LWT - Food Science and Technology 138 (2021) 110700

Contents lists available at ScienceDirect

LWT

journal homepage: www.elsevier.com/locate/lwt

Interactive effect of physicochemical and microbial variables on bioactive amines content during storage of probiotic fermented milk

Carla Paulo Vieira [a,b], Marion Pereira da Costa [c], Vitor Luiz de Melo Silva [b], Karina Frensel Delgado [a,b], Beatriz da Silva Frasão [b], Thaisa Abrantes Elias [a], Yves Eduardo Chifarelli de Oliveira Nunes [b], Maria Beatriz de Abreu Gloria [d], Carlos Adam Conte-Junior [a,b,e,*]

[a] Food Science Program, Instituto de Química, Universidade Federal Do Rio de Janeiro, 21941-909, Rio de Janeiro, Brazil
[b] Department of Food Technology, Faculdade de Veterinária, Universidade Federal Fluminense, 24230-340, Rio de Janeiro, Brazil
[c] Laboratory of Inspection and Technology of Milk and Derivatives, Escola de Medicina Veterinária e Zootecnia, Universidade Federal da Bahia, 40170-110, Bahia, Brazil
[d] Laboratory of Quality Control, Faculdade de Farmácia, Universidade Federal de Minas Gerais, 31270-901, Minas Gerais, Brazil
[e] Center for Food Analysis (NAL), Technological Development Support Laboratory (LADETEC), 21941-598, Rio de Janeiro, Brazil

ARTICLE INFO

Keywords:
Tyramine
Bioactive amines
Goat milk
Cow milk
Lactobacillus acidophilus

ABSTRACT

Bioactive amines (BAs) represent a considerable toxicological risk in fermented dairy products because they provide the ideal environment for their formation. Thus, secondary control measures to prevent or reduce BAs formation in dairy products are urgently needed. However, additional scientific knowledge about the factors affecting BAs production and the interaction among them is currently necessary to achieve this goal. In this context, Partial Least Square with Variable Importance in Projection (PLS-VIP) analysis followed by cross-validation was applied to variables to investigate their interactive effect on BAs accumulation in cow's and goat's fermented milk during refrigerated storage. *Streptococcus thermophilus* and *Lactobacillus acidophilus* LA-5 increased tyramine and total BAs content in both cow's (CFM) and goat's (GFM) fermented milks. In CFM, Maillard reaction involving galactose and increased post acidification interacted to enhance accumulation of BAs ($R^2 = 0.895$, $P = 1.11 \times 10^{-5}$); whereas for GFM, losses of consistency coefficient and viscosity were essential for BAs accumulation ($R^2 = 0.919$; $P = 2.72 \times 10^{-6}$). These findings show that by preventing Maillard reaction and delaying post acidification in CFM, as well as by controlling viscosity in GFM, there can be mitigation of BAs formation during storage.

1. Introduction

Bioactive amines (BAs) are nitrogenous organic bases of low molecular weight formed in fermented food mainly by microbial decarboxylation of free amino acids (Vieira et al., 2017). BAs represent a considerable toxicological risk in fermented dairy products (Linares et al., 2012). Tyramine in general cause acute toxicity, typically leading to symptoms like a hypertensive peak, migraine, and cardiac failure (Loizzo et al., 2013). In contrast, putrescine and cadaverine potentiate tyramine's toxicity and can cause gastric or intestinal cancer. Additionally, an increase in the physiological concentrations of spermidine and spermine can dysregulate cell proliferation, causing cancer or accelerating tumor spread (Benkerroum, 2016).

Some dairy products can accumulate high levels of BAs because they provide an ideal environment for their formation. Free amino acids, which are substrates for BAs production, are naturally present in milk, as well as they can also be released by proteolytic activities inherent to the dairy matrix. In addition, starter and probiotic cultures, as well as contaminating microorganisms, perform proteolytic activities that increase the content of free amino acids in foodstuffs (Gezginc et al., 2013). As positive-amino acid decarboxylase starters and contaminating microorganisms can be present in dairy products and/or in the milk used to produce them, these free amino acids can be converted to BAs (Linares et al., 2012). Furthermore, the optimum pH for bacterial decarboxylases lies usually in the acidic range, which is typical of these products (Smit et al., 2008).

* Corresponding author. Food Science Program, Instituto de Química, Universidade Federal Do Rio de Janeiro, 21941-909, Rio de Janeiro, Brazil.
E-mail addresses: conte@iq.ufrj.br, carlosconte@id.uff.br (C.A. Conte-Junior).

https://doi.org/10.1016/j.lwt.2020.110700
Received 27 May 2020; Received in revised form 28 November 2020; Accepted 1 December 2020
Available online 3 December 2020
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Lactic acid bacteria (LAB) are well documented as the main BAs producers in fermented dairy products. Increasing evidence has demonstrated that several LAB strains commonly used as starter cultures, such as those belonging to the genera *Streptococcus* and *Lactobacillus*, are relevant BAs producers in dairy products (Papageorgiou et al., 2017). Consistently, elevated levels of tyramine are essentially associated with high counts of LAB in starter cultures (Komprda et al., 2008). Therefore, as LAB starter cultures may contribute for BAs accumulation, conventional means to reduce the overall contamination of dairy products, such as heat treatment and fermentation, have been found to be of limited value to their control in these foodstuffs (Costa, Balthazar, et al., 2015).

In this scenario, the selection of starters not producing BAs has been reported as an action to reduce their content, but this effort has failed; several bacteria associated with food, as those belonging to the *Lactobacillus* genus, have obtained a Qualified Presumption of Safety by the EFSA (2011), even though some strains have been known to be BAs producers. Consistently, probiotic LAB and *Bifidobacteria* isolated from industrially manufactured dairy products have demonstrated decarboxylase activity and potential BAs production (Lorencová et al., 2012). Moreover, applying BA-oxidizing bacteria reduces only the levels of pre-formed BAs in dairy products. Thus, this strategy should not be envisaged as a single preventive measure (Benkerroum, 2016).

