to 113 | 155 | 147 | 3.70 | 0.48 | - |
| ADG (kg) | | | | | |
| d 20 to 113 | 1.66 | 1.58 | 0.03 | 0.47 | - |
| Feed intake (kg DM/day) | | | | | |
| d 20 to 113 | 8.84 | 8.55 | 0.38 | 0.94 | - |
| Feed efficiency (kg/kg) | | | | | |
| d 20 to 113 | 0.187 | 0.184 | 0.01 | 0.95 | - |
| Ultrasonography | | | | | |
| LEA ($cm^2$) | | | | - | 0.25 |
| d20 | 42.8 | 47.5 | 2.01 | | |
| d113 | 69.1 | 65.8 | 1.72 | | |
| Gain | 26.3[a] | 18.3[b] | 0.65 | **0.05** | - |
| SFT (mm) | | | | - | **0.01** |
| d20 | 0.24 | 0.26 | 0.14 | | |
| d113 | 3.15[a] | 2.05[b] | 0.06 | | |
| Gain | 2.91 | 1.79 | 0.05 | **0.01** | - |
| FTRC (mm) | | | | - | **0.01** |
| d20 | 0.23 | 0.24 | 0.10 | | |
| d113 | 4.18[a] | 3.13[b] | 0.04 | | |
| Gain | 3.95[a] | 2.89[b] | 0.04 | **0.01** | |
| Nutrient digestibility | | | | | |
| Dry matter | 0.68 | 0.67 | 0.01 | 0.91 | - |
| Crude protein | 0.74 | 0.72 | 0.08 | 0.93 | - |
| NDF | 0.58[a] | 0.41[b] | 0.02 | **0.01** | - |
| ADF | 0.60[a] | 0.48[b] | 0.02 | **0.08** | - |
| EE | 0.83 | 0.82 | 0.01 | 0.95 | - |

[1]Treatments were: control (215 mg monensin/animal/day) and treatment (17.2 g prebiotic g/animal/day). NOTE: additives are added to the concentrate during its production

[2]The d 20 results were removed from the data set to generate the average per treatment in the statistical analysis

[a–b]Within a row, differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$)

OBS: days 1 to 21 were the adaptation period; days 22 to 113 were the experimental period

### Hemogram

In the hemogram, there was a difference only for white cells (Table 3); where there was the effect of treatment ($P = 0.10$)

**Table 3** Hemogram of cattle consuming monensin and prebiotics

| Items | Treatments[1] | | SEM | *P* – values | |
|---|---|---|---|---|---|
| | Control | Treatment | | Treat | Treat × Day |
| Erythrocytes (×10$^6$ µL) | | | | 0.86 | 0.80 |
| | 8.55 | 8.90 | 0.08 | | |
| Hematocrit (%) | | | | 0.89 | 0.74 |
| | 37.9 | 39.3 | 0.32 | | |
| Hemoglobin (g/dL) | | | | 0.73 | 0.81 |
| | 13.7 | 14.3 | 0.08 | | |
| Leukocytes (×10$^3$ µL) | | | | **0.05** | **0.01** |
| d 20 | 6.50 | 7.21 | 0.09 | | |
| d 70 | 6.69 | 6.35 | 0.07 | | |
| d 113 | 6.02$^a$ | 4.68$^b$ | 0.07 | | |
| Granulocyte (×10$^3$ µL) | | | | **0.09** | **0.03** |
| d 20 | 1.52 | 1.26 | 0.04 | | |
| d 70 | 1.69 | 1.60 | 0.03 | | |
| d 113 | 1.73$^a$ | 1.17$^b$ | 0.03 | | |
| Lymphocytes (×10$^3$ µL) | | | | **0.10** | **0.05** |
| d 20 | 4.07 | 4.95 | 0.05 | | |
| d 70 | 3.91 | 3.76 | 0.05 | | |
| d 113 | 3.57$^a$ | 2.97$^b$ | 0.04 | | |
| Monocytes (×10$^3$ µL) | | | | 0.39 | 0.28 |
| | 0.90 | 0.76 | 0.08 | | |

[1]Treatments were: control (215 mg monensin/animal/day) and treatment (17.2 g Prebiotic g/animal/day). [2]The d 20 results were removed from the data set to generate the average per treatment in the statistical analysis

[a–b]Within a row, differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$)

and interaction treatment × day for the leukocyte count, being lower in the animals of the treatment group on day 113 (P = 0.05). The granulocyte and lymphocyte counts were lower in the animals in the treatment group on day 113. We did not find any effect of treatment or interaction between treatment and day for the red cell values (erythrocytes, hematocrit, and hemoglobin) and monocyte count (Table 3).

## Serum biochemistries

For globulin concentrations, there was an effect of treatment ($P = 0.01$) and treatment × day interaction, being higher in the treatment group on day 113 ($P = 0.04$; Table 4). There was a trend of treatment × day interaction in cholesterol levels, being lower in the treatment group on day 70 ($P = 0.06$). There were no effects of treatment or interaction for albumin, total protein, or urea levels ($P > 0.05$; Table 4).

**Table 4** Serum biochemistry and proteinogram of cattle consuming monensin and prebiotics

| Items | Treatments[1] | | SEM | *P* – values | |
|---|---|---|---|---|---|
| | Control | Treatment | | Treat | Treat × Day |
| Albumin (g/dL)[2] | | | | 0.45 | 0.21 |
| | 3.89 | 3.47 | 0.04 | | |
| Globulin (g/dL) | | | | **0.01** | **0.04** |
| d 20 | 6.43 | 6.06 | 0.04 | | |
| d 70 | 3.73 | 4.65 | 0.05 | | |
| d 113 | 3.57$^b$ | 4.60$^a$ | 0.05 | | |
| Total protein (g/dL) | | | | **0.08** | 0.29 |
| | 7.54$^b$ | 8.12$^a$ | 0.04 | | |
| Urea (mg/dL)[2] | | | | 0.35 | 0.30 |
| | 44.3 | 39.7 | 1.42 | | |
| Cholesterol (mg/dL) | | | | 0.44 | **0.06** |
| d 20 | 57.1 | 61.1 | 3.74 | | |
| d 70 | 151$^a$ | 134$^b$ | 6.10 | | |
| d 113 | 168 | 161 | 6.09 | | |
| IgA (g/dL) | | | | **0.01** | **0.01** |
| d 20 | 1.72 | 1.58 | 0.08 | | |
| d 70 | 0.95$^b$ | 1.29$^a$ | 0.06 | | |
| d 113 | 0.92$^b$ | 1.25$^a$ | 0.06 | | |
| Ig-heavy chain (g/dL) | | | | **0.05** | **0.01** |
| d 20 | 2.14 | 2.23 | 0.09 | | |
| d 70 | 1.05 | 1.20 | 0.05 | | |
| d 113 | 0.98$^a$ | 1.26$^a$ | 0.05 | | |
| Ceruloplasmin (g/dL)[2] | | | | 0.77 | 0.80 |
| | 0.68 | 0.71 | 0.05 | | |
| Haptoglobin (g/dL)[2] | | | | 0.97 | 0.95 |
| | 0.47 | 0.47 | 0.02 | | |

[1]Treatments were: control (215 mg monensin/animal/day) and treatment (17.2 g prebiotic g/animal/day)

[2]The d 20 results were removed from the data set to generate the average per treatment in the statistical analysis

[a–b]Within a row, differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$)

## Proteinogram

We verified the effect of the treatment ($P = 0.01$) and interaction treatment × day for the concentration of immunoglobulins A (IgA), which was higher in the animals of the treatment group on days 70 and 113 ($P = 0.01$ – Table 4). A higher concentration of heavy-chain immunoglobulins (IgM, IgG, IgE and IgE) was observed on day 113 ($P = 0.01$). There was no effect of treatment and interaction treatment × day for ceruloplasmin and haptoglobin concentration between groups (Table 4).

**Table 5** Oxidative status of cattle consuming monensin and prebiotics

| Items | Treatments[1] | | SEM | *P* – values | |
|---|---|---|---|---|---|
| | Control | Treatment | | Treat[2] | Treat × Day |
| TBARS (nmol MDA/mL) | | | | 0.67 | **0.10** |
| d 20 | 24.9 | 25.6 | 0.81 | | |
| d 70 | 20.7 | 22.6 | 0.60 | | |
| d 113 | 25.1[a] | 21.8[b] | 0.64 | | |
| Total thiols (nmol SH/mg protein) | | | | 0.22 | **0.07** |
| d 20 | 77.5 | 79.0 | 2.52 | | |
| d 70 | 71.8[b] | 82.0[a] | 2.43 | | |
| d 113 | 52.8 | 54.3 | 2.43 | | |
| GST (µmol CDNB/min/mg protein) | | | | **0.02** | **0.01** |
| d 20 | 465 | 489 | 10.4 | | |
| d 70 | 397[b] | 444[a] | 8.51 | | |
| d 113 | 286[b] | 330[a] | 6.34 | | |

[1]Treatments were: control (215 mg monensin/animal/day) and treatment (17.2 g prebiotic g/animal/day)

[2]The d 20 results were removed from the data set to generate the average per treatment in the statistical analysis

[a–b]Within a row, differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$)

## Oxidative status

There was a treatment effect ($P = 0.02$) and an interaction between treatment × day on GST activity that were greater in the treatment group on days 70 and 113 ($P = 0.01$ – Table 5). There was a trend of interaction between treatment × day for the concentration of TBARS and total thiols, with lower levels of TBARS (day 113) and higher levels of total thiols (day 70) in the treatment group than the control group (Table 5).

## Ruminal fluid

No difference was observed between groups for ruminal pH. There was a trend toward an effect of MBRT (P = 0.07), being smaller in the ruminal fluid of steer in the treatment group. Interaction treatment × day was observed for the protozoan count, being higher in the ruminal fluid of animals in the treatment group on day 113 (P = 0.03; Table 6).

There was an effect of treatment (P = 0.04) and interaction treatment × day for SCFA concentration, which was lower on day 70 in the ruminal fluid of animals in the treatment group than in the control group (P = 0.05). Treatment × day interaction for propionic acid was observed, being higher in the treatment group on day 70 (P = 0.03, different from the isovaleric concentration, which was lower in the treatment group on days 70 and 113 (P = 0.05). There were no differences between acetic, butyric, or valeric acid groups (Table 6).

Results of ruminal microbiota are presented in Figs. 1 and 2. The primary ten genera of bacteria in the ruminal fluid were *Actinobacter*, *Bacillus*, *Bacteroides*, *Christensenellaceae*, *Clostridium*, *Comamonas*, *Enterococcus*, *Escherichia-Shigella*, *Fusobacterium*, and *Klebsiella* (Fig. 1). We found no difference between groups for ruminal microbiota in general, regardless of the day or when evaluating the effect of treatment for each bacteria (Supplementary Material 3). We found that the abundance of *Klebsiella* and *Actinobacter* was higher in the rumen of steer in the treatment group and in the abundance of *Bacteroides* and *Escherichia-Shigella*. Many of the microorganisms identified in the ruminal fluid were also identified in the feed of the steer (Fig. 2).

In evaluating beta diversity between treatments, we verified in the principal coordinate analysis that there is no difference between the groups, and the microbiota was similar according to the period of the experiment (Figs. 3). We found no difference between treatments in alpha diversity using community richness and community diversity (invSimpson and Shannon) methodologies (Fig. 4).

## Carcass and meat

Carcass yield, meat composition, and oxidative status showed no treatment effect. In the liver, however, higher GST activity was observed in the treatment group (P = 0.01), and there was a trend towards a higher concentration of total thiols in the treatment group (P = 0.06) (Table 7).

A treatment effect was observed for oleic acid (C18:1n9c), higher in meat in the treatment group (P = 0.04), with a lower concentration of cis acid −5,8,11,14,17-eicosapentaenoic (C20:5n3) in the treatment group (P = 0.05). The treatment group had a trend toward lower niceis of arachidonic acids (C20:4n6). There was also a trend toward a higher concentration of mono-unsaturated fatty acids and a lower

**Table 6** pH, bacterial activity, number of protozoa, total short-chain fatty acids, and volatile fatty acids in cattle consuming monensin and prebiotics

| Items | Treatments[1] | | SEM | *P* – values | |
|---|---|---|---|---|---|
| | Control | Treatment | | Treat[2] | Treat × Day |
| pH | | | | 0.97 | 0.92 |
| | 6.17 | 6.12 | 0.06 | | |
| MBRT (s) | | | | **0.07** | 0.12 |
| | 60.8[a] | 51.7[b] | 2.53 | | |
| Protozoa ($\times 10^4$ / mL) | | | | 0.24 | **0.03** |
| d 20 | 84.8 | 95.0 | 10.8 | | |
| d 70 | 55.4 | 56.1 | 6.74 | | |
| d 113 | 47.0[b] | 58.3[a] | 6.52 | | |
| Total SCFA (nmol/L) | | | | **0.04** | **0.05** |
| d 20 | 97.8 | 97.4 | 5.04 | | |
| d 70 | 121[a] | 110[b] | 3.58 | | |
| d 113 | 138 | 128 | 3.47 | | |
| Acetic acid (mol 100 mol) | | | | 0.62 | 0.44 |
| | 61.4 | 59.8 | 0.68 | | |
| Propionic acid (mol 100 mol) | | | | 0.30 | **0.03** |
| d 20 | 17.4 | 17.1 | 0.87 | | |
| d 70 | 23.7[b] | 28.4[a] | 0.92 | | |
| d 113 | 22.1 | 22.3 | 0.76 | | |
| Butyric acid (mol 100 mol) | | | | 0.92 | 0.81 |
| | 12.4 | 12.5 | 0.42 | | |
| Isovaleric aci (mol 100 mol) | | | | **0.05** | **0.05** |
| d 20 | 1.54 | 1.51 | 0.07 | | |
| d 70 | 1.75[a] | 1.24[b] | 0.07 | | |
| d 113 | 1.54[a] | 1.05[b] | 0.06 | | |
| Valeric acit (mol 100 mol) | | | | 0.19 | 0.12 |
| | 1.27 | 1.20 | 0.03 | | |
| Acetic/propionic ratio | | | | 0.24 | 0.11 |
| | 2.74 | 2.48 | 0.12 | | |

[1]Treatments were: control (215 mg monensin/animal/day) and treatment (17.2 g prebiotic g/animal/day). NOTE: additives are added to the concentrate during its production

[2]The d 20 results were removed from the data set to generate the average per treatment in the statistical analysis. [a–b]Within a row, differ ($P \leq 0.05$) or tend to differ ($P \leq 0.10$). MBRT—methylene blue reduction test (Dirksen 1993) measures bacterial activity

concentration of polyunsaturated fatty acids in the treatment group (Table 8).

## Discussion

Monensin is an ionophore with an antimicrobial role widely used in ruminant nutrition as they are potential growth promoters, as they modulate the rumen microbiota; however, like any antibiotic, the problem lies in the risks to public health as they can lead to the emergence of resistant bacterial strains (Nocek et al. 2011; Vendramini et al. 2016). Oligosaccharides can enhance the immune system, optimize the absorption of nutrients, increase the breakdown of dietary fiber, and promote the synthesis of microbial proteins (Salinas-Chavira et al. 2015), enhancing ruminal functions and promote the proliferation of bacteria that favor food consumption and efficiency (Ushakova et al. 2013). In this study, animals receiving monensin or the blend of yeast and saccharides based-product demonstrated equivalent animal performance. Researchers verified that though general ADG was decreased in steers supplemented with *Saccharomyces cerevisiae* fermentation product compared to monensin, the final BW was similar among treatments (Swyers et al. 2014). Using 80 crossbred cattle in the growing phase and confined for 229 days, the researchers concluded that inclusion of yeast products (yeast cell walls) improved DMI, ADG, ruminal fiber digestion, and modified acetate:propionate ratio (Salinas-Chavira et al. 2015).

