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ANIMAL SCIENCE

Starter bacteria as producers of CLA in ripened cheese

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Abstract: The profile of polyunsaturated fatty acids in cheeses obtained through fermentation by lactic acid bacteria *Lactobacillus helveticus* and *Streptococcus thermophilus* were evaluated. The milk used to make the cheeses came from cows fed with flaxseed oil and annato. The cheeses presented microbiological and physic-chemical quality with in the standards established by the legislation for *Staphylococci* and *Listeria*. With maturation, there was a reduction in the coliform values for both treatments. Regarding the counts of lactic acid bacteria, these remained viable until the 30th day of maturation and the proteolytic bacteria decreased. For antioxidant capacity, the treatment containing the combination of the strains obtained high ABTS values. There was no significant difference between the treatments with respect to the color of the samples. For texture, there was a significant difference for the parameters cohesion and elasticity. No increase in CLA content was observed in the form of its two main isomers, however, the levels of polyunsaturated fatty acids were increased four-fold when compared to milk . The inclusion of the evaluated bacteria was effective because it promoted the development of a product with beneficial characteristics to the consumer.

Key words: Annatto, fatty acid profile, flaxseed oil, lactic acid bacteria, microbiology.

INTRODUCTION

Currently, there is a tendency to relate health problems to a diet of inferior quality. The association between the consumption of dairy products, mainly cheeses, and health is a factor that is considered in the production and trade of these foods (Terpou et al. 2017) because it determines the consumption of these products.

Cheeses are associated with high levels of saturated long chain fatty acids, which are considered harmful if consumed in high quantities in diets because they cause several metabolic disorders and diseases. However, with the applications of different production technologies, such as varying the composition of milk, breed of animals, stage of lactation and diet of the animals, this obstacle has been reduced. Due to the great diversity of dairy products, enriched or not with unsaturated fatty acids, there have been some improvements in the final product, making dairy products healthier and potentially beneficial (González-Martín et al. 2017).

The composition of fatty acids in foods is of great importance, especially polyunsaturated fatty acids, such as omega-3 and omega-6 families, which are attributed numerous benefits to humans (Perini et al. 2010). Conjugated linoleic acid (CLA) is a group of positional and geometric isomers of linoleic acid that are naturally synthesized in the rumen of animals

and mainly derived from milk-derived foods and meat from ruminants (Andrade et al. 2012).

The strategies to increase the amount of CLA in milk may involve the addition of dietary sources to animal feed (Jones et al. 2005) or by synthesis by some strains of bacteria such as bifidobacteria and lactic acid bacteria with linolenic acid as a starting material (Gorissen et al. 2010). Since cheese processing also involves bacterial fermentation, some studies have been conducted to observe the synthesis of CLA by bacteria in this type of product, as well as the effect of process steps that maintain the CLA content (Lucatto et al. 2014).

In addition, the consumption of dairy products containing probiotic bacteria, such as *Lactobacillus* and *Bifidobacterium*, also adds value to the commercial product and assists in the maintenance of the intestinal microbiota, guaranteeing health and greater well-being through its consumption.

The objective of this study was to evaluate the physicochemical and microbiological characteristics and the fatty acid profile of ripened cheese produced with milk enriched with a polyunsaturated fat source and fermentation promoted by the addition of two commercial cultures containing *Lactobacillus helveticus* and *Streptococcus thermophilus*.

MATERIALS AND METHODS

The experiment was carried out at the Experimental Farm of Iguatemi (FEI), belonging to the Univesidade Estadual de Maringá (UEM), Maringá/Paraná/BR. The experimental protocol was approved by the Committee on Ethics in Animal Use in Experimentation of the Universidade Estadual de Maringá, PR (N°. 6450240117).

The milk used for the study was from four Holstein cows within ± 120 days of lactation. The cows received a diet consisting of roughage concentrate, which included annatto seeds (1.5% DM) and flaxseed oil (3% DM). For cheese production, milk was collected for five days in four periods of animal feeding (16 feeding days and 5 days of milk collection, totaling 21 days), totaling 96 cheese units.

For the chemical analyses of the milk (Table I), the samples were conditioned in a plastic bottle containing Bronopol® (2-bromo-2-nitropropane-1,3-diol), preservative and analysed in an Ekomilk Total (Cap-Lab, São Paulo, Brazil).

To quantify the milk fatty acids, the methodologies proposed by Murphy et al. (1995) and method 5509 of ISO (1978) were used using an Agilent gas chromatograph, model 7890a (Table II).

Dairy cultures "SH type", Lyofast SH 092 F, SACCO®, containing *Streptococcus thermophilus (S. thermophilus)*, *Lactobacillus helveticus* (*L. helveticus)*, Lyofast LH 091, and SACCO® (*L. helveticus*) were used to manufacture the cheeses (05 UC/100 litres of milk).