Regarding physicochemical properties, currently there is a paucity of information in the literature on the effect of parameters, such as types and content of carbohydrates, as well as texture, about the BAs content in fermented dairy products. Furthermore, the consequences of interaction among physicochemical variables on BAs production in these products also require an investigation (Linares et al., 2012).

In this context, secondary control measures to prevent or reduce BAs formation in dairy products are urgently needed. However, additional scientific knowledge about the factors affecting BAs production and the interaction among them are necessary to reduce BAs accumulation in fermented dairy products (Benkerroum, 2016). This study evaluated the interactive effect of physicochemical and microbial variables on individual and total BAs accumulation in fermented milk from different matrices (cow and goat) during refrigerated storage for 28 days.

2. Material and methods

2.1. Reagents and chemicals

Standards of bioactive amines, carbohydrates and organic acids, all ≥98% of purity, and L-tyrosine (≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (HPLC grade) and all analytical-grade chemicals were obtained from Tedia (São Paulo, Brazil). A Milli-Q water system was used for ultrapure water (Millipore, Bedford, MA, USA).

2.1.1. Fermented milk processing

The fermented milks were processed according to Costa, Balthazar, et al. (2015). Cow's and goat's fermented milk was prepared using UHT milk (cow's milk, Macuco®, Rio de Janeiro, Brazil; goat's milk, Caprilat®, Paraná, Brazil), in triplicate. During production, lyophilized *Lactobacillus acidophilus* LA-5®, *Bifidobacterium lactis* BB-12®, and *Streptococcus thermophilus* (Biorich, Chr Hansen, Valinhos, Brazil), 4×10^{14} CFU m^{-3} each were added simultaneously in DVS form (direct vat set) to milk. Then, the samples were fermented in an incubator (Thermo Scientific, Waltham, USA) at 45 ± 2 °C until pH 4.6 was reached. Subsequently, they were stored and refrigerated at 4 ± 2 °C for 28 days.

2.2. Methods of analysis

2.2.1. Microbiological analysis

The counts of *Lactobacillus acidophilus* LA-5®, *Bifidobacterium lactis* BB-12®, and *Streptococcus thermophilus* were performed in accordance with Costa, Frasao, et al. (2015). Briefly, samples were submitted to serial dilutions and inoculated into Petri dishes using a Spiral Plater (E50, Eddy Jet 2, IUL Instruments, Barcelona, Spain). Enumeration of *Streptococcus thermophilus* was performed on M17 agar with lactose, after incubation under aerobiosis at 37 ± 1 °C for 2 days. *Lactobacillus acidophilus* counts were determined through growth on MRS agar supplemented with 1.5 kg m^{-3} bile salts, and aerobically incubated at 37 ± 1 °C for 2 days. *Bifidobacterium lactis* were enumerated on MRS agar (supplemented with neomycin sulfate, nalidixic acid, LiCl and CyHCl) after incubation at 37 ± 1 °C for 3 days under anaerobiosis. The enumeration of colonies was performed using an electronic counter (Flash & Go, IUL instruments, Barcelona, Spain) after incubation of each bacteria and expressed as log colony forming units (CFU) per gram.

2.2.2. pH determination

The pH of each sample was measured with a digital pH-meter (model PG1800 Cap-Lab Industry and Trade Ltd., São Paulo, Brazil) by direct insertion into the fermented milk. Before use, the electrode was calibrated with standard buffer solutions of pH 4.00 and 7.00.

2.2.3. Extent of proteolysis

The extent of proteolysis was estimated through determination of the concentration of total free amino acids according to Shori et al. (2013). The determination of the absorbance at 490 nm was performed using a UV-VIS spectrophotometer (Smartspec Plus; BioRad, Hercules, CA, USA). The absorbance of total free amino acids in the samples was read against L-tyrosine standard curve (0.0–0.1 mM or $0.0–1.8 \times 10^{-2}$ kg m^{-3}, $R^2 \geq 0.981$).

2.2.4. Bioactive amines quantification by RP-HPLC-DAD

The bioactive amines quantification was performed by reverse-phase HPLC with diode array detector (Shimadzu, Kyoto, Japan). The method of extraction and derivatization well as the chromatographic conditions used herein were previously described and validated by our research group following US-FDA Guidelines, being considered suitable for quality control purpose of yogurts (Vieira et al., 2017). The BAs were identified by retention times and by spiking the samples with the suspected amine and quantified by interpolation of peak area in external standard curves (1–50 mg L^{-1} or $10^{-3}–5 \times 10^{-2}$ kg m^{-3}, $R^2 \geq 0.980$) using LC Solution software.

2.2.5. Carbohydrates and organic acids quantification by HPLC-DAD-RI

The method used here for carbohydrates and organic acids quantification by high performance liquid chromatography (Shimadzu, Kyoto, Japan) was previously described and validated by our research group (Costa et al., 2016). The compounds were identified by retention times and by spiking the suspect analyte to the sample and the concentrations (mg g^{-1}) were determined by interpolation in external standard curves (0.06–60 mg g^{-1} or $6 \times 10^{-5}–6 \times 10^{-2}$ kg kg^{-1}, $R^2 \geq 0.995$) using a LC Solution software.

2.2.6. Instrumental color

The values of lightness (L^*, 100 = white, 0 = black), redness (a^*, + red, - green), and yellowness (b^*, + yellow, - blue) of the fermented milks were recorded with a Minolta CM-600D spectrophotometer (Minolta Camera Co., Osaka, Japan) according to Costa, Frasao, et al. (2015).