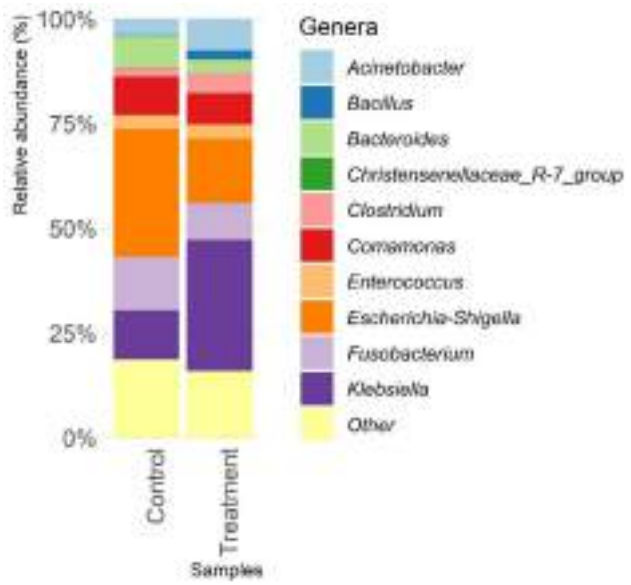



**Fig. 1** The image illustrates the top ten, referring to the ten most abundant genera in the ruminal fluid, with all others included in the "Other" category in a comparison between control (monensin) and treated (Prebiotic) animals, grouped at times 70 and 113 of the experiment

It is critical to remember that prebiotics are defined as indigestible organic compounds for animals; however, they are available for specific groups of microorganisms (Schrezenmeir and de Vrese 2001). Generally, yeast and saccharides as prebiotics can increase fiber digestion due to their affinity with bacteria (Zheng et al., 2018). Fiber digestibility in the present study was lower in animals in the yeast and saccharides group than in the monensin group, which was not expected; according to the literature, supplementation with yeast and saccharides intensifies the proliferation of fiber-degrading bacteria (Rai et al. 2013). This may have occurred because the yeast dose was not sufficient to increase fiber digestibility, requiring an even higher supplementation. Furthermore, in our study, similar to Hall et al. (2018), there was no difference in dry matter digestibility when the authors compared monensin with a fermented product from *Lactobacillus acidophilus*. However, despite the difference in digestibility that may explain the significant numerical weight gain of cattle with monensin, there was no significant difference between treatments. In the present study, digestibility in the presence of monensin was 39.5% and 26.6% higher for NDF and ADF, respectively, than in animals that consumed the yeast and saccharides based-product (Table 1).

Despite the literature reporting that monensin limits consumption (Boardman et al. 2020), we found no difference in feed intake between treatments. Similarly, De Melo et al. (2023) found no effect on intake when feeding cattle with monensin or a blend of *S. cerevisiae boulardii* and *Bacillus toyonensis*. Combining monensin and *S. cerevisiae*, strain 1026 did not affect feed intake compared to additives used individually for steer (Gomes et al. 2011).

Dry yeasts stabilize ruminal pH, stimulating populations of ciliated protozoa that rapidly consume starch and, thus, compete with amylolytic bacteria, which produce lactate (Hua et al. 2022) and reduce protozoa (Bergen and Bates 1984). These dry yeasts effects explain why, in our work, there was no difference in ruminal pH between treatments; we found a higher protozoan count in the group that received the oligosaccharides. Protozoa are critical in starch sequestration, reducing starch fermentation, maintaining a stable pH, and promoting microbial growth (Kang et al. 2016). Similar results were reported by Tripathi and Karim (2011), who supplemented lambs with dry yeast isolated or combined from the microorganism *Kluyveromyces marximanus, S. cerevisiae*, and *Saccharomyces uvarum*. The authors found a higher count of protozoa in the ruminal fluid of these animals.

Monensin can modify fermentation in the rumen by inhibiting gram-positive bacteria, producing lactate and hydrogen (Haney et al. 1947), and reducing protozoa (Bergen and Bates 1984). This activity allows the gram-negative bacteria to develop and produce propionate (Guan et al. 2006); this activity promotes a change in the production of SCFAs (Wolin et al. 1997). In the present study, we found a treatment × day interaction for the profile of SCFAs, especially on day 70, where the ingestion of monesin also increased the amount of SCFA in the rumen; however, this result did not occur due to propionic acid, which was in greater amounts in the ruminal fluid of steer that consumed prebiotic. We found lower amounts of SCFAs due to the lower proportion of isovaleric acid in the yeast and saccharides group compared to monensin group.

Branched-chain fatty acids (BCVFA), such as isovaleric and other isoacids, are degradation products of the amino acids valine, isoleucine, leucine, and proline; and in our study, they were found in quantities similar to the work of Cagliari et al. (2023), who used the same product but in an in vitro system. These acids in question are specific nutrients for ruminal cellulolytic bacteria, promoting microbial fermentation (Andries et al. 1987), and in this case, our study was in line with the values found by Cagliari et al. (2023).

It was unclear how the consumption of the blend of yeast and saccharides-based product could have raised the proportion of propionate in the rumen; however, this effect is positive because propionic derived from ruminal fermentation plays an essential role in controlling consumption in ruminants fed cereal grains. They are also the primary end-product of ruminal starch digestion and one of the vital fuels that affect satiety during meals (Maldini and Allen 2019).

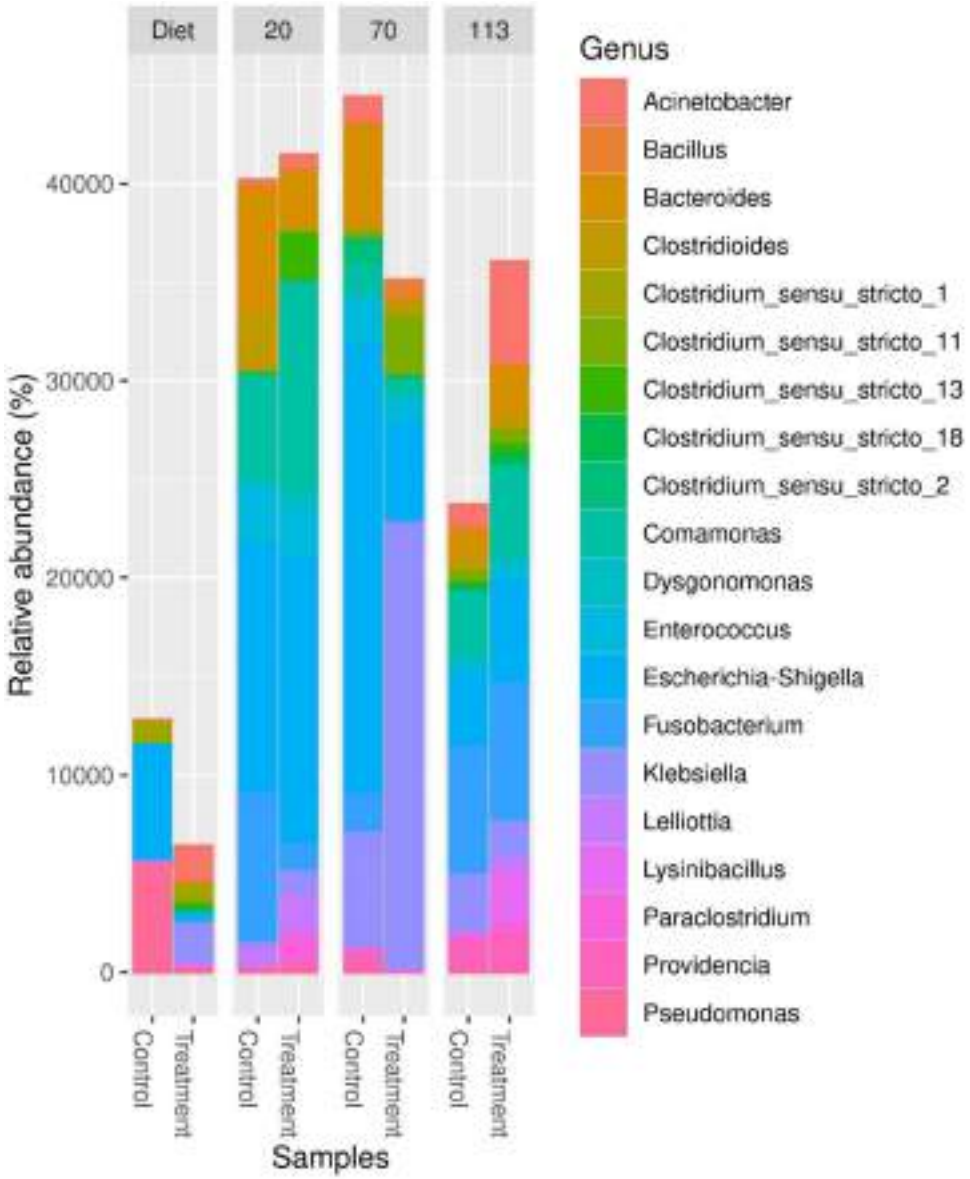



**Fig. 2** The image illustrates the top 20, referring to the 20 most abundant genera in the ruminal fluid in a comparison between control (monensin) and treated (Prebiotic) animals, at times 20, 70, and 113 of the experiment, as well as those identified in the diet

The ruminal microbiota is responsible for a large part of the breakdown of feed and its conversion into energy used for animal maintenance and development (Seymour et al. 2005). The composition of microorganisms spans several kingdoms, including bacteria, protozoa, and fungi, which perform proteolytic, lipid, and fibrolytic functions (Weston and Hogan 1968). Populations of ruminal bacteria provide microbial protein, the primary source of protein in ruminants (Houpt and Houpt 1968). In the present study, when we compared steer that consumed monensin and the blend of yeast and saccharides, we did not observe any difference; however, the numerical abundance of some microorganisms in the ruminal fluid differed.

Ionophores are used to modulate the ruminal microbiota, because they act as inhibitors to hydrogen-producing bacteria, as well as could modulate rumen fermentation toward propionate production, thereby decreasing CH4 production (Ahmed Soltan and Kumar 2022). Research with different yeast and saccharides, and other additives is being studied to improve animal feed efficiency further (Weimer et al. 2009), due to a movement in One Health in the world that has been limiting and prohibiting the use of antibiotics as a performance enhancer in livestock. In an in vitro study using a prebiotic also extracted from dry yeast walls and other compounds, the authors reported changes in the bacterial community, with emphasis on the increase in *Bacteroides* when compared to the control experimental unit and lower abundance of *Provotella* (Klassen et al. 2023). The genus *Bacteroides* includes only the clinically significant *B. fragilis*, which is common in the normal microbiota; however, when pathogenic, it is associated with abscesses and soft tissue infections in young cattle (McVey et al. 2020). Our

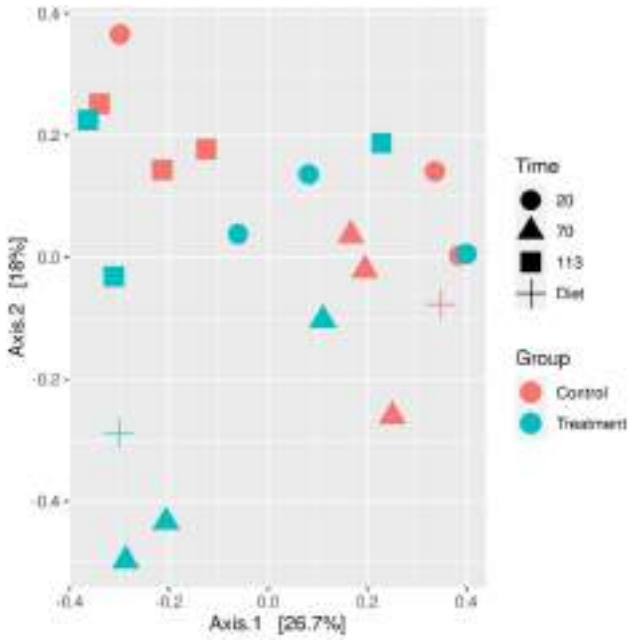



**Fig. 3** Beta diversity analysis using principal coordinate analysis obtained by the WPGMA clustering methodThe abbreviations T20, T70, and T113 indicate the sampling times; the identifications C1, C2, and C3 illustrate the control animals, and T1, T2, and T3 identify the animals in the treatment group

animals were apparently healthy, which allows us to conclude that the presence of this and other microorganisms is related to the condition of normal colonization of the ruminal environment.

In another study on dairy cows supplemented with inulin prebiotics the authors concluded that the animals in this group had a lower abundance of the genus *Escherichia-Shigella* when compared to animals in the control group (Wang et al. 2021), very similar to our results, where *Escherichia-Shigella* was numerically less abundant in ruminal fluid when consuming the prebiotic. The population of the ruminal microbiota proliferates according to the type of diet the animals consume. Some genera of bacteria are responsible for propionate fermentation, derived from the fermentation of fiber from the litter (Li et al. 2016). As found in our work, animal intestines are a natural habitat for *Acinetobacter*, especially *A. baumannii* (Doughari et al. 2011). However, they are bacteria responsible for infections in the intestine and other organs when the animal is immunosuppressed or has an unbalanced microbiota (Hou et al. 2022). The genus *Klebsiella*, one of the predominant ones in the ruminal fluid of the animals in this study, is a commensal microorganism of the digestive system of the animals and an opportunistic pathogen. Feces are significant sources of contamination and infection in young ruminants, where *K. pneumoniae* and *K. oxytoca* are the most pathogenic (McVey et al. 2020). However, these bacteria, being opportunistic, cause health problems associated with other factors when the animal is immunosuppressed.

In the present study, steer consuming the the blend of yeast and saccharides had lower total leukocyte counts due to lower lymphocyte and granulocyte counts. There was also a higher concentration of globulins, reflecting the higher concentration of immunoglobulins (IgA and other heavy-chain Ig), generally characterizing a humoral immune response stimulated by prebiotic consumption. In animal studies, the consumption of different prebiotics (oligofructose, fructooligosaccharides, and inulin) did not change the number of T lymphocytes; however, it increased the response to mitogen stimulation and cytokine secretion, stimulating a Th1 immune response and improving the functionality and memory of existing T cells (Kelly-Quagliana et al. 2003; Schouten et al. 2010; Delgado et al. 2012). Prebiotics (inulin, fructooligosaccharide, and alactooligosaccharide) can potentially modulate the host's immune system directly or indirectly (Pujari and Banerjee 2021). The effects found in this study would be welcome for production animals because of the increase in immunoglobulins without an inflammatory response. The researchers found that 1.5 g of a prebiotic composed of mannan oligosaccharides and β-glucans extracted from yeast cell walls and 2.0 g of the peptide supplement in the lamb substitute increased IgG levels (Chashnidel et al. 2020), reinforcing the hypothesis that the intake of prebiotics stimulates the humoral immune response. In human medicine, the relationship between fructooligosaccharide consumption and the pro- and anti-inflammatory response has been noted, and it is reported as having a positive effect on people with and without comorbidities (Pujari and Banerjee 2021).