For the elaboration of the cheeses, 48 litres of pasteurized milk (65°C/30 min), calcium

Table I. Physico-chemical composition of the milk used in the production of ripened cheeses.

TREATMENT				
Variables	**P1**	**P2**	**P3**	**P4**
Milky Production (Kg/day)	12.43	10.96	15.40	17.80
Total solids (% m/m)	7.49	7.83	8.05	7.66
Fat (% m/m)	3.80	3.75	4.41	3.11
Lactose (% m/m)	3.76	3.94	4.06	3.86
Protein (% m/m)	3.18	3.53	3.39	3.23
pH	6.62	6.51	6.59	6.78

*** Average values by period. P1: period 1; P2: period 2; P3: period 3; P4: period 4.**

chloride w/v (50 g for 100 litres of milk), milk cultures LH or SH and liquid coagulant v/v (*Aspergillus niger* 7 ml/10 L - HA-LA®-CHR, Denmark) were used. After cutting, the cheese cloth was heated to 45 °C and then molded (JandaPlast, model RH-1000). The curd cooking temperature can reach 55 to 60 °C for very hard cheeses (Ordóñez 2005). The cooking temperature used in this study was chosen because it was the optimal temperature for the activation of *S. thermophilus*. After 12 hours, the cheeses were salted with 2% salt (w/w) and kept in a BOD-type oven for 30 days/12°C with a relative humidity of ± 60.5%.

The water activity (Aqualab® 4TE, Decagon, São Paulo, Brazil), pH (digital pH metre, Tecnal Tec-5), titratable acidity (Lutz 2008), color (Konica Minolta), dry matter, mineral matter, crude protein (AOAC 1992) and fat content (Bligh & Dyer 1959) were determined in the cheese samples at 0, 10, 20 and 30 days of maturation.

Table II. Profile of fatty acids from milk used in ripened cheese production.

Variables	mg/g	Variables	mg/g
C6:0	0.1637	C18:2 n6c	1.4497
C8:0	0.3016	C20:0	0.1651
C10:0	1.5347	C18:3 n6	0.0409
C11:0	0.0502	C20:1	0.0503
C12:0	2.4334	C18:3 n3	0.2387
C13:0	0.1005	C21:0	0.7526
C14:0	10.5757	C20:2	0.0289
C14:1	0.7296	C20:3 n6	0.0298
C15:0	0.8831	C20:3 n3	0.0046
C15:1	0.0101	C20:4 n6	0.0944
C16:0	28.2870	C23:0	0.0042
C16:1	1.7830	C24:0	0.0129
C17:0	0.7405	C20:5 n3	0.0101
C17:1	0.2226	C24:1	0.0092
C18:0	14.0820	SFA	60.0872
C18:1 n9t	5.5840	MUFA	37.8462
C18:1 n9c	29.4287	PUFA	2.0665
C18:2 n6t	0.1981		

***SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids.**

The instrumental color was determined using a Konica Minolta chromometer (Konica Minolta, Model CR 400/410, Japan), using the CIELAB system (CIE 1996). Measurements were performed in triplicate with the previously calibrated apparatus, using pieces from the inside and outside of the cheese.

The samples were diluted in peptone water to perform the microbiological analyses (AOAC 1992), and the lactic acid bacteria (MRS agar- De Man, Rogosa e Sharpe, Himedia) were evaluated. Proteolytic mesophilic bacteria were grown on PCA-Himedia agar plus 1 % reconstituted skim milk (10 %) and coliforms were detected with VRB agar (Violet Red Bille agar, Himedia). At 30 days, the presence of *Listeria monocytogenes* and *Staphylococcus aureus* were evaluated (AOAC 1992). Samples were incubated at 35 °C for 48 hours.

To determine the lipid profile of cheeses, lipids were extracted from the samples according to Bligh & Dyer (1959). Afterwards, the lipids were esterified according to ISO (1978) method 5509 for analysis using an Agilent gas chromatograph (Trace GC 52 Ultra, Thermo Scientific, West Palm Beach, Florida, USA) self-sampler equipped with a flame ionization detector at 250 °C and a fused silica capillary column (100 m long, 0.25 mm internal diameter and 0.20 µm, Restek 2560). Fatty acid quantification of the sample was performed by comparing the retention time of fatty acid methyl esters to standard samples (Sigma Aldrich).

The antioxidant compounds were evaluated in the cheese samples after 0, 10, 20 and 30 days of storage with the radical sequestration method involving 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as described by Re et al. (1999) expressed in ET µM (Zhu et al. 2002).