2.2.7. Instrumental texture analyses

Firmness (*g*) and consistency (*g.s*) were measured according to Costa, Frasao, et al. (2015) using a texture analyzer (TA-XT.Plus, Stable Micro Systems Ltd., Surrey, UK) equipped with a 49.0 N load cell. The back-extrusion cell plunger was 3.6×10^{-2} m in diameter and set at 2×10^{-2} m above the sample surface. The test cell penetrated 2×10^{-2} m into the sample (300 mL or 3×10^{-4} m^3) at 4 °C.

2.2.8. Rheological analysis

Flow measurements were determined using a Brookfield concentric cylinder viscometer (LVDVIII, Brookfield Engineering Laboratories Inc., Stoughton, MA, USA). The measurements were performed in triplicate using a small sample adapter and SC4-31 needle with 10 mL or 10^{-5} m^3 samples at 4 °C at speeds in the range of 20–250 rpm.

The experimental data fitted the Herschel and Bulkley model (Eq. (1)), which is the best to describe the rheological behavior of fermented milk (Behnia et al., 2013).

$$\tau = \tau_0 + K\gamma^n \quad (1)$$

where: τ = shear stress (Pa), τ_0 = yield stress (Pa), K = consistency coefficient (mPa s^n), γ = shear rate (s^{-1}), and n = flow behavior index (dimensionless).

The Wingather program (Brookfield Engineering Laboratories Inc., Stoughton, MA, USA) was used to collect data and to calculate apparent viscosities at a shear rate of 25 s^{-1} (rotation at 250 rpm).

2.2.9. Estimative of Harrell's optimism on regression models using bootstrap method

The estimative of the Harrell's optimism was calculated according to Eq. (2) (Harrell et al., 1996), and the coefficient of determination of the original model after validation (Eq. (3)).

$$o = \frac{\sum_{m=1}^{M} o^{(m)}}{M} \quad (2)$$

$$R_v^2 = R_{app}^2 - o \quad (3)$$

where, for each bootstrap sample with replacement (m = 1, …, M), $R^{2(m)}_{boot}$ = bootstrap coefficient of determination obtained from fitted model to the bootstrap dataset; $R^{2(m)}_{orig}$ = original coefficient of determination obtained by applying the fitted model from the bootstrap dataset to the original dataset; o = optimism of the original model; $o^{(m)} = R^2_{boot^{(m)}} - R^2_{orig^{(m)}}$; M = number of bootstrap datasets; R^2_v: coefficient of determination of the original model after validation; R^2_{app} = apparent coefficient of determination obtained from fitted model to original data.

2.3. Statistical analysis

The experiments and the analyses were performed in analytical and experimental triplicate, and the results expressed as the mean ± standard deviation (SD). Two-way analysis of variance (ANOVA) was used at a significance level of 0.05, followed by Tukey's multiple comparison tests (two-side, $P < 0.05$). The correlation between variables was evaluated by Pearson correlation test with a significance level of 0.05. Next, the models were internally validated using the bootstrap method (confidence interval = 95%; number of simulations = 1000; size of bootstrap samples = size of original sample; and number of bootstrap samples = 200) (Harrell et al., 1996). For analysis of the interactive effects among variables, Partial Least Square with Variable Importance in Projection (PLS-VIP) data analysis at 0.05 significance level was performed and the optimal number of principal components in model was determined by cross-validation (internal validation) using the method of V-fold. All statistical analyses were performed using a commercially available statistical package (Systat 12 software, Chicago, IL, USA).

3. Results and discussion

3.1. Microbial changes

The microbiological changes in the fermented milks during refrigerated storage are indicated in Fig. 1. Regarding *S. thermophilus* and *L. acidophilus* LA-5, there was a significant increase up to 14 and 21 days of storage, respectively, for both cow and goat fermented milks (Fig. 1A and B). Afterwards, the counts of *L. acidophilus* LA-5 for both types of milk and of *S. thermophilus* for cow's fermented milk (CFM) did not change until the end of storage, whereas the counts decreased slightly in the last week of storage for goat's fermented milk (GFM). The counts of *L. acidophilus* LA-5 showed strong positive correlation with storage time ($P < 0.05$) for both matrices (Fig. 1S; Tables S1 and S2). The growth of *L. acidophilus* LA-5 and *S. thermophilus* during storage is probably associated with their proteolytic activity, which is needed as the pool of free amino acids in the milk becomes limited (Gandhi & Shah, 2014).