We observed greater GST activity in the serum and liver, a result also described by Verma et al. (2018) in rats who used a porcine blood protein hydrolysate in the diet, considered a prebiotic. The GST in the liver participates in the detoxification of liver cells, helping to protect them from reactive oxygen species, free radicals, and hydrogen peroxides that accumulate in the cell cytoplasm and are highly toxic (Vaish et al. 2020; Zhuge et al. 2020). In the present study, we observed a trend of lower serum lipid peroxidation combined with higher GST activity, positively affecting animal health.

The consumption of the blend of yeast and saccharides did not change the meat's carcass yield, chemical composition, or oxidative stability but caused small changes in the fatty acid profile. Among the differences, we highlight a higher proportion of oleic acid and a lower proportion of cis-5,8,11,14,17-eicosapentaenoic acid (both unsaturated fatty acids), which is desirable for human consumption. In serum, there was a trend toward lower concentration of total cholesterol, combined with slight changes in the fatty acid profile of the meat; changes that we do not have an

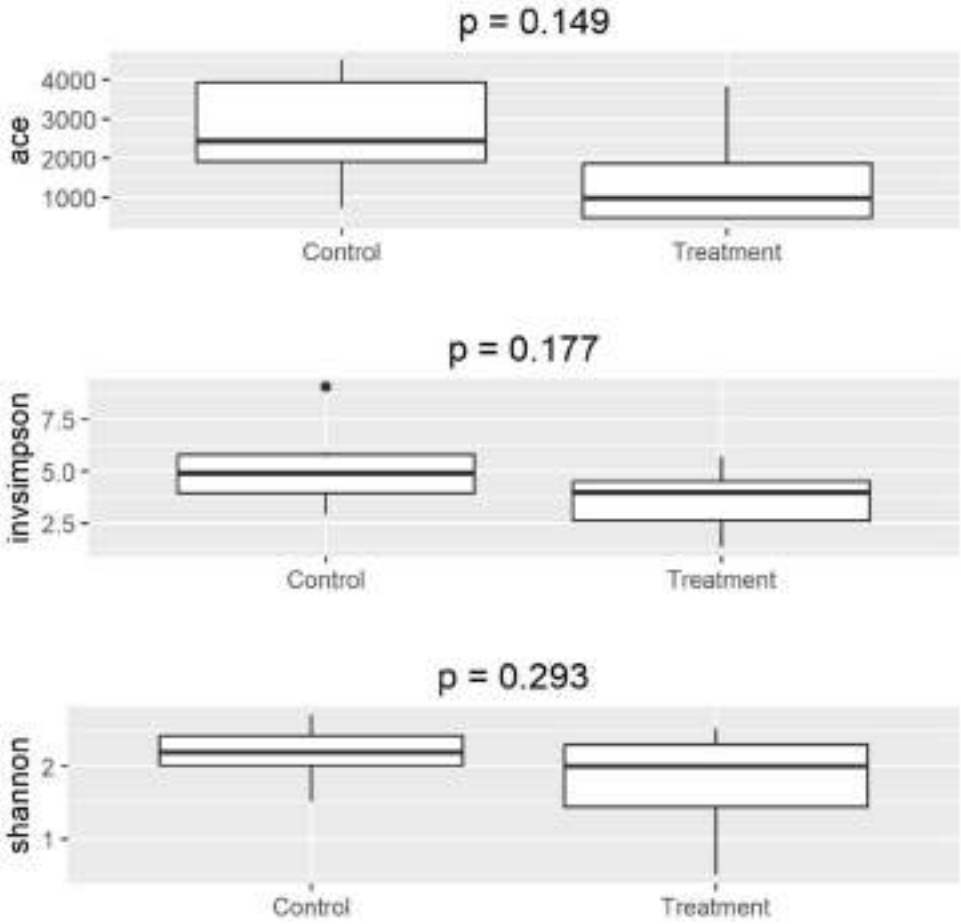



**Fig. 4** The images compare the alpha diversity of control and treated animals, grouped at times 70 and 113, using community richness (ACE) and community diversity (Simpson and Shannon)

**Table 7** Carcass and chemical composition of meat and oxidative status of meat and liver of cattle consuming monensin and prebiotics

| | Control | Treatment | SEM | *p*-value |
|---|---|---|---|---|
| Carcass yield | 53.2 | 53.1 | 0.17 | 0.97 |
| **Meat** | | | | |
| Fat, % | 1.72 | 1.95 | 0.14 | 0.11 |
| Protein, % | 21.4 | 22.5 | 0.74 | 0.80 |
| Mineral matter, % | 2.87 | 2.61 | 0.08 | 0.62 |
| TBARS (nmol MDA/mg protein) | 32.2 | 32.1 | 0.85 | 0.94 |
| Total Thyois (nmol SH/mg protein) | 1.09 | 0.97 | 0.16 | 0.72 |
| GST (µmol CDNB/min/mg protein) | 381 | 376 | 5.74 | 0.58 |
| **Liver** | | | | |
| TBARS (nmol MDA/mg protein) | 89.0 | 92.3 | 2.01 | 0.69 |
| Total thiols (nmol SH/mg protein) | 0.37[b] | 0.49[a] | 0.02 | **0.06** |
| GST (µmol CDNB/min/mg protein) | 461[b] | 492[a] | 4.25 | **0.01** |

Monensin (approximately 215 mg/animal/day) and prebiotics (about 17.2 g/animal/day) were the groups

explanation for the findings, mainly because the ruminal microbiota was very similar in steer from both groups. The prebiotic containing mannan oligosaccharides, beta-glucans, galactooligosaccharides, and fructo-oligosaccharides effect is a mechanism that deserves investigation in future studies; doses should be studied to identify the optimal amount for feed efficiency and weight gain.

## Conclusion

Under current dietary conditions, the yeast-saccharide product mixture played a similar role to monensin in productive performance. Yeast-saccharide product mixture consumption by steers stimulated the humoral immune response and increased the concentration of IgA, an essential mucosal defense antibody. In addition, there was a positive antioxidant response in steers that consumed the yeast-saccharide product mixture, since higher GST activity associated with lower serum lipid peroxidation is a health biomarker. Changes in the fatty acid profile of meat were small and related to increased monounsaturated fatty acids and reduced polyunsaturated fatty acids when consuming

**Table 8** Fatty acids profile and sum of saturated and unsaturated fatty acids in meat in meat from cattle consuming monensin and prebiotics

| Compounds, % | Control | Treatment | SEM | *P*—treat |
|---|---|---|---|---|
| C10:0 (Capric) | 0.02 | 0.02 | 0.00 | 0.98 |
| C11:0 (Undecanoic) | 0.03 | 0.02 | 0.00 | 0.97 |
| C12:0 (Lauric) | 0.04 | 0.05 | 0.00 | 0.97 |
| C14:0 (Myristic) | 1.81 | 1.83 | 0.04 | 0.94 |
| C14:1 (Myristoleic) | 0.35 | 0.38 | 0.02 | 0.92 |
| C15:0 (Pentadecanoic) | 0.22 | 0.22 | 0.01 | 0.95 |
| C16:0 (Palmitic) | 27.39 | 27.69 | 0.45 | 0.89 |
| C16:1 (Palmitoleic) | 2.36 | 2.46 | 0.12 | 0.92 |
| C17:0 (Heptadecanoic) | 0.70 | 0.68 | 0.02 | 0.94 |
| C18:0 (Stearic) | 17.30 | 16.02 | 0.13 | 0.52 |
| C18:1n9c (Oleic) | 33.80[b] | 35.73[a] | 0.21 | **0.04** |
| C18:2n6c (Linoleic) | 12.18 | 10.73 | 0.18 | 0.12 |
| C20:0 (Arachidic) | 0.15 | 0.21 | 0.04 | 0.19 |
| C18:3n6 (gamma-Linolenic) | 0.08 | 0.07 | 0.01 | 0.81 |
| C20:1n9 (cis-11-Eicosenoic) | 0.18 | 0.20 | 0.03 | 0.80 |
| C18:3n3 (alpha-Linolenic) | 0.59 | 0.55 | 0.04 | 0.42 |
| C20:2 (cis-11.14-Eicosadienoic) | 0.17 | 0.15 | 0.03 | 0.85 |
| C22:0 (Behenic) | 0.07 | 0.07 | 0.01 | 0.98 |
| C20:3n6 (cis-8.11.14-Eicosatrienoic) | 0.50 | 0.41 | 0.03 | 0.11 |
| C22:1n9 (Erucic) | 0.06 | 0.04 | 0.00 | 0.76 |
| C20:4n6 (Arachidonic) | 2.24[a] | 1.88[b] | 0.12 | **0.08** |
| C22:2 (cis-13.16-Docosadienoic) | 0.05 | 0.04 | 0.00 | 0.95 |
| C24:0 (Lignoceric) | 0.10 | 0.09 | 0.00 | 0.98 |
| C20:5n3 (cis-5.8.11.14.17-Eicosapentaenoic) | 0.43[a] | 0.34[b] | 0.02 | **0.05** |
| C24:1n9 (Nervonic) | 0.04 | 0.04 | 0.00 | 0.98 |
| C22:6n3 (cis-4.7.10.13.16.19-Docosahexaenoic) | 0.12 | 0.10 | 0.02 | 0.76 |
| ∑ Saturated fatty acids (SFA) | 46.83 | 47.38 | 0.47 | 0.35 |
| ∑ Unsaturated fatty acids (UFA) | 53.17 | 52.62 | 0.56 | 0.66 |
| ∑ Monounsaturated fatty acids (MUFA) | 36.79[b] | 38.35[a] | 0.34 | **0.10** |
| ∑ Polyunsaturated fatty acids (PUFA) | 16.38[a] | 14.26[b] | 0.23 | **0.07** |
| UFA/SFA | 1.15 | 1.11 | 0.08 | 0.77 |
| ∑ ω6 | 2.83 | 2.36 | 0.12 | 0.24 |
| ∑ ω3 | 1.15 | 0.98 | 0.05 | 0.70 |
| ω6/ω3 | 2.45 | 2.35 | 0.02 | 0.16 |

The groups were control (215 mg monensin/animal/day) and treatment (17.2 g prebiotic g/animal/day)

the yeast-saccharide product mixture compared to monensin. Lower fiber digestibility may be related to ruminal fermentation, since lower bacterial activity and concentration of short-chain fatty acids were observed.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11250-025-04353-3.

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**Data availability** Raw data are held by the authors and may be available upon request.

## Declarations

**Informed consent** Not applicable.

**Conflicts of interest** The authors declare no conflict of interest.

**Institutional review board statement** The Animal Research Ethics Committee of the Santa Catarina State University (UDESC) approved the project under number 6212210822, following guidelines from National Council for the Control of Animal Experimentation (CONCEA), Brazil.

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# Effects of Adding Guanidinoacetic Acid to the Diet of Jersey Cows on Ruminal Fermentation, Milk Efficiency, Milk Quality and Animal Health

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**Abstract:** The aim of this study was to investigate the effects of including guanidinoacetic acid (GAA) in the diet of Jersey cows during their first lactation, evaluating the effects on the ruminal environment, productivity, milk composition and quality, and hematological variables. The study used 18 Jersey animals in their first lactation with 230 days of lactation. The study lasted 28 days: 14 days of adaptation, and 14 days for data and sample collection. The animals were housed in a compost barn confinement system and milking was performed by a robotic system. The cows were randomly divided into two groups of nine each, a control group and a treatment group (guanidinoacetic acid at a dose of 10 g animal/day). The animals were fed individually provided at three times, with weighing of the leftovers whenever necessary. The diet was formulated to meet the nutritional requirements of the animals, using the Nutrient Requirements of Dairy 2001 and considering an average daily production of 20 L/cow. The animals in the treatment group showed higher feed intake compared to the control group, and consequently, milk production was also higher in the treatment group. No difference was observed between the groups for milk efficiency, since the increase in production is directly related to the increase in intake of the treated animals. The cows that consumed GAA had a higher percentage of total milk solids; however, there was no significant effect on the percentage of fat, protein, lactose and fatty acid profile. Greater bacterial activity was observed in the ruminal fluid, which may have improved degradation and increased the rate of passage of the feed, resulting in greater milk production. There was no effect of the treatment on the volatile fatty acid profile in the rumen. A treatment × day interaction for beta-hydroxybutyrate concentration in the blood was observed, with lower levels observed in the animals in the treatment group. The total leukocyte count was lower in the blood of cows that consumed GAA as a result of the lower lymphocyte count. On days 21 and 28, a higher concentration of total cholesterol was observed, associated with lower activity of the enzymes creatine kinase, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT) in the serum of cows in the treatment group compared to the control group. On day

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28, lower lipid peroxidation (TBARS) associated with an increase in antioxidant enzymes (superoxide dismutase and glutathione S-transferase) was observed in cows that consumed GAA. The results allow us to conclude that the addition of GAA to the diet of dairy cows has positive effects on zootechnical performance.

**Keywords:** additive; dairy cows; productivity; ruminal environment

## 1. Introduction

The COVID-19 pandemic has caused major changes in the supply and demand of several commodities [1]. Milk production was affected by the costs of inputs used in the production of concentrate and fertilizers, which occurred sharply from the second half of 2020. The cost of milk production in 2021 increased by 80% compared to 2020 [2]. As a result, producers' profit margins decreased and one of the alternatives to increase profitability was investing in genetics (a slower way) or nutrition (a faster way). Thus, the use of additives comes into play to enhance animal performance, improving efficiency in food use and free of growth promotors [3].

The market demand for antibiotic-free products and growth promoters increases the search for differentiated additives, such as guanidinoacetic acid (GAA), which has been gaining ground in animal nutrition, as its responses are positive in productivity and health. A preliminary study carried out by Al-Qudah et al. [4] in the diet of dairy cows with GAA reported an improvement in milk production and composition, as well as in the metabolic profile of dairy cows. When used in the diet of beef cattle, researchers found greater weight gain [5].

GAA is a molecule produced endogenously in the animal organism from the amino acid arginine. Specifically, GAA is formed by the methylation of arginine with the donation of a methyl group from S-adenosylmethionine (SAM) and subsequent decarboxylation [6]. According to Wyss and Kaddurah-Daouk [7], creatine, in turn, is synthesized in the liver, kidneys and pancreas from three amino acids known as glycine, arginine and methionine. Creatine is stored in skeletal muscles and is an important source of energy during intense physical activity or reduced feeding intake. Wallimann et al. [8] reported that when muscle cells need energy, creatine is converted to phosphocreatine, which can donate a phosphate group to regenerate ATP (adenosine triphosphate), the main source of cellular energy. GAA is a precursor of creatine, and its availability limits the synthesis of creatine itself. This means that the addition of GAA can increase the availability of creatine in the body and, consequently, improve the muscle's ability to produce energy.

Another study found that GAA intake in the diet of lactating Holstein dairy cows had higher milk production and better feed efficiency [9]. Jersey and Holstein cows are metabolically different, with the Holstein being known to produce a larger quantity of milk, but with a smaller amount of solids; while the Jersey can produce as much as a Holstein cow and still have milk with a high concentration of solids; this means that protein, lipid and carbohydrate metabolism is different. Therefore, the objective was to evaluate whether the addition of GAA to the diet of Jersey cows has positive effects on the ruminal environment, productivity, milk composition and quality and blood variables.