For the analysis of the texture profile (TPA), the Brookfield texture analyzer-CT III (Engineering Laboratories, INC., Middleboro, MA, USA) was used in the following configurations: TPA; test speed, 1 mm/s; compression distance, 5 mm; and a TA4 cylindrical acrylic probe of 38 mm. The variables measured for TPA were hardness, chewing, cohesiveness and elasticity.

The data were analysed by ProcMixed of SAS 9.3 (2013), which tested the interaction between treatment and time, and a linear and quadratic contrast study was performed for storage times.

RESULTS

For the evaluated times, the dry matter (DM) contents increased as a function of maturation day, with a consequent decrease in moisture values (P<0.001). The values of dry matter were significant for time (P<0.001) and for the treatment x time interaction (P<0.0261), presenting linear contrast (P<0.0001) (Table III).

With regard to moisture, there was a linear effect, and the levels were reduced during maturation. For ash content, there was a significant effect for treatment (P=0.0312) and for time (<0001) but not for the treatment x time interaction (P<0.6919), presenting a linear behaviour (P=0. 0033).

The values for ethereal extract increased with time P<0.0001 because with the decrease in moisture and the increase in the dry matter, there was an increase in the concentration of the cheese components, thus linearly increasing the amount of EE present.

There was no significant difference between the treatments and the time x treatment interaction (P>0.10) for the pH and Aw values; however, the pH and Aw values had significant differences for time. The values of titratable acidity and pH were not congruent, and acidity values were significant for the time and the treatment x time interaction (Table IV).

High coliforms counts were found (Table V). There were significant differences for the treatments (P=0.0352) and for maturation times (P<0.0001), with a reduction in counts during maturation. For lactic acid bacteria counts, significant differences were observed for the maturation times (P=0.0107) and for the treatment x time interaction (P=0.0212), which exhibited quadratic behaviour. The microbiological count of proteolytic bacteria showed no significant difference for the treatments (P>0.05) but presented significant differences for time, decreasing throughout storage. *Listeria* and

Table III. Bromatological composition of ripened cheeses.

	Treatment Lh+ST				Treatment Lh				SE	P				Contrast
	0	10	20	30	0	10	20	30		Treat	Time	Treat x Time	L	Q
DM	44.96 ^{c}	65.38 ^{ab}	69.22 ^{ab}	72.80 ^{a}	46.08 ^{c}	67.08 ^{ab}	67.43 ^{ab}	62.07 ^{b}	2.349	0.0930	<.0001	0.0261	<.0001	<.0001
M	55.17 ^{a}	34.61 ^{bc}	30.58 ^{bc}	27.19 ^{c}	52.74 ^{a}	35.41 ^{bc}	35.07 ^{bc}	40.42 ^{b}	2.661	0.0930	<.0001	0.0119	<.0001	<.0001
CP	22.62	23.22	23.66	24.16	28.05	30.36	17.51	22.43	4.099	0.8008	0.1662	0.0808	0.1783	0.7521
ASH	4.12	5.09	6.17	5.02	3.29	4.84	5.31	4.03	0.374	0.0312	<.0001	0.6919	0.0033	<.0001
EE	18.65	30.64	23.53	29.15	17.08	25.52	23.73	29.21	2.931	0.3945	<.0001	0.4786	0.1783	0.1103

DM: dry matter in %; M: moisture in %; CP: crude protein in %; ASH: ashes in % EE: ethereal extract in %; SE: mean standard error; P treat: level of significance for treatment; P time: significance level for time; P treat x P Time: level of significance for iteration between treatment and time; L: linear; Q: quadratic. P<0.10; averages evaluated in the lines. Treatment Lh + ST: *Lactobacillus helveticus* and *Streptococcus thermophilus*; Treatment Lh: *Lactobacillus helveticus.

Staphylococcus aureus were not present at 30 days of maturation.

For color (Table VI), there was no difference for the parameters a* and b*, and no color change was observed as a function of cheese fermentation by *Streptococcus* or *L. helveticus*. For the treatment x time interaction, the color parameter L* was significant (P=0.0096).

The values obtained for the texture were significant for treatment, time and the treatment x time interaction, only for cohesiveness and elasticity. For the chewable characteristic, the values were significant for the treatment and time, with an increase in values for the storage time (linear effect P<0.001) (Table VII).

The values found for the antioxidant capacity of the ABTS radical in the equivalent Trolox (ETμM) showed that there were significant differences for the treatment and for the treatment x time interaction (P<0.001), the highest value was for the Lh + St treatment (669.5 ETμM).

Fatty acids (Table VIII), such as C20:3 n6; C20:3 n3; C22:1 n9; C20:4 n6; C23:0; C22:2; C24:0; C20:5 n3; C24:1 and C22:6 n3, were found sporadically but were used to calculate the values of mono-unsaturated, saturated and polyunsaturated fatty acids.