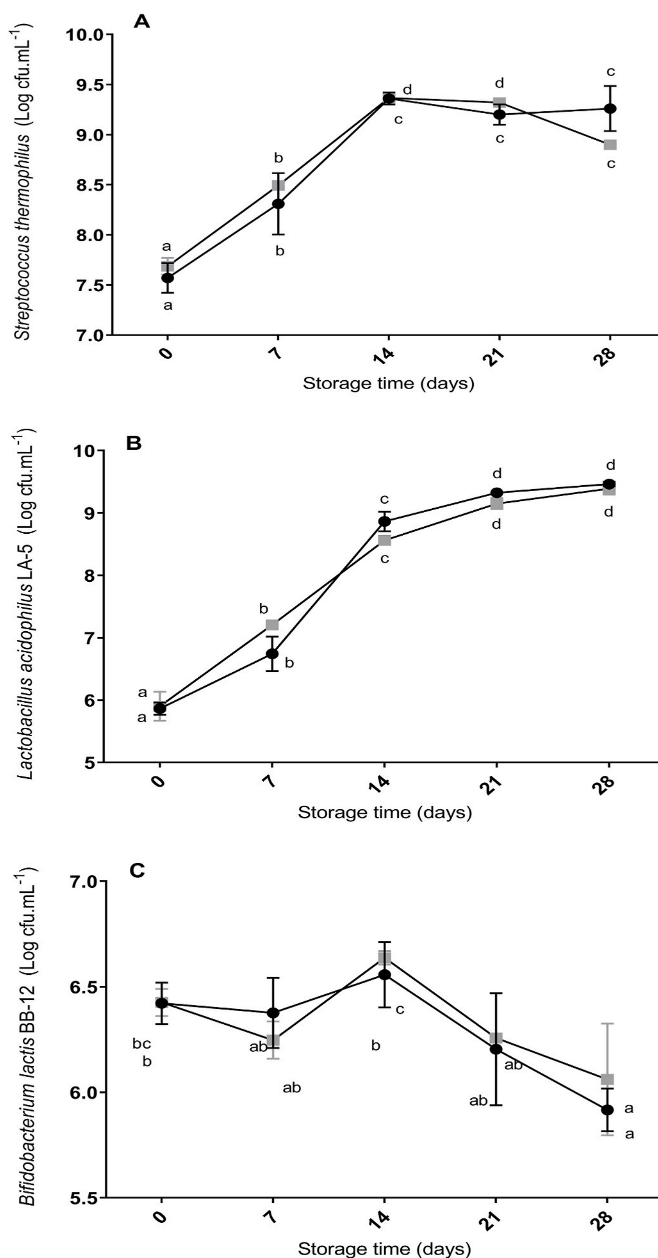

Fig. 1. Number of surviving cells (Log CFU mL^{-1}) during storage (28 days) at 4 °C for (A) *Streptococcus thermophilus*, (B) *Lactobacillus acidophilus* LA-5 and (C) *Bifidobacterium lactis* BB-12 in cow's fermented milk (black line) and goat's fermented milk (grey line). Analysis were performed in triplicate and the data are reported as the means ± SD, $P < 0.05$, two-way ANOVA and post-hoc Tukey test: [a,b,c] ≠ among storage days.

On the other hand, the counts of *B. lactis* BB-12 changed slightly until the 14th day, declining afterwards until the end of storage (Fig. 1C). The lower viability of *B. lactis* BB-12 compared to the lactic acid bacteria (LAB) was also reported previously for commercial dairy products

during refrigerated storage (Shin et al., 2000). Such lower viability may be attributed to the smaller proteolytic activity and to the higher sensibility inherent to *Bifidobacterium* to post acidification (pH ≤ 4.0) and to hydrogen peroxide, which result from LAB metabolism (Shin et al., 2000).

There was no significant difference for the microbial counts between the cow and goat milks (Fig. 1).

3.2. pH changes

The changes on pH values of the fermented milks during 28 days of storage are reported in Table 1. In both cow and goat products, there was a substantial decrease in pH in the first week. Afterwards, only slight pH changes were observed until the end of storage. The reduction in pH is due to post acidification during storage, linked to the progressive transformation of lactose into lactic acid by LAB, needed for the maintenance of the metabolic activity of these bacteria during cold storage (Olson & Aryana, 2008).

GFM had a more acidic pH than CFM throughout the storage period (Table 1). This can be attributed to differences in the post acidification rate and/or lipolytic activity of LAB typical of each milk, as some starters are more active in goat milk while others are more active in cow milk (Güler & Gürsoy-Balcı, 2011).

3.3. Proteolysis in the fermented milk samples

Proteolysis during 28 days of storage of the fermented milk is presented in Table 1. The free amino acids (FAAs) content increased linearly with storage time in CFM (Fig. 1S; Table S1). However, the FAAs content increased in GFM only until the 14th day, decreasing afterwards. According to the literature (Gandhi & Shah, 2014), LAB cannot synthesize essential amino acids, therefore, they require exogenous source, which result from the proteolytic activity of LAB enzymes on milk casein.

The difference in behavior between GFM and CFM, the latter presenting a continuous increase of FFAs, may be attributed to the fact that *S. thermophilus*, which was predominant in GFM, has lower proteolytic activity than *L. acidophilus* (Gandhi & Shah, 2014). Additionally, the different profile of amino acids that makes up the proteins in each matrix can also contribute to differences on proteolysis (Folkenberg et al., 2006).

3.4. Changes on carbohydrates and organic acids

As shown in Table 1, the levels of **lactose** decreased gradually throughout storage (until 21st day) in CFM, whereas for GFM, there was a sharp reduction in the first week of storage, and then, it remained constant until almost the end of storage (21st day). On the other hand, glucose and galactose contents increased sharply in the first week (Table 1). The decrease in lactose and increase in glucose and galactose during storage can be explained by the action of β-galactosidase released by LAB, an enzyme which hydrolyzes lactose to galactose and glucose (Kailasapathy & Sultana, 2003).

There was a sharp increase on **galactose** levels in the first week of storage, remaining constant afterwards, until the end of storage for CFM, while GFM presented similar behavior to CFM until the 14th day of storage. However, with the galactose content slightly decreasing afterwards (Table 1). Low utilization of galactose by LAB could be attributed to intricacies in the induction of enzymes present or which participate in the Leloir pathway (Srinivas et al., 1990). However, these high levels of residual galactose can react steadily with milk proteins; therefore, galactose has been implicated in Maillard reaction in fermented dairy product (Joubran et al., 2017).

The levels of **formic acid** increased in CFM and GMF until the 21st and the 14th days, respectively (Table 1). Although *S. thermophilus* metabolizes carbohydrates, mainly glucose into lactic acid (homolactic), it also possesses pyruvate-formate-lyase which can produce formic acid from glucose (Nishimura et al., 2015).

The contents of **lactic and citric acid** fluctuated ($P < 0.05$) during storage, suggesting production and consumption by LAB. Citric acid is the main substrate for acetoin and diacetyl formation, while lactic acid is the main substrate for acetaldehyde production by LAB, compounds which are relevant for the aroma of dairy products (Papagianni, 2012).