## 2. Materials and Methods

### *2.1. Experiment Location*

The study was conducted at the experimental farm of the Centro Superior de Educação do Oeste (FECEO), located in Guatambu—Santa Catarina/Brazil. The Research Unit is located 23 km from the headquarters of the UDESC in Chapecó—SC/Brazil. The animals were housed in a confined system of compost barns, with bedding composed of sawdust, turned daily and with an area of 15.3 $m^2$ per animal. The barn consists of a bedding area, used by the animals as a resting area, a waiting room, a milking room, a feeding area equipped with individual feeders and drinkers, animal restraint pens, a feed room, and a cooler room; as well as a robotic milking area detailed below.

The resting area has two MX300 (MX do Brasil®, Tigrinhos, SC, Brazil) collective tilting drinkers, with a capacity of 300 L of water each, ensuring ad libitum consumption by the animals. The area also has 4 Eco Fan MX 160 fans (MX do Brasil®) to control the microclimate inside the barn and prevent humidity in the bedding. A Delaval robotic milking system is installed in the center of the facility.

The track where the animals feed has 2 Eco Fan MX 160 fans (MX do Brasil®, Tigrinhos—SC, Brazil), individual feeders equipped with automatic closing cans and plastic drinkers distributed among the feeders. The floor of the facilities is made of concrete, with grooves to increase the animals' grip and provide greater safety. Animal waste and cleaning residues from these areas are taken through gutters to the waste lagoons, which are distributed in agricultural areas in the form of organic fertilizer.

### *2.2. Animals and Experimental Design*

Eighteen primiparous Jersey cows (pregnant) with an average weight of 395 ± 8.9 kg, 31 months of age and 230 days of lactation were used. The cows were divided into two groups, with 9 cows each, among the control and treatment groups. The treatment group received a daily addition of 10 g of the GAA additive mixed in the concentrate, while the control group did not receive any additive in its diet. The experiment period lasted 28 days, 14 days of adaptation and 14 days for data collection.

The GAA ($C_3H_7N_3O_2$) used in this experiment was purchased from Nutriquest and sold commercially as Creamino®—the purity of at least 96% GAA and a maximum of 1% water. Creamino® is white to slightly beige granulated powder, with a bulk density of 600 kg/$m^3$ and solubility of approx. 4.0 g/L water at 20 °C.

### *2.3. Diet*

The animals' diet was formulated using the NRC 2021 to meet the animals' nutritional requirements for the production of 20 L of milk per day. The basal diet of both groups was the same (Table 1), with the only difference being that the treatment group received GAA during its production, which resulted in a GAA consumption of 10 g per cow per day. The animals were fed at three times (6:00 am; 11:00 am; 4:00 pm), in individual feeders where the cow was restrained by yokes. Between each two feeders, there was a drinker to which two cows had access. This drinker was closed and opened to the animals 45 min after the start of feeding, which lasted 2 h per treatment. Samples of the feed and diets provided to the cows were collected throughout the experiment for later analysis in the laboratory. This material was stored frozen (−20 °C).

**Table 1.** Calculated nutritional composition of the animals' diet during the experimental period.

| Variables, % | Silage | Concentate | Hay | TMR: Control | TMR: Treatment |
|---|---|---|---|---|---|
| Dry matter | 25.1 | 89.4 | 88.0 | 40.8 | 40.8 |
| Crude protein | 11.0 | 20.6 | 12.0 | 15.5 | 15.6 |
| EE [1] | 3.50 | 5.71 | 1.10 | 4.30 | 4.32 |
| ADF [1] | 53.7 | 9.20 | 31.8 | 21.6 | 21.8 |
| NDF [1] | 32.9 | 18.7 | 67.5 | 38.7 | 38.5 |

The proportion of the daily total mixed ration (TMR) was 46.39; hay, 7.19%; corn grain, 12.27%; soybean meal, 11.83%; soybean hulls, 2.48%; wheat bran, 4.67%; robot concentrate, 11.44%; palm oil, 1.2%; sodium bicarbonate, 0.91%; mineral/vitamin supplement, 1.62% (animals received 250 g per day). The guaranteed levels were as follows: Calcium (min.) 230.45 g/kg, Calcium (max.) 249.00 g/kg, Phosphorus (min.) 40.00 g/kg, Sulfur (min.) 20.00 g/kg, Magnesium (min.) 25.00 g/kg, Potassium (min.) 10.00 g/kg, Sodium (min.) 70.00 g/kg, Cobalt (min.) 15.00 mg/kg, Copper (min.) 750.00 mg/kg, Chromium (min.) 20.00 mg/kg, Iodine (min.) 40.00 mg/kg, Manganese (min.) 2000.00 mg/kg, Selenium (min.) 22.00 mg/kg, Zinc (min.) 2850.00 mg/kg, Vitamin A (min.) 350,000.00 IU/kg, Vitamin D3 (min.) 100,000.00 IU/kg, Vitamin E (min.) 2000.00 IU/kg, Monensin 1000.00 mg/kg. Note 1: ethereal extract (EE), acid detergent fiber (ADF) and neutral detergent fiber (NDF).

### *2.4. Milking Management*

The animals were milked using a robotic milking system (Delaval Brasil®, Jaguariúna, São Paulo, Brazil) in a guided flow, twice a day (starting at 5:00 a.m. and in the afternoon at 3:00 p.m.). The animals were placed on the compost barn bed, then entered the waiting room, awaiting the opening of the robotic milking gates, more specifically the robot. The procedures for milk extraction began with pre-dipping, cleaning of the teats with the fifth teat cup (washing, removing the three jets and subsequently drying the teat), then the robot places the teat cups and after the milking is finished, automatic extraction occurs, followed by post-dipping and release of the cow. It is important to emphasize that, during milking, the cows received commercial pelleted concentrate (limited to a maximum of 2 kg/cow/day), specific for the robot. All procedures described were performed automatically by robotic milking. All cows have a collar that identifies their daily activity (high activity can indicate heat and low activity indicates illness). They also have an ear tag, which allows the robot to recognize them.

### *2.5. Data Collection and Samples*

The blood collected on days 1, 14, 21 and 28 of the experiment was allocated into tubes without an anticoagulant for biochemical analyses; and into tubes with an anticoagulant (EDTA) for hematological analyses. After clot formation, samples were centrifuged at 3500 rpm for 10 min, then the serum was removed, allocated to microtubes and frozen at −20 °C for later analyses.

Ruminal fluid was collected to measure pH, bacterial activity, and volatile fatty acid profile. Fluid samples were collected using an esophageal tube, two hours after feeding the cows on days 1, 14 and 28.

Milk collection was performed using the automatic collection system of the robotic system, with the sample being taken from the total volume of milk produced by the cows, i.e., a homogeneous sample of the entire lactation of the animal. The milk was then transferred to tubes sterilized with Bronopol preservative for analysis of chemical composition, so that they could be sent to the laboratory.

We evaluated the daily production of individual milk by the robotic milking system (DeLaval VMS™ V300), which was obtained individually. The milk was collected using a collector from the robot to evaluate the composition of protein, fat, lactose, minerals and total solids, as well as somatic cell count (SCC). The individual SCC was performed by the robot itself, daily, i.e., every time the cow was milked. The body score was performed using a specific camera on the robotic system, every time the cow passed by the robot.

### *2.6. Ruminal Liquid: pH and Bacterial Activity*

To determine the pH of the ruminal fluid, we used a Kasvi® benchtop pH meter (Pinhais, PR, Brazil) immediately after collection; then, 15 mL of ruminal fluid from each animal was stored in a Falcon tube and frozen at −20 °C to measure the VFA profile. The methylene blue reduction test (MBRT) was also applied to fresh samples, and the methodology involves assessing how long it takes bacteria to consume methylene blue [10]. According to the methodology, the shorter the time to consume methylene blue, the greater the bacterial activity in the ruminal fluid.

### *2.7. Hematological and Biochemical Analyses*

The complete blood count was measured using an automatic device, through the VET3000 automatic hematology analyzer (EQUIP®, Itatiba, SP, Brazil), which determines the following variables: leukocytes, lymphocytes, granulocytes, monocytes, erythrocytes and platelet count in cells $\times 10^3/\mu L$. It also determines the hemoglobin concentration (g/dL), hematocrit (%), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and red blood cell distribution width (RDW-SD).

Serum levels of total protein, albumin, urea, C-reactive protein, cholesterol, and ureic acid were evaluated in the blood serum, and the activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyltranspeptidase (GGT) were determined. For this purpose, Analisa® kits were used with automatic equipment (Zybio EXC-200®, Apparat Brasil Comercio De Reagentes Importadora Ltda, Itatiba, SP, Brazil). The globulin concentration was calculated using the equation: total protein–albumin.

Lipoperoxidation is a highly rapid reaction formed by the breakdown of polyunsaturated fatty acids, which are usually measured by their products, mainly TBARS, among which malondialdehyde (MDA) is the primary product [11]; and the results are expressed in nanomolar MDA/mL. Glutathione S-transferase activity was measured according to Habig et al. [12]. SOD activity measurement was based on the inhibition of $O_2$ reaction with adrenalin as described by McCord and Fridovich [13].

The measurement of ketosis by beta-hydroxybutyrate (BHB) was performed using a Free Style Optium Neo human line device (Drogasil, Butantã, Brazil), which measures the concentration of ketone bodies through a drop of blood placed on the device's strip. Values used as reference for BHB are <1.1 mmol/L absence of ketosis, >1.2 mmol/L subclinical ketosis, and above 3.5 mmol/L clinical ketosis, according to Duffield et al. [14].

### *2.8. Milk Analysis*

Analyses of chemical composition, and SCC were performed by the Commercial Milk Quality Laboratory, Curitiba, PR, accredited to the Ministry of Agriculture, Livestock, and Supply by MAPA ordinance. The quantification of fat, protein, lactose, total solids, and the defatted dry extract was performed using the Mid-Infrared Spectrometry Method, according to ISO 9622—IDF Standart 141:2013 (Brasilia, Brazil, 2013). The SCC by the Flow Cytometry Method, according to ISO 13366-2—IDF Standart 148-2, (Brasilia, Brazil, 2006).

### *2.9. Determination of Short-Chain Fatty Acids (SCFA) in Ruminal Liquid*

Ruminal fluid samples were thawed at 5 °C and manually shaken for homogenization. Aliquots of 1 mL of the supernatant of ruminal fluid samples were collected in polypropylene microtubes (2 mL) and then centrifuged for 5 min (12,300× *g*). Then, 250 µL of the supernatant was transferred to a new microtube containing 250 µL of formic acid. The mixture was manually shaken and centrifuged for 3 min. After centrifugation, 250 µL of the supernatant of the mixture were collected in another polypropylene tube previously con-

taining 500 µL of isoamyl alcohol (3-metil-1-butanol) solution (638.28 µg $ml^{-1}$ in methanol), used as an internal standard, and were homogenized and centrifuged again. In total, 650 µL of sample was inserted into a 2 mL injection vial. Then, 1 µL of extract was injected into a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3400, Palo Alto, Sudbury, MA, USA) and an autosampler (Varian 8200CX, Palo Alto, Sudbury, MA, USA) in split mode (1:10) at 250 °C. The carrier gas used was hydrogen at a constant pressure of 20 psi. The analytes (acetic, propionic, butyric, valeric and isovaleric acids) were separated by a CP-Wax 52CB capillary column (60 m × 0.25 mm; 0.25 µm stationary phase thickness). The initial column temperature was set at 80 °C for 1 min and increased to 120 °C at a rate of 8 °C $min^{-1}$, then to 230 °C for 20 °C $min^{-1}$, where it remained for 1 min. The detector temperature was set to 250 °C. Method validation comprised the following parameters: selectivity, linearity, linear range, repeatability, precision, limit of detection (LOD) and limit of quantification (LOQ) for acetic, propionic, butyric, and isovaleric acids. Linearity was assessed by calculating a regression equation using the method of least squares. LOD and LOQ values were obtained by sequential dilutions up to signal-to-noise ratios of 3:1 and 6:1, respectively. Precision was assessed by analyzing the repeatability of six replicated samples. Accuracy was determined by recovering known amounts of the standard substances added to a diluted sample (Table S1). Valeric acid was expressed as equivalent of isovaleric acid. The results were expressed in mol 100 $mol^{-1}$ of each SCFA in the ruminal fluid.

### *2.10. Statistical Analysis*

A descriptive analysis of the data was performed by applying the normality test (Shapiro–Wilk) and evaluating the residual error. Data without a normal distribution were transformed to logarithms and normalized for subsequent analyses. All data were analyzed using the SAS 'MIXED procedure' (SAS Inst. Inc., Cary, NC, USA; version 9.4) with Satterthwaite's approximation to determine the denominator degrees of freedom for the fixed-effects test. Mean milk production and feed efficiency variables were tested for a fixed effect of treatment using animals (treatment) as a random effect. The remaining data were analyzed as repeated measures and tested for fixed treatment effects, day effect, and treatment × day, with animal (treatment) as a random effect. The d1 results were included as independent covariate. Additionally, for these variables, the d1 scores were removed from the dataset to generate the mean per treatment but were retained as covariates. The first-order autoregressive covariance structure was selected according to the lowest Akaike information criterion. Means were separated using the PDIFF method (Student test), and all results were reported as LSMEANS followed by standard error (SEM). Significance was defined as $p \leq 0.05$, and a trend when $p > 0.05$ and $\leq 0.10$.

## 3. Results

The results of productive performance were presented in Table 2 and Figure 1. Cows that consumed GAA consumed a greater amount of feed intake (6%) compared to the control during the experimental period, and there was an interaction between treatment × day for feed intake (Figure 1). The highest milk production (5.3%) and fat-corrected milk production (7.63%) were higher in cows that consumed GAA when compared to the control. There was an interaction between treatment and day for milk production, which was higher at some points during the experiment in cows in the treatment group (Figure 1). There was no effect of treatment on feed efficiency; nor was there an interaction between treatment and day for body condition score.

**Table 2.** Productive performance of Jersey cows fed guanidinoacetic acid (treatment group) and without additive (control group).

| Variables | Control | Treatment | SEM | P: Treat [2] | P: Treat × Day [1] |
|---|---|---|---|---|---|
| Feed intake (kg DM/day) | | | | | |
| d1 to 14 | 14.4 | 15.3 | 0.21 | 0.24 | 0.18 |
| d15 to 28 | 14.9 [b] | 15.8 [a] | 0.22 | 0.05 | 0.03 |
| Milk production (kg/cow/day) | | | | | |
| d -14 to -1 | 17.9 | 17.9 | 0.21 | 0.94 | - |
| d1 to 14 | 16.6 | 17.1 | 0.17 | 0.25 | 0.12 |
| d15 to 28 | 17.0 [b] | 17.9 [a] | 0.18 | 0.05 | 0.02 |
| Fat-corrected milk (4%FCM) [3] (kg/cow/day) | | | | | |
| d15 to 28 | 18.8 [b] | 20.2 [a] | 0.20 | 0.02 | - |
| Feed efficiency, kg/kg | | | | | |
| d15 to 28 | 1.26 | 1.27 | 0.02 | 0.92 | - |
| Body score | | | | | |
| d1 to 14 | 3.43 | 3.49 | 0.05 | 0.98 | 0.97 |
| d15 to 28 | 3.45 | 3.48 | 0.04 | 0.96 | 0.96 |

[1] The interactions between treatment × day were presented in Figure 1. [2] Treatment effect: consecutive means with different subscript letters (a,b) in the same row illustrate the difference between groups when considering $p \leq 0.05$. [3] Milk production corrected for 4% fat (4% FCM) was estimated by the equation proposed by the NRC (2001): FCM = 0.4 × (kg of milk produced) + 0.15 × (% fat) × (kg of milk produced).