There was a significant difference in the fatty acid profiles between the treatments (P<0.05), with the treatment containing *S. thermophilus* + *L. helveticus* having the highest levels of C18:0 (stearic acid) at 30 days of maturation and C19:1 n9 (oleic acid) at 10 days of maturation. The n3:n6 ratio was significant (P<0.05) for treatment, with the highest values observed in the Lh + St treatment cheese.

The highest levels of C18:3 n3 (alpha-linolenic acid) 1.2187 mg/g at the 20 days of maturation, C18:2 n6 t (linoleic acid) 2.0335 mg/g at the 20 days of maturation, fatty acid ratio polyunsaturated 0.1023 mg/g at the 20 days of maturation were observed for the treatment containing only *L. helveticus*. The saturated fatty acids presented a lower content in the cheese produced with *L. helveticus*, with an average of 30.6662 mg/g in relation to the cheese produced with the other bacteria, with a value of 64.8814 mg/g.

In the present study, the cheese produced with *L. helveticus* showed no increase in the CLA (conjugated linoleic acid) content in the form of its two major isomers: cis-9, trans 11 and trans 10, cis 12. However, the treatment containing only *L. helveticus* exhibited a higher concentration of C18:3 n3 (alpha-linolenic), presenting a higher value at 20 days of maturation (1.2187 mg/g).

The milk presented an average of 0.2387 mg/g C18:3 n3 (alpha-linolenic), and the cheese produced with Lh had an average concentration of 0.8138 mg/g. This concentration was four times higher, indicating the production of this fatty

Table IV. Physico-chemical composition of ripened cheese.

	Treatment Lh+ST				Treatment Lh				SE	P			P Contrast	
	0	10	20	30	0	10	20	30		Treat	Time	Treat x Time	L	Q
pH	5.88	5.34	5.29	5.45	5.97	5.78	5.65	5.92	0.220	0.1072	0.0119	0.5102	0.0678	0.0036
Aw	0.976	0.930	0.903	0.884	0.976	0.938	0.930	0.893	0.013	0.1647	<.0001	0.4452	<.0001	0.2458
Acidity	1.025^{b}	2.435^{ab}	1.652^{b}	2.876^{ab}	1.062^{b}	2.188^{ab}	3.225^{a}	2.498^{ab}	0.377	0.1972	<.0001	0.0017	<.0001	0.0073

Aw: water activity; SE: mean standard error; P treat: level of significance for treatment; P time: significance level for time; P treat x P Time: level of significance for iteration between treatment and time; L: linear; Q: quadratic. P<0.05; means evaluated on the lines; Treatment Lh + ST: *Lactobacillus helveticus* and *Streptococcus thermophilus*; Treatment Lh: *Lactobacillus helveticus.

acid by metabolic routes during fermentation, which can improve the characteristics of the final product. These results appear to indicate that during ripening, CLA can be formed from linoleic acid through the action of the primary or secondary cultures besides the fact that the continuous release of free linoleic acid during cheese ripening and, as a consequence, the possible formation of yet additional CLA (Sieber et al. 2004).

DISCUSSION

Evaluated cheeses are classified as medium and low humidity (Brasil 1996). According to Salazar-Montoya et al. (2018), by evaluating the use of *Lactococcus lactis* during the maturation of Manchego cheese, a gradual decrease in moisture emerged after 15 days of maturation as a result of the syneresis provided by the rearrangement of the protein network, resulting in large amounts of whey expulsion, a behaviour similar to that observed in these experiments. The moisture of the cheese is responsible for directly influencing its consistency, and during maturation, the intensity of dehydration depends on the size and shape of the cheese, as well as on the environmental conditions in which the maturation takes place (Beresford et al. 2001).

The ash contents were in agreement with the literature recommended for ripened cheese, which ranges from 1.0% to 6.0% (Gomes 1997). Ferreira & Freitas Filho (2008) and Uliana & Rosa (2010) studied artisanal cheeses (colonial and rennet) and obtained ash contents from 3.85% to 4.31% and 2.77% to 2.87%, respectively.

The oscillation in protein values may have occurred because of microbial activity and casein degradation may have been the result of rennet enzymes (Hynes et al. 2003). *L. helveticus* has proteases attached to the cell envelope and intracellular peptidases that can be released into the cheese matrix during autolysis. Consequently, *L. helveticus* has also been used to accelerate protein degradation and improve flavour development during cheese maturation (Moser et al. 2017).