In addition, the differences in the rates of carbohydrates fermentation and organic acids production in both products (Table 1) can be attributed to differences in the post acidification rate of LAB with the type of milk (Güler & Gürsoy-Balcı, 2011).

3.5. Bioactive amines changes

The levels of BAs obtained by RP-HPLC-DAD during storage of the fermented milks are indicated in Table 1. Among the BAs investigated, tyramine was the prevalent amine. In fact, tyramine is the predominating BA in fermented dairy products (Costa, Balthazar, et al., 2015).

The concentration of putrescine increased showing a peak on the 14th day in GFM decreasing afterwards. In CFM, putrescine showed the same trend, although it did not reach statistical significance. Such a pattern of change – increase followed by reduction, can be explained by the fact that putrescine is a catabolic product of ornithine or arginine pathways and can be converted into spermidine which, in turn, can form spermine (Papageorgiou et al., 2017). Consistently, spermidine increased until the 14th day for both milks, decreasing afterwards until the end of storage in GFM and only in the last week in CFM (Table 1). Spermine, the final product of the polyamine's pathway, accumulated in CFM, presenting linear increase with storage time (Table S1 and Fig. 1S), but it accumulated in GFM until the 14th day, showing subsequent fluctuations (Table 1). Cadaverine was not detected in GFM. However, the concentration of cadaverine increased and showed a peak on the 14th day in CFM decreasing afterwards (Table 1). Cadaverine is produced from lysine through one-step decarboxylation reaction, and its subsequent reduction during storage may be attributed to its catabolism by *Lactobacillus* strains (Costa, Balthazar, et al., 2015). Regarding tyramine, it increased linearly up to 28 days of storage in both products (Tables S1 and S2; Fig. 1S). Tyramine is synthesized from tyrosine by one-step decarboxylation (Benkerroum, 2016).

The concentration of total BAs increased showing a peak on the 14th day in CFM, decreasing afterwards. This can be attributed to the increase of individual amines observed, including putrescine, cadaverine and spermidine until the 14th day of storage (Table 1). On the other hand, for GFM, total BAs remained constant in the first week, increasing from the 7th up to the 21st day reducing afterwards. The content of total BAs was positively correlated with the count of *S. thermophilus* for CFM and *L. acidophilus* LA-5 for GFM (Fig. 2; Table S3). This can be explained by the fact that BAs production depends on positive-amino acid decarboxylase bacteria activity (Papageorgiou et al., 2017).

Furthermore, total BAs were inversely correlated with pH values for CFM, while none correlation was observed for GFM (Fig. 2; Table S3). Bacterial decarboxylases show maximum activity in an acid environment (pH 4–5.5), similar to those found in fermented milk (Lazárková et al., 2012). However, BAs accumulation also depend on other factors, such as precursor amino acid concentration, as well as factors which influence bacteria growth and enzyme activity (Smit et al., 2008). This fact suggests that acidity can be a relevant factor for BAs formation in CFM, but not in GFM.

In addition, the concentration of galactose correlated directly with total BAs only in CFM (Fig. 2; Table S3). Monosaccharides, as galactose, can induce bacteria growth and BAs production in acidic pH (Lazárková et al., 2012). This indicates that galactose is a relevant monosaccharide for the accumulation of BAs in CFM, but not for GFM.

Table 1
Physicochemical properties of cow and goat's fermented milk over 28 days of storage at 4 °C.