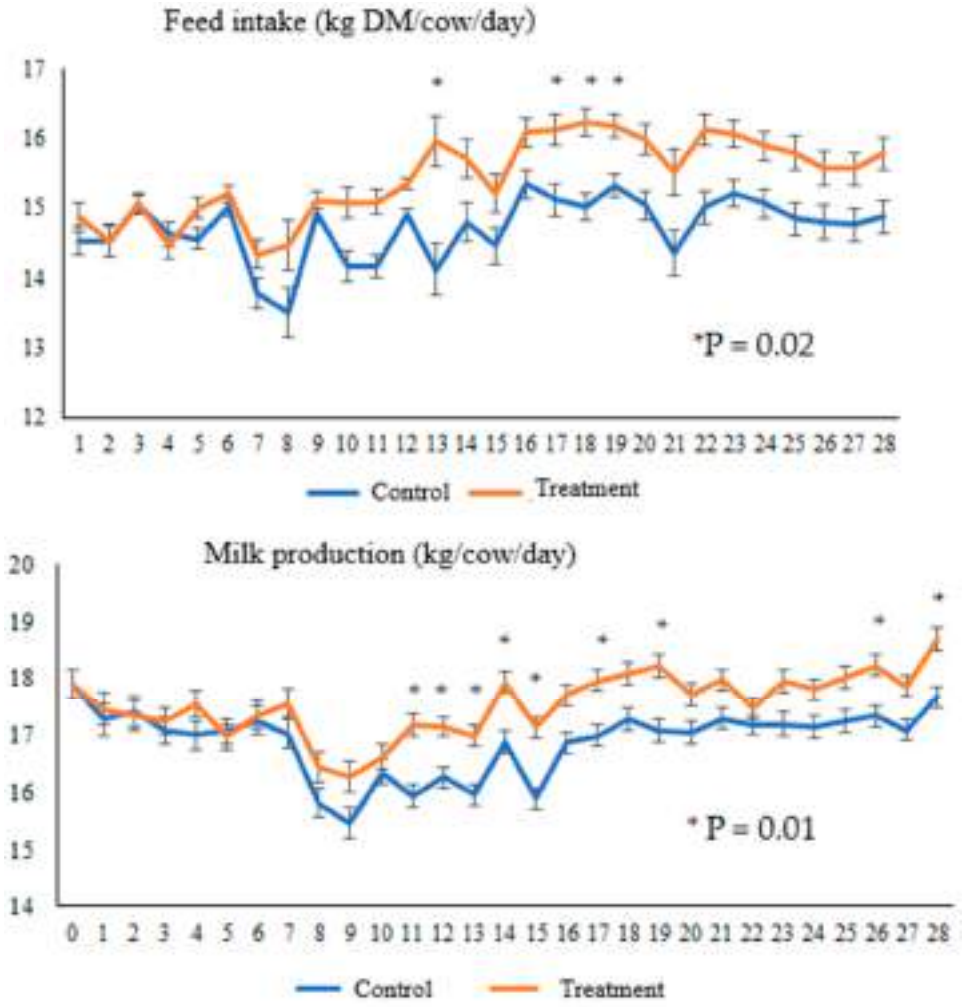



**Figure 1.** Images show that there was a treatment × day interaction for feed intake ($p = 0.02$) and milk production ($p = 0.01$) during some moments of the 28-day experimental period. We used the asterisk (*) to identify the days when the difference occurred.

Milk analysis results were presented in Tables 3 and 4. No treatment effect or treatment × day interaction was observed for the amount of fat, protein and lactose in the milk. There was a treatment × day interaction for total solids, being 8.75% higher in the milk of cows that consumed GAA compared to the control. There was no effect of treatment or treatment × day interaction for somatic cell counts. There was no effect of treatment or treatment × day interaction on the fatty acid profile in the milk of cows increased or not with GAA; with the exception of oleic acid (treatment effect: $p = 0.05$). Also was no difference between groups for the sum of saturated and unsaturated fatty acids; in addition to omegas (Table 4).

**Table 3.** Centesimal composition of milk from cows fed guanidinoacetic acid and control group.

| Variables | Control | Treatment | SEM | P: Treat [1] | P: Treat × Day [2] |
|---|---|---|---|---|---|
| Fat (g/100 g) | 4.72 | 4.87 | 0.11 | 0.72 | 0.66 |
| Protein (g/100 g) | 3.66 | 3.80 | 0.15 | 0.31 | 0.25 |
| Lactose (g/100 g) | 4.92 | 5.16 | 0.18 | 0.23 | 0.18 |
| Total solid (g/100 g) | | | | 0.56 | 0.05 |
| d1 | 13.6 | 13.5 | 0.21 | | |
| d14 | 14.3 | 14.9 | 0.26 | | |
| d21 | 14.1 | 13.7 | 0.17 | | |
| d28 | 13.7 [b] | 14.9 [a] | 0.18 | | |

Note 1: there was no treatment effect on these variables ($p > 0.05$); Note 2: significant treatment × day. interaction ($p \leq 0.05$) was illustrated by different letters in the same line (a,b).

**Table 4.** Fatty acid profile in milk from cows fed guanidinoacetic acid.

| Fatty Acid | Control | Treatment | SEM | P-Treat [1] | P-Treat × Day [1] |
|---|---|---|---|---|---|
| C6:0 (Caproic) | 0.60 | 0.59 | 0.02 | 0.95 | 0.92 |
| C8:0 (Caprylic) | 0.55 | 0.54 | 0.01 | 0.96 | 0.89 |
| C10:0 (Capric) | 1.96 | 1.99 | 0.03 | 0.95 | 0.97 |
| C11:0 (Undecanoic) | 0.19 | 0.19 | 0.00 | 0.98 | 0.98 |
| C12:0 (Lauric) | 2.98 | 3.06 | 0.06 | 0.87 | 0.91 |
| C13:0 (Tridecanoic) | 0.14 | 0.15 | 0.01 | 0.96 | 0.93 |
| C14:0 (Myristic) | 11.5 | 11.6 | 0.29 | 0.97 | 0.94 |
| C14:1 (Myristoleic) | 0.70 | 0.66 | 0.05 | 0.89 | 0.86 |
| C15:0 (Pentadecanoic) | 1.16 | 1.21 | 0.04 | 0.84 | 0.76 |
| C16:0 (Palmitic) | 46.5 | 45.5 | 0.41 | 0.56 | 0.42 |
| C16:1 (Palmitoleic) | 1.32 | 1.15 | 0.02 | 0.18 | 0.12 |
| C17:0 (Heptadecanoic) | 0.50 | 0.53 | 0.03 | 0.92 | 0.80 |
| C17:1 (cis-10-Heptadecenoic) | 0.17 | 0.15 | 0.01 | 0.95 | 0.92 |
| C18:0 (Stearic) | 11.3 | 11.4 | 0.12 | 0.97 | 0.98 |
| C18:1n9t (Elaidic) | 1.21 | 1.20 | 0.02 | 0.97 | 0.99 |
| C18:1n9c (Oleic) | 16.3 [b] | 17.7 [a] | 0.16 | 0.05 | 0.11 |
| C18:2n6c (Linoleic) | 1.74 | 1.80 | 0.04 | 0.90 | 0.68 |
| C20:0 (Arachidic) | 0.18 | 0.19 | 0.02 | 0.97 | 0.98 |
| C18:3n6 (?-Linolenic) | 0.03 | 0.02 | 0.00 | 0.98 | 0.99 |
| C20:1n9 (cis-11-Eicosenoic) | 0.06 | 0.05 | 0.00 | 0.98 | 0.99 |
| C18:3n3 (a-Linolenic) | 0.14 | 0.15 | 0.01 | 0.98 | 0.98 |
| C21:0 (Henicosanoic) | 0.26 | 0.26 | 0.03 | 0.99 | 0.96 |
| C20:2 (cis-11,14-Eicosadienoic) | 0.02 | 0.03 | 0.00 | 0.97 | 0.99 |
| C22:0 (Behenic) | 0.09 | 0.09 | 0.00 | 0.99 | 0.99 |
| C20:3n6 (cis-8,11,14-Eicosatrienoic) | 0.07 | 0.07 | 0.00 | 0.99 | 0.99 |
| C20:4n6 (Arachidonic) | 0.05 | 0.05 | 0.00 | 0.99 | 0.99 |

**Table 4.** *Cont.*

| Fatty Acid | Control | Treatment | SEM | P-Treat [1] | P-Treat × Day [1] |
|---|---|---|---|---|---|
| Other variables | | | | | |
| ∑ Saturated fatty acids (SFA) | 78.0 | 77.4 | 0.62 | 0.79 | 0.65 |
| ∑ Unsaturated fatty acids (UFA) | 21.9 | 22.5 | 0.20 | 0.73 | 0.59 |
| ∑ Monounsaturated fatty acids (MUFA) | 19.8 | 20.3 | 0.22 | 0.87 | 0.91 |
| ∑ Polyunsaturated fatty acids (PUFA) | 2.07 | 2.14 | 0.13 | 0.71 | 0.82 |
| UFA/SFA | 0.28 | 0.29 | 0.03 | 0.96 | 0.98 |
| ∑ ω6 | 1.89 | 1.95 | 0.06 | 0.77 | 0.89 |
| ∑ ω3 | 0.16 | 0.16 | 0.01 | 0.98 | 0.99 |
| ω6/ω3 | 12.1 | 11.9 | 0.65 | 0.92 | 0.81 |

Note 1: there was no treatment effect and treatment versus day on these variables ($p > 0.10$), with the exception of oleic acid ($p = 0.05$) illustrated by different letters on the same line (a,b).

The blood count of the cows was presented in Table 5. Lower leukocytes count due to lower lymphocyte count was observed in cows that consumed GAA compared to the control, with treatment effect and treatment × day interaction (d21 and d28). No treatment effect or interaction between groups was observed for hematocrit, platelets, erythrocytes, hemoglobin, granulocytes and monocytes ($p > 0.05$).

**Table 5.** Hemogram of cows fed with guanidinoacetic acid (treatment group) and without additive (control group).

| Variables | Control | Treatment | SEM | P: Treat [1] | P: Treat × Day [2] |
|---|---|---|---|---|---|
| Leukocyte ($\times 10^3/\mu L$) | | | | 0.05 | 0.04 |
| d1 | 6.70 | 6.50 | 0.21 | | |
| d14 | 7.10 | 6.37 | 0.28 | | |
| d21 | 8.71 [a] | 7.47 [b] | 0.25 | | |
| d28 | 8.13 [a] | 7.16 [b] | 0.22 | | |
| Lymphocyte ($\times 10^3/\mu L$) | | | | 0.04 | 0.05 |
| d1 | 4.07 | 3.89 | 0.17 | | |
| d14 | 4.59 | 3.40 | 0.16 | | |
| d21 | 4.55 [a] | 3.45 [b] | 0.19 | | |
| d28 | 5.06 [a] | 4.21 [b] | 0.16 | | |
| Hematocrit (%) | 27.9 | 26.8 | 0.48 | 0.96 | 0.92 |
| Platelets ($\times 10^3/\mu L$) | 243 | 277 | 15.5 | 0.85 | 0.74 |
| Erythrocytes ($\times 10^6/\mu L$) | 6.00 | 6.58 | 0.15 | 0.81 | 0.73 |
| Hemoglobin (mg/dL) | 10.5 | 10,0 | 0.21 | 0.89 | 0.82 |
| Granulocyte ($\times 10^3/\mu L$) | 3.15 | 2.95 | 0.22 | 0.79 | 0.50 |
| Monocyte ($\times 10^3/\mu L$) | 0.43 | 0.39 | 0.08 | 0.80 | 0.84 |

Note 1. Treatment effect, when $p < 0.05$. Note 2: Treatment × day interaction, illustrated by different letters (a,b) on the same line.

Serum biochemistry results were presented in Table 6. No treatment effect or treatment × day interaction was observed for albumin, total protein, globulin, C-reactive protein, glucose, uric acid and urea. There was a treatment effect for cholesterol levels, as well as a treatment × day interaction; cholesterol levels were higher in the serum of cows that consumed GAA on days 21 and 28. Creatine kinase and gamma glutamyl transferase activities were lower in the serum of cows that consumed GAA when compared to the control, as a treatment effect and interaction on days 14, 21 and 28 of the experiment. Treatment × day interaction for AST (d21 and d28) and ALT (d28) activities were lower in the serum of cows in the treatment group compared to the control.

**Table 6.** Metabolic, biochemical, enzymatic and oxidative stress markers in cows fed guanidinoacetic acid (treatment group) and without additive (control group).

| Variables | Control | Treatment | SEM | P: Treat [1] | P: Treat × Day [2] |
|---|---|---|---|---|---|
| Serum biochemistry | | | | | |
| Albumin (g/dL) | 3.32 | 3.26 | 0.12 | 0.95 | 0.92 |
| Total protein (g/dL) | 7.15 | 7.13 | 0.23 | 0.97 | 0.94 |
| Globulin (g/dL) | 3.82 | 3.87 | 0.14 | 0.95 | 0.96 |
| C-reactive protein (mg/dL) | 5.82 | 6.12 | 0.23 | 0.82 | 0.61 |
| Glucose (mg/dL) | 67.5 | 67.4 | 1.20 | 0.96 | 0.91 |
| Uric acid (mg/dL) | 0.96 | 1.00 | 0.02 | 0.89 | 0.93 |
| Urea (mg/dL) | 40.9 | 41.4 | 0.95 | 0.96 | 0.89 |
| Cholesterol (mg/dL) | | | | 0.05 | 0.01 |
| d1 | 146 | 148 | 2.51 | | |
| d14 | 148 | 153 | 2.54 | | |
| d21 | 156 [b] | 178 [a] | 2.53 | | |
| d28 | 164 [b] | 188 [a] | 2.54 | | |
| Creatine kinase (U/L) | | | | 0.01 | 0.01 |
| d1 | 142 | 138 | 3.94 | | |
| d14 | 185 [a] | 136 [b] | 3.95 | | |
| d21 | 155 [a] | 118 [b] | 3.54 | | |
| d28 | 134 [a] | 99.8 [b] | 3.50 | | |
| GGT (U/L) | | | | 0.03 | 0.05 |
| d1 | 50.4 | 46.2 | 2.08 | | |
| d14 | 35.7 [a] | 22.1 [b] | 2.08 | | |
| d21 | 36.2 [a] | 28.4 [b] | 2.11 | | |
| d28 | 35.5 [a] | 30.5 [b] | 1.89 | | |
| ALT (U/L) | | | | 0.36 | 0.05 |
| d1 | 30.7 | 34.4 | 1.41 | | |
| d14 | 30.8 | 30.8 | 1.36 | | |
| d21 | 30.3 | 30.2 | 1.25 | | |
| d28 | 33.4 [a] | 26.1 [b] | 1.21 | | |
| AST (U/L) | | | | 0.10 | 0.01 |
| d1 | 128 | 116 | 3.41 | | |
| d14 | 141 | 149 | 3.39 | | |
| d21 | 119 [a] | 107 [b] | 3.42 | | |
| d28 | 132 [a] | 112 [b] | 3.40 | | |
| Oxidative status | | | | | |
| SOD (U SOD/mL) | | | | 0.54 | 0.02 |
| d1 | 0.93 | 0.95 | 0.05 | | |
| d14 | 0.96 | 0.96 | 0.05 | | |
| d21 | 0.95 | 0.91 | 0.05 | | |
| d28 | 1.08 [b] | 1.66 [a] | 0.07 | | |
| GST (U GST/mL) | | | | 0.25 | 0.01 |
| d1 | 1.35 | 1.32 | 0.08 | | |
| d14 | 1.46 | 1.23 | 0.08 | | |
| d21 | 1.51 [b] | 2.66 [a] | 0.09 | | |
| d28 | 1.12 [b] | 1.79 [a] | 0.07 | | |
| TBARS (nmol MDA/mL) | | | | 0.38 | 0.05 |
| d1 | 0.84 | 0.81 | 0.04 | | |
| d14 | 0.89 | 0.75 | 0.04 | | |
| d21 | 1.09 | 0.99 | 0.05 | | |
| d28 | 1.74 [a] | 1.09 [b] | 0.05 | | |
| BHB (mmol/L) | | | | 0.05 | 0.05 |
| d1 | 1.08 | 1.02 | 0.04 | | |
| d14 | 1.81 [a] | 1.40 [b] | 0.07 | | |
| d28 | 1.80 [a] | 1.38 [b] | 0.07 | | |

Note 1. Treatment effect, when $p < 0.05$. Note 2: Treatment × day interaction, illustrated by different letters (a,b) on the same line.