The variation in pH may be dependent on the buffering capacity of the cheese and due to the amount of proteins and minerals (Narimatsu et al. 2003) (Table IV). The process of acidification continued during ripening, which, except for the high production of lactic acid, is related to the low buffering capacity of the mass of the cheese. This is a consequence of the demineralization undergone during coagulation and the removal of the whey (Havranek et al. 2014).

In relation to the increasing levels of acidity, these can be justified by the possible action of lactic acid bacteria (Faion et al. 2015).

Table V. Parameters of microbiological quality of ripened cheese.

	Treatment Lh+ST				Treatment Lh				SE	P			Contrast	
	0	10	20	30	0	10	20	30		Treat	Time	Treat x Time	L	Q
COL	6.23	6.18	5.29	4.73	6.39	6.83	6.59	5.72	0.381	0.0352	<0.0001	0.1516	<0.0001	0.014
PA	6.31	6.63	6.39	5.51	6.33	4.78	5.88	5.23	0.864	0.5039	0.0392	0.1422	0.0374	0.6793
LAB	6.15 b	7.70 a	6.19 b	6.08 b	6.41 ab	6.61 ab	6.63 ab	6.62 ab	0.430	0.8928	0.0107	0.0212	0.4922	0.0157

***SE: standard error of the mean; P treat: level of significance for treatment; P time: level of significance for time; P treat x P Time: level of significance for iteration between treatment and time; L: linear; Q: quadratic. P<0.05; the means are evaluated in the lines; Treatment Lh + ST: *Lactobacillus helveticus* and *Streptococcus thermophilus*; Treatment Lh: *Lactobacillus helveticus*. COL= coliforms, PA= proteolytic bacteria, LAB= latic acid bacteria.**

Artisanal cheeses are usually hand-pressed, thus presenting whey retention, interfering with the amount of lactose eliminated in the serum (Alinovi et al. 2018). In addition, there is less elimination of lactose and higher amounts of substrates for fermentation; therefore, a greater amount of lactic acid can be produced, which may justify the lack of standardization presented for acidity and, consequently, influence the percentages of these parameters.

Another preponderant factor in the physical-chemical composition of cheeses is the Aw value, which presented a rapid decrease that can be justified as a function of water loss by evaporation and the hydrolysis of proteins to peptides and amino acids and of triglycerides to glycerol and fatty acids (Beresford et al. 2001).

Lactic acid bacteria (LAB) produce lactic acid, which accelerates the coagulation of milk, aiding in serum syneresis and contributes to taste, form and texture (Awad et al. 2007). During cheese manufacturing and maturation, the composition of the lactic microbiota undergoes several changes, according to changes in environmental conditions, such as increased lactic acid and decreased pH (Di Cagno et al. 2006).

Delamare et al. (2012), evaluated Serrano cheese for mesophilic bacteria, a group to which lactic acid bacteria belong, and stated that there may be a variation of 4.0 to 9.0 log CFU/g in cheeses produced with unpasteurized milk. Paiva et al. (2015) evaluated the natural starter addition of Minas artisanal cheese from Serro for over 60 days of maturation and noticed a decrease in LAB during the storage period of 8.20 log CFU/g to 7.90 log after 30 days in storage. Gursoy et al. (2018) evaluated cheeses produced from goat and sheep milk and obtained pH values of approximately 5.2 and values of lactic acid bacteria of 7.84 log CFU/g at 3 months of maturation.

The proteolytic microorganisms did not present significant differences between the evaluated treatments (P>0.05) since both treatments contained *L. helveticus,* a bacterium belonging to this microbial group. Proteolysis is an important factor to be considered during cheese maturation.

Although typically proteolytic microorganisms are undesirable, certain lactic acid bacteria have proteolytic activity, which is very important during maturation (Perry 2004). *L. helveticus* has biochemical pathways responsible for producing flavour compounds. Proteolysis is a pre-requisite for the growth of lactic acid bacteria and the subsequent degradation of milk proteins (casein), leading to the release of peptides and free amino acids (Forsythe 2002, Moulay et al. 2006). This effect

Table VI. Instrumental colors of ripened cheese.

	Treatment Lh+ST				Treatment Lh					P			Contrast	
	0	10	20	30	0	10	20	30	SE	Treat	Time	Treat x Time	L	Q
color l*	78.96^{ab}	80.43^{ab}	81.52^{ab}	72.28^{b}	87.11^{a}	79.10^{ab}	71.03^{b}	79.10^{ab}	4.064	0.5836	0.0576	0.0096	0.0107	0.5176
color a*	0.965	0.580	0.611	-0.232	0.449	0.850	-0.155	-0.225	0.433	0.5992	0.0201	0.3215	0.0029	0.3026
color b*	12.47	15.54	14.84	16.48	14.82	13.77	16.15	16.16	2.156	0.8432	0.1683	0.2894	0.0322	0.9098

SE: standard error of the mean; P treat: level of significance for treatment; P time: level of significance for time; P treat x P Time: level of significance for iteration between treatment and time; L: linear; Q: quadratic. P<0.05; means are analyzed in the lines; Treatment Lh + ST: *Lactobacillus helveticus* and *Streptococcus thermophilus*; Treatment Lh: *Lactobacillus helveticus.

is of great importance for the development of flavour, aroma and texture of the finished product (Forsythe 2002) if the bacteria do not produce bitter peptides.