Parameters	Storage time (days) at 4 °C									
	Cow's fermented milk					Goat's fermented milk				
	0	7	14	21	28	0	7	14	21	28
BAs (mg L^{-1})										
Putrescine	2.4 ± 0.3	2.8 ± 0.5	3.7 ± 0.8	3.5 ± 0.3	2.6 ± 0.4	$3.0^{a}\pm0.3$	$3.2^{a}\pm0.0$	$5.3^{b} \pm 0.2$	$3.6^{a}\pm0.8$	$3.6^{a}\pm0.3$
Cadaverine	$1.2^{a}\pm3 \times 10^{-2}$	$3.0^{a}\pm0.9$	$7.5^{b} \pm 1.6$	$3.9^{a}\pm1.2$	ND	ND	ND	ND	ND	ND
Spermidine	$2.4^{abA}\pm0.8$	$3.4^{abA}\pm1.4$	$5.0^{bA} \pm 2.3$	$5.6^{bA}\pm2 \times 10^{-2}$	$0.5^{aA}\pm3 \times 10^{-2}$	$4.2^{aA} \pm 1.0$	$3.6^{aA} \pm 0.4$	$13.1^{bB} \pm 6.5$	$5.7^{abA}\pm1.6$	$0.6^{cA} \pm 0.1$
Spermine	$2.2^{a}\pm0.4$	$2.9^{ab} \pm 0.5$	$3.8^{b} \pm 0.8$	$3.4^{b} \pm 0.2$	$4.8^{c}\pm1.0$	$3.4^{a}\pm0.5$	$3.3^{a}\pm0.18$	$6.2^{b} \pm 0.9$	$4.7^{c}\pm0.5$	$5.2^{bc}\pm0.5$
Tyramine	$13.3^{a}\pm0.7$	$17.1^{b} \pm 0.1$	$17.9^{b} \pm 0.4$	$16.8^{b} \pm 1.0$	$23.3^{c}\pm1.3$	$16.2^{a}\pm0.4$	$18.2^{ab} \pm 1.8$	$20.0^{ab} \pm 2.3$	$25.0^{b} \pm 4.5$	$26.1^{c}\pm2.7$
Total BAs	$20.3^{aA} \pm 0.2$	$30.1^{bA} \pm 1.3$	$35.9^{cA} \pm 3.0$	$30.1^{bA} \pm 1.8$	$30.1^{bA} \pm 1.7$	$26.9^{aB} \pm 1.9$	$26.0^{aA} \pm 0.3$	$33.9^{bA} \pm 4.4$	$42.5^{cB} \pm 1.5$	$36.8^{bB} \pm 0.1$
Total free AA (mM)	$0.41^{aA} \pm 0.03$	$0.54^{bA} \pm 0.05$	$0.53^{bA} \pm 0.03$	$0.62^{bcA} \pm 0.01$	$0.69^{cA} \pm 0.02$	$0.49^{aA} \pm 0.05$	$0.57^{abA}\pm0.06$	$0.85^{cB} \pm 0.05$	$0.62^{abA}\pm0.01$	$0.66^{bA} \pm 0.01$
pH	$4.39^{cB} \pm 0.01$	$3.80^{aB} \pm 0.01$	$3.81^{aB} \pm 0.01$	$3.84^{bB} \pm 0.01$	$3.85^{bB} \pm 0.01$	$4.29^{dA} \pm 0.01$	$3.67^{cA} \pm 0.01$	$3.72^{aA} \pm 0.01$	$3.72^{aA} \pm 0.01$	$3.73^{bA} \pm 0.01$
Carbohydrates (mg g^{-1})										
Lactose	$51.20^{cB} \pm 1.03$	$50.36^{bcB} \pm 0.01$	$49.63^{abB}\pm0.32$	$48.22^{aB} \pm 1.45$	$53.42^{dB} \pm 0.63$	$46.71^{bA} \pm 1.57$	$42.53^{aA} \pm 0.27$	$42.40^{aA} \pm 0.43$	$43.23^{aA} \pm 2.27$	$44.14^{abA}\pm1.79$
Glucose	$0.034^{aA} \pm 0.011$	$0.350^{bB} \pm 0.001$	$0.347^{bB} \pm 0.002$	$0.342^{bB} \pm 0.018$	$0.048^{aB} \pm 0.022$	$0.030^{bA} \pm 0.001$	$0.278^{cA} \pm 0.005$	$0.284^{cA} \pm 0.005$	$0.016^{abA}\pm0.001$	$0.012^{aA} \pm 0.011$
Galactose	$8.19^{a}\pm0.20$	$10.27^{b} \pm 0.01$	$10.23^{b} \pm 0.06$	$9.93^{b} \pm 0.38$	$10.22^{b} \pm 0.17$	$8.50^{a}\pm0.28$	$10.19^{c}\pm0.07$	$10.19^{c}\pm0.08$	$9.59^{b} \pm 0.46$	$9.74^{bc}\pm0.34$
Organic acids (mg g^{-1})										