Results of oxidative biomarkers were presented in Table 6. There was treatment × day interaction for SOD and GST activity, as well as TBARS levels. Higher SOD (d28) and

GST (d21 and d28) activity in the serum of cows that consumed GAA; concomitantly with lower TBARS levels on day 28. Beta hydroxide butyrate (BHB) in whole blood of cows that consumed GAA was lower when compared to the control.

Results of ruminal fluid biomarkers were presented in Table 7. Lower ruminal fluid pH was observed when cows consumed GAA compared to the control. Treatment × day interaction was observed for bacterial activity (MBRT), with greater bacterial activity being observed on day 14 in cows in the treatment group compared to the control. No effect of treatment or treatment × day interaction was observed for levels of short-chain fatty acids (SCFA), acetic, propionic, butyric, isovaleric and valeric acid.

**Table 7.** pH and methylene blue reduction test (MBRT) and volatile fatty acids in ruminal fluid of cows fed guanidinoacetic acid (treatment group) and without additive (control group).

| Variables | Control | Treatment | SEM | P: Treat [1] | P: Treat × Day [2] |
|---|---|---|---|---|---|
| pH | | | | 0.05 | 0.05 |
| d1 | 6.43 | 6.42 | 0.04 | | |
| d14 | 6.67 [a] | 6.47 [b] | 0.04 | | |
| d28 | 7.08 [a] | 6.87 [b] | 0.05 | | |
| MBRT (s) | | | | 0.21 | 0.05 |
| d1 | 266 | 225 | 20.1 | | |
| d14 | 298 [a] | 187 [b] | 16.4 | | |
| d28 | 400 | 381 | 25.9 | | |
| SCFA (mmol/L) | 96.3 | 96.6 | 4.21 | 0.95 | 0.97 |
| Acetic acid (mmol/L) | 64.9 | 64.7 | 3.14 | 0.96 | 0.92 |
| Propionic acid (mmol/L) | 17.3 | 17.7 | 2.02 | 0.95 | 0.97 |
| Butiryc acid (mmol/L) | 11.5 | 11.6 | 1.12 | 0.94 | 0.94 |
| Isovaleric acid (mmol/L) | 1.27 | 1.34 | 0.08 | 0.67 | 0.52 |
| Valeric acid (mmol/L) | 1.12 | 1.12 | 0.02 | 0.96 | 0.98 |

Note 1. Treatment effect, when $p < 0.05$. Note 2: Treatment × day interaction, illustrated by different letters (a,b) on the same line.

## 4. Discussion

The inclusion of GAA in the diet of lactating Jersey cows (230 days) increased feed intake (dry matter), unlike what had occurred in Holstein cows [15]; however, it is worth noting that the cows used in the experiment by Liu et al. [15] were 18 days into lactation, and the experiment lasted for 80 days, that is, a study at peak production; but also a period of negative energy metabolism of the cow, with different and individual responses, which may explain the different results of the two studies for feed intake. In addition, the daily intake of a Holstein cow is generally greater than that of Jersey cows, as well as the size/weight of Holstein cows has greater variability, and this may reflect on the cow's feed consumption. Another study with GAA reports that the consumption of the additive stimulated the feed consumption [5] of Angus beef cattle. The technical explanation for the improvement in feed consumption is not known, but it is desired, because it is reflected in greater milk production, as occurred in the present study.

Liu et al. [15] also found that the addition of GAA increased the milk production of Holstein cows, which, according to the authors, is mainly related to the increase in total nutrient availability, due to the increase in short-chain fatty acids (VFA) in the rumen and increased creatine in the blood, which can provide more energy for milk production. In the present study, the higher milk production of Jersey cows that consumed GAA appears to be related to higher feed intake, as there was no effect of the treatment on milk efficiency, nor did it change the proportion of AVG in the rumen. However, we cannot disregard other indirect effects related to animal health and regulation of energy metabolism, which will be discussed below and may have influenced the higher daily milk production.

In our research, we found lower pH of the ruminal fluid and higher bacterial activity in the rumen of cows that consumed GAA. This lower pH at both times is a result that is repeated in GAA intake, as it has already been described in Angus cattle [5]. This is because pH changes are generally related to the increase in VFA as a consequence of the increase in propionate, leading to a slight decrease in pH important for fiber degradation. It is important to highlight that Jersey cows that consumed GAA did not have any change in the AVG profile, unlike the study with Holstein cows, because when the level of GAA addition increased in their dose study, the ruminal pH, the molar percentages of butyrate, isobutyrate and isovalerate and the acetate–propionate ratio decreased linearly, and the total concentration of volatile fatty acids and the molar percentage of propionate increased linearly. According to Vázquez-Mendoza et al. [16], an in vitro study, GAA is susceptible to degradation in the rumen, is used as a source of energy and nitrogen by microorganisms for protein synthesis; as well as having the potential to increase the activities of fibrolytic enzymes, amylase and protease combined with the total increase in the bacterial population and the improvement in feed degradation. These researchers describe that the higher fermentation content is related to the positive modulation of the population of ruminal microorganisms, an increase in microorganisms capable of increasing the digestibility of nutrients, which directly influences AVGs [16]. Researchers have already described that the addition of GAA to the diet of beef cattle increases crude protein digestibility, as well as increases the proportion of propionate [5], similar to what was described in another recent study [17] where it was found that the concentrations of acetate and the acetate–propionate ratio were significantly lower in the cattle that consumed GAA, while propionate concentration was significantly higher. However, the intake of GAA at a dose of 9.9 g/animal/day reflected in a higher concentration of acetic, propionic and butyric acids in the ruminal fluid of beef cattle [18]. Therefore, these articles show that changes occur; but these are not absolute in the different animal categories.

The highest bacterial activity in the ruminal environment occurred when cows consumed GAA, which may be related to the fact that the product is partially degraded in the rumen and can thus be used by the microbial population for its growth and population, which influences the higher milk production of these cows. In Holstein cows, researchers investigated several ruminal biomarkers and found that in the control group, enzymatic activities increased with the addition of GAA [15], as did the protozoan population. Studies with GAA in the diet of ruminants have already shown that the presence of this additive in the rumen has the property of modulating the ruminal microbiota [5,15,17], which may explain these effects on ruminal fermentation.

In the milk of Jersey cows, a higher concentration of total solids was observed, which is desired for milk quality and also for better use in the industry. However, it is important to remember that this increase in total solids does not reflect just one component of the milk, but rather all three main components (fat, protein and lactose) that were numerically in higher proportions in the milk of cows that consumed GAA. The fatty acid profile had a slight change, with only a greater amount of oleic acid being observed in the milk of cows fed GAA; cattle that consumed GAA had an increase in the proportion of linoleic acid, reflecting greater PUFA in the meat [18].

The lowest lymphocyte count was observed in the blood of cows that consumed GAA, combined with lower levels of beta-hydroxybutyrate and lipid peroxidation, combined with higher activity of antioxidant enzymes (SOD and GST) are indicators of the health benefits of the additive to cows, which can be interpreted as an anti-inflammatory effect and capable of minimizing metabolic disorders and oxidative stress. The reduction in total leukocytes due to the reduction in lymphocytes had already been described by Giraldi et al. [18] when they supplied it to beef cattle, without altering globulin levels, as also

occurred in the present study. The recurrence of this finding needs to be better understood in order to understand the mechanism involved in future studies by our research group.

The creatine synthesis process occurs in two stages, catalyzed by L-arginine:glycine amidinotransferase and guanidinoacetate N-methyltransferase, which occur mainly in the kidney and liver, respectively [19]. According to the literature, this molecule plays an important energy/pH buffer function in tissues and, to ensure the maintenance of its total body pool, lost creatine must be replaced through the diet or de novo synthesis. When creatine is administered, there is a reduction in the consumption of sadenosyl methionine, reducing the production of homocysteine in the liver, decreasing fat accumulation, as well as resulting in beneficial effects on the liver [19]. This mechanism probably occurred in the Jersey cows in this study, which reflected in lower activity of AST, AST and GGT, enzymes that indicate liver disorders and injury, reflecting an improvement in lipid metabolism, providing a greater quantity of serum cholesterol, an important lipid for several physiological functions [20–22].

Creatine kinase (CK) is an enzyme that plays a fundamental role in energy metabolism and is found in various tissues, particularly skeletal and cardiac muscles and the brain [23,24]. CK plays several functions in the body, being responsible for energy production during muscle contraction, ATP regeneration and phosphorylation, and energy transport between mitochondria and cytoplasm [24,25]. In the Jersey cows in this study, CK activity was lower in serum, which was unexpected, since the addition of GAA increases blood creatine levels [5].

## 5. Conclusions

We conclude that the addition of GAA stimulates feed intake and increases milk production in Jersey cows after the animals' adaptation period. Cows fed GAA had a higher total solids content at the end of the experiment, combined with a higher proportion of oleic acid in the milk. GAA intake does not alter the volatile fatty acid profile but reduces pH and increases bacterial activity in the rumen of Jersey cows during experimental period. GAA intake in cows showed anti-inflammatory and antioxidant effects, and in the final days of the experiment, it exhibited hepatoprotective effects and minimized physiological oxidative stress in dairy cows during the experimental period.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation11020085/s1, Table S1: Standardization of reading of volatile fatty acid profile in ruminal fluid; Table S2: Fatty acid profile in the total mixed ration (TMR) used in this experiment.

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# Addition of Exogenous Fibrolytic Enzymes to the Feed of Confined Steers Modulates Fat Profile in Meat

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**Simple Summary:** We used male dairy cattle (Holstein) as an experimental model for testing exogenous enzymes. The addition of fibrolytic enzymes to the diet of feedlot cattle did not significantly enhance digestibility, but there was a tendency for a higher NDF coefficient. The consumption of fibrolytic enzymes altered the ruminal fatty acid profile, especially a higher amount of volatile fatty acids (VFA). A higher quantity and proportion of acetic acid in the ruminal fluid may have been precursors of higher fat content in the meat, in addition to modulating the fatty acid profile, reducing SFA and increasing UFA in the meat.

**Abstract:** The objective of this study was to evaluate whether the addition of fibrolytic enzymes to the diet of cattle in confinement improves feed digestibility weight gain; as well as evaluating the profile of fatty acids in the ruminal environment and in meat fatty acids profile. In total, 24 male dairy cattle (Holstein) aged 8 months and weighing an average of 212 kg, were divided into 2 groups: control (without additive) and treatment (0.25 g of enzymes/kg of dry matter of total diet). The experiment lasted 120 days. The first 20 days are allocated for the adaptation period. During the study, samples of blood, ruminal fluid, and feces were collected, as well as weighing the cattle and measuring their daily feed consumption. There was no effect of treatment on body weight, feed intake, feed efficiency, and nutrient digestibility ($p > 0.05$). Cholesterol levels were higher in the serum of cattle that consumed the enzyme; serum amylase activity was higher in cattle that received the additive only on day 120 of the experiment ($p < 0.05$). There was a greater amount of volatile fatty acids in the ruminal fluid, combined with a greater amount of acetic acid. The amount of fat in the meat of cattle that consumed fibrolytic enzymes was higher compared to the control group ($p < 0.05$). Meat from cattle in the treatment group had lower amounts of saturated fatty acids and higher amounts of unsaturated fatty acids ($p < 0.05$). The fibrolytic enzymes addition altered rumen fermentation in such a way that lipid metabolism was changed, which had a serious impact on cholesterol and tissue levels, that is, in the meat that had a greater amount of total lipids, an unsaturated fat.

**Keywords:** rumen fermentation; volatile fatty acids; lipid metabolism; meat quality

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## 1. Introduction

Feed additives are adopted in 99.8% of confinement systems in Brazil [1]. With the primary objective of modulating rumen fermentation, the main group of additives are ionophores, which act preferentially on Gram-positive bacteria, favoring the growth of Gram-negative bacteria [2]. Research on the use of exogenous fibrolytic enzymes in ruminants dates back to the 1960s [3]. These additives aim to improve nutrient availability in the digestive system and break specific chemical bonds in raw material [4]. Previous studies have highlighted the positive effects of fibrolytic enzymes in ruminant diets, resulting in improvements in animal performance [5–7]. Exogenous fibrolytic enzymes, such as xylanases and cellulases, are widely used in ruminant feeding, with a focus on optimizing the digestibility of fiber present in low quality roughages [8]. Bureenok et al. [9] concluded that the addition of fibrolytic enzymes improves the digestibility of fiber and proteins.

Several studies have demonstrated that adding fibrolytic enzymes to ruminant diets can increase milk production in cows [7]. Liu et al. [10] observed that the combination of cellulase, xylanase, β-glucanase, and amylase in dairy cows at the beginning of lactation increases enzymatic activity in the rumen, resulting in improvements in productive performance, with a positive trend for feed efficiency. However, animal responses to enzyme addition vary considerably [5]. This variation can be attributed to a number of factors, such as variations in enzymatic activity, rate and method of application, substrate specificity, and physiological stage of the animals [11]. Therefore, the main objective of this study was to evaluate whether the addition of fibrolytic enzymes improves digestibility and growth performance, as well as the meat quality of steers finished in confinement.

## 2. Materials and Methods

### *2.1. Enzymatic Blend*

The commercial product (Smizyme Multi-Nzymes—Salus) contains corn starch, 2.000 U/g of beta glucanase (*Trichoderma longibrachiatum*—CGMCC 7290), 300 U/g of cellulase (*Trichoderma longibrachiatum*—CGMCC 7289), and 20.000 U/g of xylanase (*Aspergillus oryzae*—CGMCC 7.70).

### *2.2. Animals, Installation and Experimental Design*

The experiment was carried out at the Experimental Farm of the State University of Santa Catarina (UDESC), in the municipality of Guatambu, Santa Catarina, southern Brazil. Twenty-four Holstein cattle (not-castrated) aged 8 months, and weighing approximately 212 (±4.62) kg were confined in a shed with individual stalls, with feeders and drinkers, which allowed daily measurement of feed intake. The feeding of these animals was based on corn silage and concentrate (ground corn, soybean meal, soybeans, wheat bran and mineral, and vitamin core) in a proportion of 30:70%, respectively. The diet was calculated in BRCORTE 2016 [12], in order to meet the animal's nutritional demands so that it has an average daily gain of 1.5 kg during the experimental period of 120 days. It is important to make it clear that the cattle's diet was calculated individually for each animal, based on its initial body weight, and provided in a restrictive manner throughout the experiment, that is, what was necessary for the animal to gain 1.5 kg per day. Adjustments to the quantity of feed available to the animals were made at intervals of 15 days, to meet physiological demands, and also allow the animal to gain weight and accumulate fat in the carcass. The adaptation period lasted 20 days, followed by 100 experimental days. The animals were divided into two homogeneous groups with twelve animals each, considering body weight to form the groups: Control group (n = 12—without additive) and treatment group (n = 12–0.25 g of enzyme blend/kg DM of total mixed ration (TMR)). The enzymes were added to the animals' diet via mashed concentrate, being added during production.