Coliforms are considered important parameters to evaluate the microbiological quality of cheeses and are affected mainly by the decrease in water activity, increase in acidity due the lactic acid bacteria and secondary microbiota (Alexandre et al. 2002, Manolopoulou et al. 2003 and Caridi et al. 2003). The coliforms found in the product indicate contamination after pasteurization (Birollo et al. 2001), which may have occurred in the present study because although pasteurized milk was used, recontamination may have occurred.

The use of annatto and lactic acid bacteria was not effective in the decimal reduction of this microbial group (Table V), although studies conducted by Gonçalves et al. (2005) confirmed the antimicrobial action of annatto "in vitro". In addition, the bioprotective effect of LAB on cheese was verified by authors such as Favaro et al. (2015) and (Montel et al. 2014) and Al-Gamal (2019), who found a reduction of up to 95% in food pathogens such as *Escherichia coli, Staphylococcus, Salmonella, Pseudomonas aeruginosa and Bacillus cereus* by the *L. helveticus* strain CNRZ32. The cheeses color (Table VI) may be related to different internal and external factors, and the opacity of the cheeses (L*) can be affected by the degree of matrix hydration.

The variations may be due mainly to the initial composition of milk and whey and the cheese making technology (Pena-Serna et al. 2016). According to Ginzinger et al. (1999), the color parameter b* is strongly correlated with the yellowish color and may be linked with the maturation time. In this study annato did not influence b*. Buffa et al. (2001) analysed the color change in cheeses with and without the pasteurization process during the 60-day maturation and found that the a* value remained constant up to 30 days (0.55), the L-value decreased (91.53) and the value b* increased (8.51).

The elasticity is a measure of the recovery of the original, undeformed condition after the first compressive force is removed and the cohesiveness is the amount a cheese can be deformed before breaking (Ong et al. 2012). These attributes are affected by milk composition, cheese production and procedures, microorganisms, maturation and moisture, pH and soluble calcium (Lucey et al. 2003, McMahon et al. 2005). The elasticity shows if the biochemical reactions taking place inside the cheese are not enough to modify the final structure, giving more or less flexibility. Treated cheeses containing St+Lh were less cohesive at 20 days; that is, they had lower internal bond

Table VII. Antioxidant capacity of the ABTS radical (ET µM) and texture parameters of ripened cheese.

	Treatment Lh+ST				Treatment Lh					P				Contrast
	0	10	20	30	0	10	20	30	SE	Treat	Time	Treat x Time	L	Q
ABTS	542.7^{ab}	433.9^{b}	381.2^{b}	669.5^{a}	248.8^{b}	386.0^{b}	537.8^{ab}	309.8^{b}	48.31	0.0005	0.2091	<0.0001	0.0403	0.8175
Cohe	0.94^{a}	0.79^{a}	0.48^{b}	0.74^{a}	0.88^{a}	0.85^{a}	0.82^{a}	0.91^{a}	0.052	0.0021	0.0003	0.0054	0.0133	0.0012
Elast	4.24^{a}	3.90^{a}	3.07^{b}	4.14^{a}	4.15^{a}	4.18^{a}	4.30^{a}	3.95^{a}	0.127	0.0022	0.0044	<0.0001	0.0596	0.0088
Gum	1382.7	1460	6726	15562	1132	2811	12890	11991	2335.2	0.5813	<0.0001	0.2388	<0.0001	0.3588
Chew	57.52	56.65	222.10	631.8	46.20	113.5	545.57	444.55	88.607	0.04754	<0.0001	0.0579	<0.0001	0.3434

*** Treatment Lh+ST: *Lactobacillus helveticus* and *Streptococcus thermophilus*; Treatment Lh: *Lactobacillus helveticus.* Cohe: cohesiveness; Elast: elasticity; Gum: gum; Chew: Chewiness.**

strength and thus no resistance to structural disintegration.

For the lowest value for the elasticity (Lh + ST on the 20th day of maturation), the moisture showed a decrease of 52.65% at 10 days to 34.61%. Pinho et al. (2004) evaluated the texture profile of Terrincho cheese during 60 days of maturation and observed that in the first 20 days of maturation, there was an increase in the gumminess and chewing and a decrease in the adhesiveness, elasticity, cohesiveness of the cheese. According to the authors, the change in texture was attributed to a decrease in pH below 5.5.