Citric	$0.07^{bA} \pm 0.01$	$0.11^{cB} \pm 0.01$	$0.04^{aA} \pm 0.01$	$0.03^{aA} \pm 0.01$	$0.11^{cA} \pm 0.01$	$0.14^{bB} \pm 0.01$	$0.02^{aA} \pm 0.01$	$0.02^{aA} \pm 0.01$	$0.13^{bB} \pm 0.02$	$0.13^{bA} \pm 0.01$
Lactic	$1.92^{abA}\pm0.04$	$2.00^{abA}\pm0.30$	$1.68^{aA} \pm 0.01$	$1.66^{aA} \pm 0.04$	$2.10^{bA} \pm 0.10$	$1.93^{bA} \pm 0.05$	$1.71^{aB} \pm 0.01$	$1.69^{aA} \pm 0.01$	$1.99^{bB} \pm 0.08$	$1.97^{bA} \pm 0.05$
Formic	$0.44^{aA} \pm 0.01$	$0.45^{aA} \pm 0.01$	$0.78^{bA} \pm 0.01$	$0.79^{bB} \pm 0.07$	$0.45^{aA} \pm 0.01$	$0.38^{aA} \pm 0.00$	$0.72^{bB} \pm 0.02$	$0.70^{bA} \pm 0.01$	$0.39^{aA} \pm 0.00$	$0.39^{aA} \pm 0.01$
Color										
L^*	79.72 ± 0.06	78.00 ± 0.16	78.50 ± 1.80	79.00 ± 1.50	78.02 ± 0.08	80.93 ± 0.03	80.70 ± 0.60	81.00 ± 0.40	81.37 ± 0.02	81.20 ± 0.30
a^*	$-0.78^{bA} \pm 0.01$	$-0.68^{abA}\pm0.03$	$-0.69^{abA}\pm0.04$	$-0.65^{aA} \pm 0.03$	$-0.67^{abA}\pm0.06$	$-0.72^{aA} \pm 0.07$	$-0.86^{abB}\pm0.06$	$-0.89^{bB} \pm 0.05$	$-0.89^{bB} \pm 0.03$	$-0.89^{bB} \pm 0.03$
b^*	$7.74^{B} \pm 0.02$	$7.03^{B} \pm 0.02$	$7.20^{B} \pm 0.40$	$7.20^{B} \pm 0.30$	$7.02^{B} \pm 0.01$	$4.86^{A} \pm 0.10$	$5.01^{A} \pm 0.09$	$5.19^{A} \pm 0.12$	$5.10^{A} \pm 0.18$	$5.01^{A} \pm 0.06$
AV (mPa.s)	$2397.57^{cB} \pm 0.01$	$2000^{bcB} \pm 200$	$1400^{abB}\pm500$	$1300^{abB}\pm500$	$700^{aB} \pm 300$	$254^{cA} \pm 8$	$157^{bA} \pm 3$	$111.34^{aA} \pm 0.01$	$94^{aA} \pm 8$	$81^{aA} \pm 6$
Texture										
Firmness (*g*)	$45^{cB} \pm 5$	$40^{bB} \pm 4$	$39^{bB} \pm 4$	$37^{bA} \pm 3$	$32.1^{aA} \pm 1.8$	$34.3^{A} \pm 0.6$	$34.3^{A} \pm 0.4$	$34.2^{A} \pm 0.6$	$34^{A} \pm 0.7$	$34.2^{A} \pm 0.5$
Consistency (*g*.s)	$475.79^{dA} \pm 37.01$	$430^{cdA}\pm50$	$400^{bcA} \pm 50$	$381^{bA} \pm 4$	$323^{aA} \pm 22$	$366^{aB} \pm 5$	$372.0^{bB} \pm 2.4$	$372^{bA} \pm 4$	$374^{bA} \pm 8$	$371^{aB} \pm 4$
Rheological										
τ_0 (Pa)	$34^{cA} \pm 9$	$18^{bcA} \pm 11$	$2.5^{abA}\pm0.5$	$0.08^{aA} \pm 5$	$7^{bA} \pm 3$	$0.90^{aB} \pm 0.3$	$0.80^{aB} \pm 0.5$	$0.54^{aA} \pm 0.2$	$0.80^{aB} \pm 0.3$	$0.50^{aA} \pm 0.1$
K (mPa s^n)	$8000^{aA} \pm 4000$	$8580^{aA} \pm 80$	$8408^{aA} \pm 0.01$	$12586^{aA} \pm 0.01$	$11000^{aA} \pm 2000$	$3982^{cB} \pm 7$	$1200^{bB} \pm 300$	$423^{aB} \pm 8$	$250^{aB} \pm 90$	$300^{aB} \pm 140$
n	$0.61^{cA} \pm 0.11$	$0.47^{bcA} \pm 0.13$	$0.33^{abA}\pm0.09$	$0.22^{aA} \pm 0.09$	$0.34^{abA}\pm0.01$	$0.30^{aB} \pm 0.03$	$0.47^{bA} \pm 0.02$	$0.63^{cB} \pm 0.01$	$0.72^{cB} \pm 0.09$	$0.66^{cB} \pm 0.11$