### *2.3. Data and Sample Collection*

The animals were weighed at 30-day intervals; feed intake was measured daily. Based on weight gain and consumption information, feed efficiency and feed conversion were calculated.

Blood collection was carried out on days 1, 20, 60, and 120 of the experiment, in order to evaluate the health of the steers. Blood samples were collected from the coccygeal vein, in tubes with anticoagulant for blood count analysis; another blood sample was stored in tubes without anticoagulant to obtain the serum that was used in metabolic biochemistry analyses. To obtain serum, the tubes were centrifuged (800× *g*) for 10 min; then the serum was collected and stored in Eppendorf tubes (−20 °C) until analysis.

Rumen fluid collection was performed using an esophageal probe coupled to the vacuum system at the end of the adaptation period (day 20), as well as on days 60 and 120 of the study. The first 100 mL of the collected liquid were discarded. This material was filtered using a double layer of gases and then frozen (−20 °C) to measure the volatile fatty acid profile.

During 5 consecutive days (between days 101 and 105 of the experiment), feces were collected directly from the rectum. During the collection period, samples were collected at different times of the day (08:00, 10:00; 13:00; 15:00, and 17:00) to have a homogeneous final sample. At the end of the collection, a pool of five collections per animal was made. The material was used for analyses to determine the apparent digestibility coefficient of nutrients.

At the end of the experiment, the animals were sent to the slaughterhouse, and the hot carcass weight was obtained on the slaughter line, as well as a fragment of muscle (Longissimus thoracic) was collected to evaluate the fat and fatty acid profile in the meat.

### *2.4. Feed Composition Analysis*

The feed and feces samples were pre-dried in a forced ventilation oven at 55 °C for 72 h, after being removed from the oven, weighed to determine the partial dry matter content, and then ground in a Wiley mill (Marconi, model: MA340), using a 2 mm mesh sieve. The pre-dried and ground samples were heated at 105 °C to obtain the MS and the mineral material in a muffle furnace at 600 °C [13]. Samples were analyzed according to AOAC [14]: dry matter (DM), method 930.15; crude protein (CP), method 976.05; ether extract (EE), method 920.39 and ash, method 942.05. The concentrations of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined according to the methodology of Van Soest et al. [15] without the addition of sodium sulfite or alpha-amylase.

### *2.5. Hemogram*

The total erythrocyte and leukocyte count, hemoglobin concentration, hematocrit percentage, and leukocyte differentiation were performed with an automatic hematologic analyzer VET3000 (EQUIP®), obtained using blood tubes with EDTA. In addition to the variables mentioned above, the determination of hemoglobin concentration (g/dL), hematocrit (%), mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and amplitude red blood cell distribution (RDW-SD).

### *2.6. Serum Biochemistries*

The concentrations of total protein, albumin, cholesterol, fructosamide, urea, and uric acid were evaluated, as well as the activity of amylase in a semi-automatic analyzer (Bio-2000®, Bioplus Produtos para Laboratórios Ltd.a, Barueri, SP, Brazil) and using specific commercial kits for each variable (Analisa®, Belo Horizonte, Brazil). Globulin levels were obtained using the formula total protein—albumin.

### *2.7. Determination of Short Chain Fatty Acids (SCFA)*

The preparation of samples for analysis and measurement of the SCFA profile used in this study was described in detail by Brunetto et al. [16]. A volume of 650 µL of the sample was inserted into a 2 mL injection vial; 1 µL of extract was injected into a gas chromatograph equipped with a flame ionization detector (GC-FID; Varian Star 3400, Palo Alto, CA, USA) and an autosampler (Varian 8200CX, Palo Alto, CA, USA) in split mode (1:10) up to 250 °C to measure acetic, propionic, butyric, valeric, and isovaleric acids. Accuracy was determined by recovering known amounts of the standard substances added to a diluted sample (Table S1). The results were showed in mol 100 mol/L of each short-chain SCFA in the ruminal fluid.

### *2.8. Apparent Digestibility Coefficient*

Indigestible neutral detergent fiber (iNDF) was used as an internal marker to calculate apparent feed digestibility [17,18]. The feed and feces samples were weighed in bags with 16 µm porosity and incubated in the rumen of cattle for 288 h. Then, they were washed with tap water, treated with neutral detergent in an autoclave [19], and dried in a forced-air ventilation oven at 55 °C. Digestibility was calculated as 1 − (iNDF in feed (% of DM)/iNDF in feces (% of DM)).

### *2.9. Fatty Acid (FA) Profile in Meat and Feed*

To analyze the fatty acid profile in meat and feed, extraction was performed using the Bligh and Dyer [20] method with some modifications. FA methylation was also performed using a transesterification method proposed by Hartman and Lago [21]. Both methodologies have been previously described in detail by Brunetto et al. [16]. A TRACE 1310 gas chromatograph with a flame ionization detector was used to determine FAME, where one microliter of sample was injected into a split less injector operated in 1:10 split mode at 250 °C [16]. The chromatographic separation was performed using a CP-Sil 88 capillary column (100 m × 0.25 mm i.d., 0.20 µm film thickness) with helium as the carrier gas at a flow rate of 1 mL/min. The oven temperature program consisted of an initial temperature of 50 °C, held for 1 min, followed by an increase to 175 °C at 13 °C/min, held for 27 min, and then increased to 215 °C at 4 °C/min, held for 30 min. The results were presented as a percentage of each FA identified in the lipid fraction to according with Visentainer and Franco [22]. The results of FA at feed (TMR) are presented in Table 1.

**Table 1.** Feed composition and fatty acid profile of diets fed to experimental animals.

| Variables, % | TMR: Control | TMR: Treatment |
|---|---|---|
| Dry matter (%) | 90.25 | 90.44 |
| Organic matter, % MS | 93.38 | 93.36 |
| Crude protein, % MS | 15.78 | 15.76 |
| Ether extract, % MS | 2.87 | 3.07 |
| Ash, % MS | 6.62 | 6.64 |
| NDF, % MS | 28.80 | 25.68 |
| **Fatty acid, %** | | |
| C14:0 (Myristic) | 0.313 | 0.331 |
| C14:1 (Myristoleic) | 0.003 | 0.022 |
| C15:0 (Pentadecanoic) | 0.174 | 0.192 |
| C16:0 (Palmitic) | 21.84 | 21.78 |
| C16:1 (Palmitoleic) | 0.300 | 0.318 |
| C17:0 (Heptadecanoic) | 0.295 | 0.313 |
| C18:0 (Stearic) | 4.679 | 4.682 |
| C18:1n9t (Elaidic) | 0.346 | 0.364 |
| C18:1n9c (Oleic) | 32.09 | 32.00 |

**Table 1.** *Cont.*

| Variables, % | TMR: Control | TMR: Treatment |
|---|---|---|
| C18:2n6c (Linoleic) | 34.47 | 34.37 |
| C20:0 (Arachidic) | 0.793 | 0.810 |
| C20:1n9 (cis-11-Eicosenoic) | 0.304 | 0.321 |
| C18:3n3 (a-Linolenic) | 2.407 | 2.417 |
| C20:2 (cis-11,14-Eicosadienoic) | 0.121 | 0.139 |
| C22:0 (Behenic) | 0.789 | 0.806 |
| C22:1n9 (Erucic) | 0.071 | 0.089 |
| ∑ Saturated fatty acids (SFA) | 29.88 | 29.95 |
| ∑ Unsaturated fatty acids (UFA) | 70.12 | 70.05 |

Note: The concentrate was formulated based on ground corn (63.04%), soybean hulls (25.5%), soybean meal (7.45%), mineral core (2.87%), and urea (1.15%).

### *2.10. Statistical Analysis*

The experimental data were first analyzed descriptively; measures of central tendency (median) and data dispersion (range that stands for the interval between the minimum and maximum values in the data) were computed. Further, all variables were subjected to the Shapiro–Wilk test, which revealed a normal data distribution. Skewness, kurtosis, and homogeneity were evaluated using the Levene test, and linearity was used using linear regression. All data were analyzed using the 'MIXED procedure' of SAS (SAS Inst. Inc., Cary, NC, USA; version 9.4, 2013), with Satterthwaite approximation to determine the denominator degrees of freedom for the test of fixed effects. The weight gain and feed efficiency were tested for treatment-fixed effects using a pen (treatment) and animal (pen) as random variables (i.e., pen within treatment and animal within pen). The data of body weight, all blood, and rumen fluid results were analyzed as repeated measurements and tested for fixed effects of treatment, day, and treatment × day, and pen (treatment) and animal (pen) as random variables. Post-mortem variable analysis (meat, carcass, and fatty acid profile) was tested using animal (treatment) as random variables for treatment-fixed effects. The d1 results were included as an independent covariate. The first-order autoregressive covariance structure was selected according to the lowest Akaike information criterion. Means were separated using PDIFF method (Student test) and all results were reported as LSMEANS followed by SEM. Significance was defined when $p \leq 0.05$.

## 3. Results

### *3.1. Performance*

The results of weight and intake measurements are presented in Table 2. No difference was observed between groups for body weight, average daily gain, feed intake, feed conversion, and feed efficiency ($p > 0.05$).

**Table 2.** Fibrolytic enzymes impact on experimental animal's performance.

| Variables | Control | Treatment | SEM | *p*: Treat | *p*: Treat × Day |
|---|---|---|---|---|---|
| Body weight, kg | | | | 0.80 | 0.72 |
| Initial | 208 | 216 | 4.62 | | |
| Final | 389 | 399 | 4.29 | | |
| Average daily gain, kg | 1.56 | 1.57 | 0.25 | 0.94 | - |
| Feed intake, kg DM | 8.18 | 8.39 | 0.18 | 0.66 | 0.48 |
| Feed convertion | 5.23 | 5.34 | 0.15 | 0.79 | - |
| Feed efficiency | 0.19 | 0.18 | 0.02 | 0.91 | - |

Note: There was no treatment effect or treatment × day interaction ($p > 0.05$).

### *3.2. Hemogram and Biochemistry Seric*

Hemogram results are presented in Table 3. There was no effect of treatment and treatment interaction for the variables: total leukocytes, lymphocytes, monocytes, erythrocytes, hematocrit and hemoglobin, and platelets ($p > 0.05$). However, there was an interaction effect for granulocyte count, which was lower in the blood of cattle in the treatment group on day 120.

**Table 3.** Impact of fibrolytic enzymes on blood hemogram and seric biochemistry of the experimental animals.

| Variables | Control | Treatment | SEM | *p*: Treat | *p*: Treat × Day |
|---|---|---|---|---|---|
| **Hemogram** | | | | | |
| Leukocytes ($\times 10^3$ µL) | 10.1 | 9.19 | 0.74 | 0.63 | 0.31 |
| Granulocyte ($\times 10^3$ µL) | | | | 0.59 | 0.05 |
| D20 | 1.30 | 1.29 | 0.21 | | |
| D60 | 1.67 | 1.38 | 0.21 | | |
| D120 | 3.77 [a] | 2.56 [b] | 0.25 | | |
| Lymphocytes ($\times 10^3$ µL) | 6.33 | 6.23 | 0.52 | 0.87 | 0.91 |
| Monocyte ($\times 10^3$ µL) | 1.47 | 1.30 | 0.41 | 0.72 | 0.80 |
| Erythrocyte ($\times 10^6$ µL) | 7.13 | 6.98 | 0.08 | 0.89 | 0.73 |
| Hemoglobin | 11.4 | 11.5 | 0.36 | 0.95 | 0.97 |
| Hematocrit, % | 30.1 | 30.3 | 0.76 | 0.92 | 0.95 |
| Platelet ($\times 10^3$ µL) | 241 | 268 | 9.74 | 0.41 | 0.13 |
| **Seric biochemistry** | | | | | |
| Amylase (U/L) | | | | 0.25 | 0.05 |
| D20 | 88.1 | 97.3 | 4.74 | | |
| D60 | 106 | 105 | 4.68 | | |
| D120 | 116 [b] | 131 [a] | 4.71 | | |
| Globulin (g/dL) | 4.13 | 4.06 | 0.35 | 0.74 | 0.81 |
| Albumin (g/dL) | 2.71 | 2.69 | 0.12 | 0.91 | 0.85 |
| Total protein (g/dL) | 6.85 | 6.75 | 0.39 | 0.88 | 0.79 |
| Fructosamine (mg/dL) | 2.11 | 2.12 | 0.09 | 0.96 | 0.98 |
| Cholesterol (mg/dL) | 87.6 | 98.9 | 1.25 | **0.05** | 0.11 |
| Uric acid (mg/dL) | 0.88 | 0.90 | 0.06 | 0.96 | 0.92 |
| Urea (mg/dL) | 15.9 | 16.1 | 0.63 | 0.97 | 0.99 |

Note: Different letters on the same line show statistical differences between groups ($p \leq 0.05$) and also trend ($p > 0.05$ and $p \leq 0.10$).

The results regarding serum biochemistry are presented in Table 3. There was no effect of treatment and treatment interaction for the variables: globulins, albumin, total protein, fructosamine, uric acids, and urea ($p > 0.05$). However, there was an interaction effect for amylase activity, which was greater in the serum of cattle in the treatment group on day 120. The effect of treatment for cholesterol concentration was observed; being higher in the serum of cattle that consumed the enzymes.

### *3.3. VFAs in Ruminal Liquid*

Volatile fatty acid (VFA) profile results in rumen fluid are presented in Table 4. Treatment effect and treatment × day interaction for total VFA were observed, being higher in the rumen fluid of cattle that consumed the enzyme blend on days 60 and 120. The concentration (nmol/mL) and proportion (%) of acetic acid in the rumen was higher in cattle that consumed exogenous enzymes on days 60 and 120 compared to the control. There was no treatment or interaction effect for the concentration (nmol/mL) of propionic acid; but there was a treatment × day interaction in the proportion (%) of this acid, being higher in the rumen fluid of the animals in the treatment group on day 20. A treatment × day interaction

(day 20) was observed for the concentration and proportion of butyric acid, being lower in cattle who consumed the fibrolytic enzyme blend. The concentration of isovalaric acid was higher in animals in the treatment group on day 120, which was not observed in the proportion analysis. The proportion (%) of valeric acid only had a treatment × day interaction, with the percentage of valeric acid being lower on days 60 and 120 in the rumen fluid of cattle in the treatment group. The acetic/propionic ratio was higher in the rumen fluid of cattle that consumed the fibrolytic enzyme blend on days 60 and 120.