The ABTS radical sequestration method measures the antioxidant activity of compounds of hydrophilic and lipophilic nature (Gülçin et al. 2010, Karadag et al. 2009). The annatto seed provided for animals in the diet presents the carotenoid bixin (*Bixa orellana L*), which has an antioxidant potential (Nozière et al. 2006), conferring greater reactivity of these molecules to oxidizing agents, thus providing greater stability (Kiokias & Gordon 2013).

Carotenoids can be degraded when exposed to light or when subjected to high temperatures during colonial cheese processing, which can

Table VIII. Profile of fatty acids in samples of ripened cheese (mg/g fat).

	Treatment Lh+St				Treatment Lh				P	
	0	10	20	30	0	10	20	30	P Treat	P Time
C6:0	0.3594	0.753	0.736	0.8955	0.6176	0.7798	1.1753	0.7333	0,3507	0.0879
C8:0	0.4722 ^{b}	0.7365 ^{b}	0.7913 ^{b}	0.9075 ^{b}	2.0034 ^{ab}	2.4413^{ab}	3.5030 ^{a}	2.2152 ^{ab}	<.001	0.2215
C10:0	1.7693 ^{b}	2.1600 ^{ab}	2.4188 ^{ab}	2.6720 ^{a}	0.1491 ^{bc}	0.2398 ^{bc}	0.3728 ^{bc}	0.2063 ^{bc}	<.001	0.0573
C12:0	2.7666 ^{ab}	2.8910 ^{ab}	3.3075 ^{a}	3.5600 ^{a}	0.1308 ^{bc}	0.1612 ^{bc}	0.2390 ^{bc}	0.1517 ^{bc}	<.001	0.118
C16:0	31.593 ^{a}	29.461^{a}	30.674 ^{a}	32.335 ^{a}	1.9729 ^{b}	2.0487 ^{b}	2.4410 ^{b}	2.1915 ^{b}	<.001	0.4731
C18:0	12.2865^{a}	110150^{a}	12.2863^{a}	12.5875^{a}	6.4212 ^{b}	5.9856 ^{b}	6.3376 ^{b}	5.8685 ^{b}	<.001	0.3976
C18:1 n9t	6.3077 ^{b}	5.7142 ^{b}	5.9328 ^{b}	5.7173 ^{b}	25.7443 ^{a}	21.2828 ^{a}	15.5183^{ab}	21.1745 ^{a}	<.001	0.3261
C19:1 n9c	23.8830^{a}	27.1178^{a}	22.9053^{a}	20.0052^{a}	0.3137 ^{b}	0.3052 ^{b}	0.3900 ^{b}	0.3463 ^{b}	<.001	0.3734
C18:2 n 6t	0.3358 ^{b}	0.3272^{b}	0.3253^{b}	0.3385 ^{b}	1.3389 ^{a}	1.3922 ^{a}	2.0335 ^{a}	1.4412 ^{a}	<.001	0.1675
C18:2 n6c	1.5204 ^{a}	1.3760 ^{a}	1.3463 ^{a}	1.3750 ^{a}	0.1254 ^{a}	0.1318 ^{b}	0.2025 ^{b}	0.1417 ^{b}	<.001	0.9222
C20:0	0.1524 ^{a}	0.1245 ^{a}	0.1333 ^{a}	0.1343 ^{a}	0.05147 ^{b}	0.04675 ^{b}	0.06800^{b}	0.05575 ^{b}	<.001	0.2929
C18:3 n6	0.05598	0.04225	0.0445	0.03975	0.06723	0.07489	0.1313	0.06925	0.2501	0.3452
C18:3 n3	0.2526 ^{b}	0.1945 ^{b}	0.2085 ^{b}	0.2107 ^{b}	0.6306 ^{ab}	0.6740 ^{ab}	1.2187 ^{a}	0.7320 ^{ab}	0.0002	0.3679
SFA	63.9723^{a}	60.8100 ^{a}	65.8012^{a}	69.5422^{a}	28.2383 ^{b}	28.9915 ^{b}	37.5497 ^{b}	27.8855 ^{b}	<.0001	0.3694
MUFA	33.3540^{b}	36.5080 ^{b}	32.3630^{b}	27.6770^{b}	69.6677 ^{a}	67.9425 ^{a}	83.1800 ^{a}	68.8740 ^{a}	<.0001	0.514
PUFA	2.3187	2.064	2.0668	2.0845	2.1041	2.3105	3.7518	2.487	0.1083	0.3178
PUFA:SUFA	0.03360^{b}	0.03400 ^{b}	0.03175^{b}	0.02975^{bc}	0.07594 ^{ab}	0.08000^{ab}	0.1023 ^{a}	0.09050 ^{a}	<.0001	0.6885
n3: n6	0.2878 ^{a}	0.2267 ^{ab}	0.2075 ^{ab}	0.1965 ^{ab}	0.08181 ^{b}	0.08141 ^{b}	0.1003 ^{b}	0.1143 ^{b}	0.0006	0.7457