**Table 4.** Ruminal fluid in bovines fed with fibrillytic enzyme blend: results presented in percentage and absolute values.

| Variables | Control | Treatment | SEM | *p*: Treat | *p*: Treat × Day |
|---|---|---|---|---|---|
| Volatile fatty acids (mmol/L) | | | | 0.05 | 0.01 |
| D20 | 75.6 | 70.2 | 2.29 | | |
| D60 | 87.5 $^{b}$ | 102.7 $^{a}$ | 2.88 | | |
| D120 | 79.0 $^{b}$ | 90.9 $^{a}$ | 1.76 | | |
| **Quantity results** | | | | | |
| Acetic acid (mmol/L) | | | | 0.05 | 0.01 |
| D20 | 52.6 | 48.3 | 1.50 | | |
| D60 | 53.6 $^{b}$ | 66.7 $^{a}$ | 1.52 | | |
| D120 | 51.2 $^{b}$ | 61.3 $^{a}$ | 1.33 | | |
| Butyric acid (mmol/L) | | | | 0.35 | 0.05 |
| D20 | 7.79 $^{a}$ | 6.11 $^{b}$ | 0.28 | | |
| D60 | 11.9 | 13.8 | 0.68 | | |
| D120 | 9.46 | 10.7 | 0.36 | | |
| Isovaleric acid (mmol/L) | | | | 0.68 | 0.02 |
| D20 | 1.24 | 1.02 | 0.17 | | |
| D60 | 2.59 | 2.64 | 0.18 | | |
| D120 | 1.00 $^{b}$ | 1.37 $^{a}$ | 0.09 | | |
| Propionic acid (mmol/L) | 15.8 | 16.3 | 0.66 | 0.83 | 0.46 |
| Valeric acid (mmol/L) | 1.05 | 0.91 | 0.05 | 0.65 | 0.71 |
| **Proportion results** | | | | | |
| Acetic acid (%) | | | | 0.05 | 0.03 |
| D20 | 69.5 | 69.1 | 0.42 | | |
| D60 | 61.6 $^{b}$ | 65.1 $^{a}$ | 0.47 | | |
| D120 | 64.0 $^{b}$ | 67.7 $^{a}$ | 0.39 | | |
| Propionic acid (%) | | | | 0.70 | 0.05 |
| D20 | 16.9 $^{b}$ | 19.1 $^{a}$ | 0.66 | | |
| D60 | 20.8 | 17.9 | 0.81 | | |
| D120 | 20.8 | 18.3 | 0.48 | | |
| Butyric acid (%) | | | | 0.86 | 0.03 |
| D20 | 10.3 $^{a}$ | 8.73 $^{b}$ | 0.22 | | |
| D60 | 13.4 | 13.4 | 0.51 | | |
| D120 | 11.9 | 11.7 | 0.33 | | |
| Isovaleric acid (%) | 1.91 | 1.84 | 0.11 | 0.55 | 0.69 |
| Valeric acid (%) | | | | 0.12 | 0.01 |
| D20 | 1.56 | 1.53 | 0.01 | | |
| D60 | 1.29 $^{a}$ | 0.87 $^{b}$ | 0.08 | | |
| D120 | 1.04 $^{a}$ | 0.85 $^{b}$ | 0.05 | | |
| Acetic/propionic | | | | 0.79 | 0.01 |
| D20 | 4.28 | 3.72 | 0.29 | | |
| D60 | 3.13 $^{b}$ | 3.66 $^{a}$ | 0.14 | | |
| D120 | 3.14 $^{b}$ | 3.73 $^{a}$ | 0.11 | | |

Note: Different letters on the same line show statistical differences between groups ($p \leq 0.05$) and also trend ($p > 0.05$ and $p \leq 0.10$).

### *3.4. Apparent Digestibility Coefficient (ADC)*

ADC results are presented in Table 5. There was no treatment effect for ADC of dry matter, organic matter, crude protein, ethereal extract, and NDF ($p > 0.05$). However, if we consider the trend ($p > 0.05$ to $p \leq 0.10$), we have a difference between groups for the dry matter and NDF coefficient.

**Table 5.** Impact of fibrolytic enzymes addition on animal's apparent digestibility coefficient.

| Variables | Control | Treatment | SEM | *p*: Treat |
|---|---|---|---|---|
| Dry matter | 0.41 | 0.49 | 0.05 | 0.10 |
| Organic matter | 0.46 | 0.50 | 0.04 | 0.40 |
| Crude protein | 0.45 | 0.48 | 0.03 | 0.42 |
| Ether extract | 0.52 | 0.50 | 0.02 | 0.85 |
| NDF | 0.29 | 0.37 | 0.05 | 0.07 |

Note: There was no treatment effect to variables ($p > 0.05$).

### *3.5. Meat Quality*

The results of total lipids and fatty acid profile in meat are presented in Table 6. Cattle in the treatment group had a higher amount of lipids in meat compared to the control. Lower percentages of myristic and palmitic acids, as well as a higher proportion of oleic and arachidonic acids, were observed in beef from cattle that consumed fibrolytic enzymes. As a result, we found a lower amount of saturated fatty acids and a higher amount of unsaturated fatty acids in the meat of cattle in the treatment group compared to the control group. Both MUFA and PUFA sums were higher in the meat of cattle that consumed enzyme blends. A higher UFA/SFA ratio was observed in the meat of cattle in the treatment group.

**Table 6.** Total lipids and fatty acid in meat of bovines fed with the fibrolytic enzyme blend.

| Variables | Control | Treatment | SEM | *p*-Value |
|---|---|---|---|---|
| Total lipids (g/kg) | 12.2 [b] | 14.7 [a] | 0.64 | 0.02 |
| Fatty acid (%) | | | | |
| C14:0 (Myristic) | 2.07 [a] | 1.66 [b] | 0.072 | 0.02 |
| C14:1 (Myristoleic) | 0.37 | 0.32 | 0.024 | 0.35 |
| C15:0 (Pentadecanoic) | 0.22 | 0.21 | 0.005 | 0.96 |
| C16:0 (Palmitic) | 30.00 [a] | 25.75 [b] | 0.166 | 0.01 |
| C16:1 (Palmitoleic) | 3.71 | 3.33 | 0.104 | 0.43 |
| C17:0 (Heptadecanoic) | 1.27 | 1.44 | 0.176 | 0.65 |
| C18:0 (Stearic) | 15.22 | 15.15 | 0.128 | 0.97 |
| C18:1n9t (Elaidic) | 1.83 | 1.71 | 0.096 | 0.82 |
| C18:1n9c (Oleic) | 34.08 [b] | 37.66 [a] | 0.276 | 0.01 |
| C18:2n6c (Linoleic) | 7.18 | 8.12 | 0.436 | 0.73 |
| C20:0 (Arachidic) | 0.07 | 0.07 | 0.002 | 0.99 |
| C18:3n6 (Linolenic) | 0.05 | 0.06 | 0.003 | 0.95 |
| C20:1n9 (cis-11-Eicosenoic) | 0.16 | 0.17 | 0.003 | 0.96 |
| C18:3n3 (a-Linolenic) | 0.32 | 0.35 | 0.018 | 0.26 |
| C21:0 (Henicosanoic) | 0.29 | 0.32 | 0.012 | 0.15 |
| C20:2 (cis-11.14-Eicosadienoic) | 0.10 | 0.11 | 0.005 | 0.95 |
| C20:3n6 (cis-8,11,14-Eicosatrienoic) | 0.47 | 0.51 | 0.029 | 0.36 |
| C20:4n6 (Arachidonic) | 2.27 [b] | 2.69 [a] | 0.055 | 0.01 |
| C22:2 (cis-13,16-Docosadienoic) | 0.03 | 0.03 | 0.002 | 0.99 |
| C24:0 (Lignoceric) | 0.04 | 0.04 | 0.002 | 0.99 |
| C20:5n3 (cis-5,8,11,14,17-Eicosapentaenoic) | 0.16 | 0.19 | 0.012 | 0.16 |
| C24:1n9 (Nervonic) | 0.04 | 0.04 | 0.003 | 0.98 |
| C22:6n3 (cis-4.7.10.13.16.19-Docosahexaenoic) | 0.05 | 0.06 | 0.006 | 0.95 |

**Table 6.** *Cont.*

| Variables | Control | Treatment | SEM | *p*-Value |
|---|---|---|---|---|
| Other variables | | | | |
| ∑ Saturated fatty acids (SFA) | 49.17 [a] | 43.64 [b] | 0.388 | 0.01 |
| ∑ Unsaturated fatty acids (UFA) | 50.83 [b] | 55.36 [a] | 0.388 | 0.01 |
| ∑ Monounsaturated fatty acids (MUFA) | 40.19 [b] | 43.24 [a] | 0.270 | 0.05 |
| ∑ Polyunsaturated fatty acids (PUFA) | 10.64 [b] | 12.12 [a] | 0.249 | 0.09 |
| UFA/SFA | 1.04 [b] | 1.27 [a] | 0.011 | 0.01 |

Note: Different letters on the same line show statistical difference between groups ($p \leq 0.05$) and also trend ($p > 0.05$ and $p \leq 0.10$).

## 4. Discussion

In the current study, the ingestion of fibrolytic enzymes by cattle altered the fatty acid profile of the meat. Although we did not identify a significant impact on the animals' weight gain, the addition of enzymes resulted in modulation of rumen fermentation. Abid et al. [23] stated that the effectiveness of fibrolytic enzymes is influenced by the chemical composition of feed; as fibrolytic enzymes are most effective for sorghum straw, molasses grass, elephant grass, and sugar cane, respectively [24]. Furthermore, it was known that the greatest effects of fibrolytic enzymes are in diets composed of low quality forage; which would not be the case here in the present work, as the diet is based on concentrate (70%) and good quality silage (30%).

Short-chain fatty acids are the main source of energy for ruminant animals, increasing the production of these acids in the rumen has a positive correlation with performance. In our research, an increase in the total production of short-chain fatty acids and acetic and isovaleric acids was observed for animals fed with exogenous enzymes. Giraldo et al. [25] observed that in diets rich in forage, the addition of fibrolytic enzyme cellulase increases the production of butyrate and isovalerate. Another study demonstrated that the addition of exogenous fibrolytic enzymes improved the total digestibility [8] of NDF and ADF, but without affecting the total production of short-chain fatty acids [26–28]. With the addition of fibrolytic enzymes [8], they found an increase in ruminal ammonia due to better use of nitrogen for microbial growth. Added fibrolytic enzymes is a strategy to mitigate methane emissions without compromising meat quality [29].

Neutrophils, which are granulocyte leukocyte cells of fundamental importance, are considered a front line in the defense of animal organisms. They perform antimicrobial, pro- and anti-inflammatory functions. They perform antimicrobial, pro- and anti-inflammatory functions [30,31]. Neutrophils have the ability to attract and activate others of their species, modulating the secretions of mediators, such as platelet-activating factor, leukotriene B4 [32], and acid glycoprotein [33]. Immunosuppression, including neutrophil dysfunction, is considered a contributing cause and a target for therapeutic intervention [34–36]. Based on our observations, the greater number of granulocytes in the animals in the control group at the end of the experimental period suggests that the animals fed with the fibrolytic enzyme blend had a less activated immune system, consequently with lower energy expenditure. This could have resulted in better zootechnical performance.

Acetate is a precursor substance for fatty acid synthesis in bovine mammary epithelial cells (BMECs), and the mTOR signaling pathway plays an important role in milk fat synthesis. However, the mechanism of the regulatory effects of acetate on lipogenic genes via the mTOR signaling pathway in BMEC remains unknown. The ingestion of fibrolytic enzymes mixture (cellulase, xylanase, pectinase, and laccase) in the diet of Holstein bulls resulted in a lower rumen pH value and the ratio of acetate to propionate, and a higher concentration of VFA, but the content of ammonia-N was not influenced with increasing dose of enzyme mixture [37]. Based on our results, it is clear that the ingestion of fribrolytic

enzymes changes the profile of volatile fatty acids in the rumen, with emphasis on the increase in acetic acid, which leads to the hypothesis that a similar mechanism occurs in lipid metabolism that impacts fat in meat. The modulation of the fatty acid profile in meat was beneficial to consumer health, since cattle that consumed fibrolytic enzymes had a lower proportion of saturated fatty acids, and a greater amount of unsaturated fatty acids; i.e., it increased the omegas. The recommendation for ingesting a smaller amount of saturated fat is to protect the cardiovascular system, preventing the deposition of fat in the blood vessels.

The animals in this study consumed restrictive feed, formulated to meet physiological and nutritional requirements, as well as an average daily weight gain of 1.5 kg. It is important to make it clear that if more feed was provided, the Holstein cattle would consume it. However, production costs are unsustainable according to research already carried out by our group when using cattle of the Holstein breed (unpublished data). We argue that most of the studies that tested fibrolytic enzymes and had positive results provided feed ad libitum. In dairy cows, researchers found that the ingestion of fibrolytic enzyme enhances the digestibility of DM, NDF, potentially digestible NDF (pdNDF), and organic matter [38]; but in our study we only found a trend towards better digestibility of NDF.

The fatty acid profile of meat not only reveals the nutritional value of the product, but also directly influences its flavor and contributes to the promotion of human health [39]. In ruminant animals, polyunsaturated fatty acids in the diet undergo the biohydrogenation process in the rumen, being converted into monounsaturated and saturated fatty acids. Only about 10% of polyunsaturated fatty acids remain available for incorporation into lipid tissues [39]. Based on the changes observed in our study, it is possible to state that fibrolytic enzymes modulate ruminal fermentation, improving the lipid profile of the meat. Simon et al. [40] observed a significant increase in the percentage of polyunsaturated fatty acids with exogenous enzyme fed. Furthermore, they concluded that the inclusion of digestive enzymes not only increased the amount of unsaturated fatty acids, and reduced saturated ones, but also had a positive effect on the oxidative stability of the meat and improved the animals' performance.

The mechanisms that lead to changes in the fatty acid profile in meat are indirect, involving the modulation of rumen modification. biohydrogenation and, consequently, lipid metabolism [40]. In agreement with our data. Simon et al. [40] concluded that the addition of exogenous enzymes to the cattle diet increases polyunsaturated fatty acids and reduces saturated ones. This is extremely beneficial for human health as these acids have a potential protective effect against cancer [41]. Enriching meat with polyunsaturated fatty acids not only improves human nutrition but also has the potential to contribute to the health and well-being of impoverished populations [41]. Among saturated fatty acids, palmitic acid (C16:0) is the most common in diets, followed by stearic acid (C18:0) and myristic acid (C14:0) [42]. There is strong evidence that even-numbered saturated fatty acids increase the concentration of total and LDL cholesterol, in addition to promoting clotting, inflammation and insulin resistance, and are strongly associated with a greater risk of cardiovascular disease and type 2 diabetes [42].

Oleic acid (C18:1) is the most abundant monounsaturated fatty acid in the human diet [42]. Replacing saturated fatty acids with oleic acid has a lowering effect on total and LDL cholesterol [43,44], in addition to potentially reducing blood pressure [45]. Replacing saturated fatty acids with oleic acid can also improve glucose control and insulin sensitivity [46]. These findings prove the improvement in the quality of meat from animals fed with exogenous enzymes for human consumption, since there was a reduction in palmitic acid and the sum of saturated fatty acids. Therefore, the addition of exogenous fibrolytic

enzymes, by improving the fatty acid profile of the meat, provides better quality food for human consumption.

## 5. Conclusions

In conclusion, under these experimental conditions, there was no effect of adding fibrolytic enzymes to the diet of cattle in the confinement phase, which may be related to not having improved nutrient digestibility. However, the consumption of fibrolytic enzymes changed the rumen fatty acid profile, especially a greater amount of VFA. A greater quantity and proportion of acetic acid in rumen fluid may have been the precursor to greater fat in meat; as well as modulating the fatty acid profile, reducing SFA and increasing UFA in meat.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ruminants5020023/s1. Table S1. Standardizations carried out to carry out profile analyzes of volatile fatty acids in ruminal fluid.

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