***the means are presented in the lines for interaction x treatment time; C13:0, C14:0, C14:1, C15:0, C15:1, C16:1, C17:0, C17:1, C21:0, C22:0, C24:0 3 C20:1 are not present in the table. Treatment Lh+ST: *Lactobacillus helveticus* and *Streptococcus thermophilus*; Treatment Lh: *Lactobacillus helveticus.* Different lowercase letters on the same line differ from each other.**

influence the antioxidant capacity (Rocha Garcia et al. 2012).

During maturation, CLA can be formed from linoleic acid through the action of primary or secondary cultures (Collomb et al. 2003), with a change in lipid profile due to lipolysis reactions. Some bacteria have this characteristic including *Butyrivibrio fibrisolvens*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium brevis*, *Bifidobacterium longum*, *Propionibacterium acnes*, *Propionibacterium freudenreichii* and *Propionibacterium sporogenes* (Yang et al. 2017).

A number of CLA-producing species and strains have been reported; however, the mechanisms for CLA bioconversion have not been elucidated for each species, and the individual potential is unknown (Yang et al. 2017).

Lactobacillus reuteri was the first species of lactic acid bacteria related to the high production capacity of CLA (Rosson et al. 1999) besides *Lactobacillus plantarum* AKU1009 with a conversion rate of up to 85% of linoleic acid (LA) converted to c9, t11-CLA (Kishino et al. 2002). *L. helveticus* and *S. thermophilus* also have the ability to produce CLA according to studies conducted by Pariza & Yang (1999) and Lin et al. (1999).

According to Ribeiro et al. (2018), CLA values can be related to factors such as temperature, fermentation time, linoleic acid content (LA). Some authors have shown a positive correlation between CLA formation and the ability to tolerate free LA, suggesting that is a detoxification mechanism (Adamczak et al. 2008, Wang et al. 2007).

Production of CLA in cheeses was also verified by Chamba & Perread (2002), who studied Emmental, and by Woo et al. (1984), who studied Roquefort and blue cheeses. Zlatanos et al. (2002) reported an increase in CLA with maturation times in hard cheeses when compared to fresh cheeses. Some strains are inhibited by LA, suggesting the need to study specific producer strains, as well as cheese processing conditions and maturation (Sieber et al. 2004).

Perotti et al. (2008) evaluated the free fatty acid profile (C6: 0 to C18:2) at different maturation times with different strains of *L. helveticus*, which showed no significant differences.

The cheese produced showed high values of saturated fatty acids (SUFA: 63.1508 mg/g fat) similar to those observed by Carafa et al. (2019), who evaluated cheeses produced with *Lactococcus lactis subsp. lactis, S. thermophilus* and *Lactobacillus rhamnosus*. The higher production of palmitic (14: 0), myristic (16: 0) and stearic (18: 0) fatty acids led to the high values obtained for saturated fatty acids (SUFA) at 7 months of maturation.

The recommended n6:n3 ratio for humans in diets should be 2:1 to 3:1 (Masters 1996). Diets based on lower ratios inhibit the transformation of linoleic acid into very long chain polyunsaturated fatty acids (Martin et al. 2006).

In general, cheeses are related to a high concentration of long chain fatty acids; however, it is also known that they contain unsaturated fatty acids, such as oleic acid and CLA, that are important for health. The composition of fatty acids in cheeses varies according to the animal breeds, time of year, animal and species diet and manufacturing processes (González-Martín et al. 2017).

CONCLUSIONS

Ripened cheeses produced with *L. helveticus* and *S. thermophilus* showed physicochemical characteristics according to the standards for low and medium moisture cheeses. Cheeses

containing *L. helveticus* had lower saturated fatty acid content and higher monounsaturated fatty acid content with a higher PUFA:SUFA ratio.

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Paula Martins Olivo: planning and execution, Geraldo Tadeu dos Santos: experiment with animals, Bruna Moura Rodrigues: aid in the preparation and analysis of cheeses, Milene Puntel Osmari: statistical analysis, Grasiele Scaramal Madrona texture analysis, Bruna Calvo Agostinho: analysis of antioxidants, Francilaine Heloise de Marchi: analysis of fatty acids, Magali Soares dos Santos Pozza: supervisor.