BAs: bioactive amines; AA: amino acids; L^*: lightness; a^*: greenness; b^*: yellowness; AV: apparent viscosity; τ_0: yield stress; K: consistency coefficient; n: flow behavior index; ND: not detected. Analysis were performed in triplicate and the data are reported as the means ± SD.

[a,b,c] Means within the same row with different superscripts indicate significant difference among storage times (Tukey's post-hoc test, $P < 0.05$).

[A,B] Means within the same row with different superscripts indicate significant difference between matrices (Tukey's post-hoc test, $P < 0.05$).

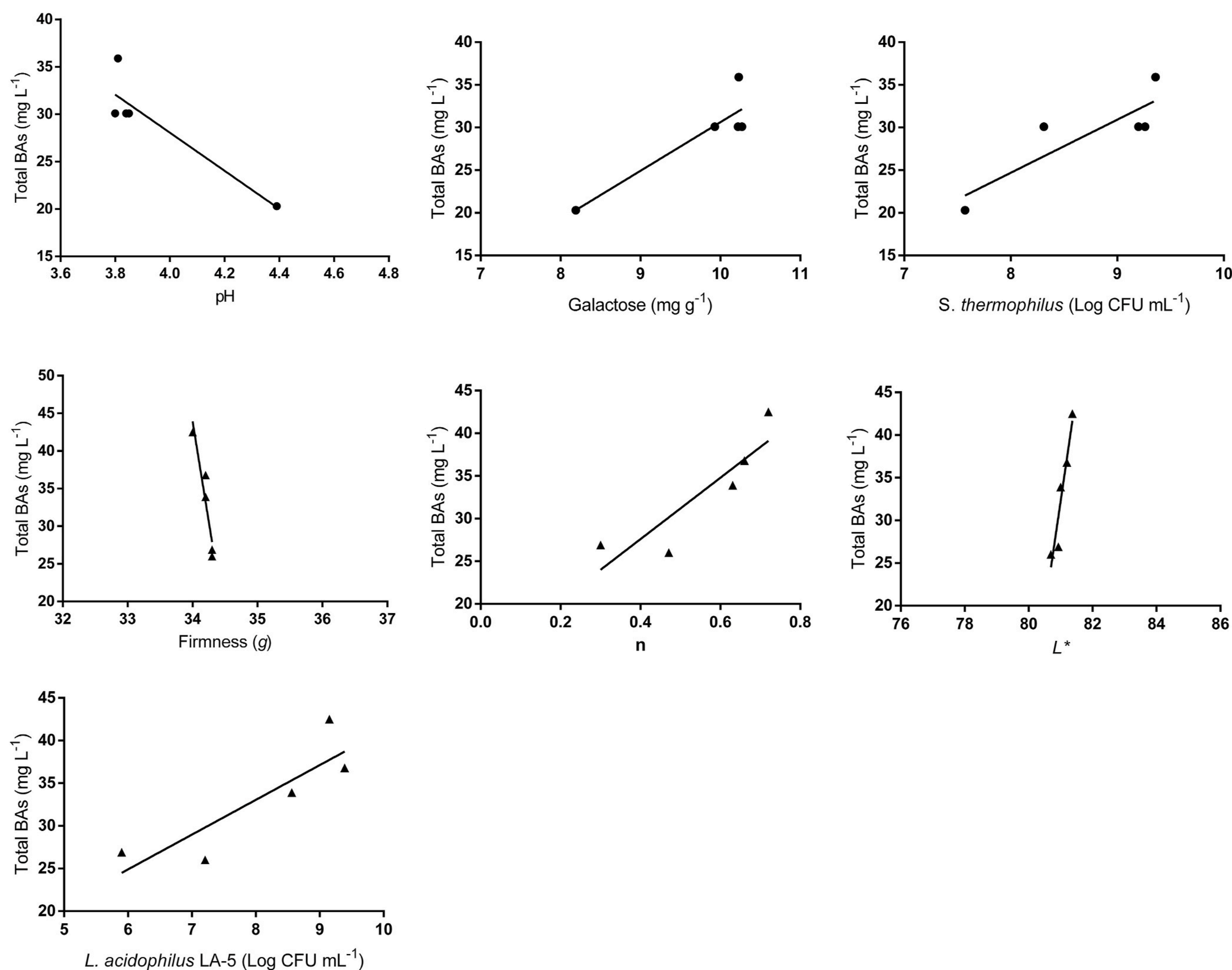

Fig. 2. Significant correlations (P < 0.05) and internally validated by Bootstrap method of total bioactive amines in relation to physicochemical and microbial parameters for cow's fermented milk (-●-) and goat's fermented milk (-▲-) stored at 4 °C. BAs: bioactive amines; L^*: lightness; n: flow behavior index. Analysis were performed in analytical and experimental triplicate.

3.6. Instrumental color measurements

The L^*, a^*, and b^* values of the fermented milks during storage are indicated in Table 1. The L^* value, which measures whiteness, results from colloidal particles in milk, including fat globules and casein micelles, which can scatter light in the visible spectrum (García-Perez et al., 2005). There was no significant difference on L^* values with storage time. Nevertheless, for GFM, the total BAs content correlated with L^* values (Fig. 2; Table S3). This can be attributed to proteolytic activity releasing free amino acids which are substrates for BAs production (Costa, Frasao, et al., 2015). The reduction of the size of the casein micelles during proteolysis can increase L^* values due to an increase on scattering light (Vargas et al., 2008).

In addition, there was a significant influence of storage time on a^* values, which increased (Table 1) in CFM. This increase in a^* values is typical of non-enzymatic browning (Maillard) reactions (Bassey et al., 2013). Consistently, correlation between galactose and a^* values ($R = 0.905$; $P = 0.034$) was found only for CFM (data no shown).

On the other hand, for GFM there was a significant decrease on a^* values with storage. It is well established that the color of fermented milk also depends on pH (García-Perez et al., 2005). Consistently, correlation between pH and a^* values ($R = 0.965$; $P = 0.008$) was found for GFM (data no shown). In addition, b^* value was lower in GFM than CFM ($P < 0.05$). It is well established that the absence of β-carotene in GFM makes the product less yellow, which leads to lower b^* values (Vargas et al., 2008).

3.7. Apparent viscosity and instrumental texture

Apparent viscosity, firmness and consistency values are shown in Table 1. For both products, there was a linear reduction on viscosity with storage. However, only in CFM, the reduction on viscosity was accompanied by a significant linear decrease in firmness and consistency during storage (Fig. 1S; Tables S1 and S2). These results are in agreement with Kamble and Kokate (2015), who reported a decrease on apparent viscosity, firmness, and consistency for cow yogurts during cold storage. In contrast, firmness remaining essentially constant in GFM during storage, as observed herein, was previously reported (Costa, Frasao, et al., 2015).

L. acidophilus has proteolytic activity higher than *S. thermophilus*: therefore, the higher the proteolytic activity of *L. acidophilus* in milk, the greater the breakdown of the protein network, resulting in significant reductions on texture (Gandhi & Shah, 2014). On the other hand, *S. thermophilus* has lower proteolytic activity and it can also produce exopolysaccharides, which can contribute to a firmer and more consistent fermented milk (Folkenberg et al., 2006).

For GFM, there was a negative correlation between firmness and total BAs content; the lower the firmness, the higher the BAs concentration (Fig. 2; Table S3). Indeed, proteolysis can affect firmness as there is breakdown of the protein network. Proteolysis can also cause a release of free amino acids, which are substrates for BAs production (Smit et al., 2008). This suggests that the breakdown of the protein network during storage can be a factor relevant for BAs accumulation in GFM, but not for CFM.

Finally, lower values of viscosity and texture parameters in GFM compared to CFM can be attributed to differences in the gel mechanical properties and casein aggregation behavior between these matrices (Vargas et al., 2008).

3.8. Rheological analysis of fermented milks

The correlation coefficient for the fitted model (Herschel-Bulkley) was above 0.968 in all cases (data not showed). Values of yield stress (τ_0), consistency coefficient (K) and flow behavior index (n) of the fermented milks during cold storage are reported in Table 1.

The τ_0 (yield stress) is the minimum stress value to detect a deformation of the material (Behnia et al., 2013). For CFM, it reduced until the 21st day ($P < 0.05$). This result shows that CFM lost resistance to shear rate during storage, thus presenting a weaker structure with time. This result is in agreement with the reduction of texture parameters values observed herein for this matrix.

The flow behavior index (n) indicates the degree of deviation from Newtonian flow ($n = 1$). If $n > 1$, the fluid presenting shear-thickening, and if $0 > n < 1$ the fluid exhibiting shear-thinning; the values of n for the samples were below 1 ($n \leq 0.720$) for all cases, presenting a pseudoplastic behavior, which is typical of yogurt (Behnia et al., 2013). For CFM, the values of n decreased until the 21st day (Table 1). In this matrix, counts of *L. acidophilus* showed negative correlation with n (R = -0.94; $P = 0.02$) (data not shown); due to the high proteolytic activity of *L. acidophilus* (Gandhi & Shah, 2014), there can be increased total solids during yogurt storage, what contributes to the higher susceptibility to shear thinning decrease (Elhamid & Elbayoumi, 2017).

However, for GFM, there was a linear elevation of n with storage time (Fig. 1S and Table S2). In addition, the counts of *S. thermophilus* exhibited a positive correlation with n ($R = 0.95$; $P = 0.01$) (data not shown), probably due to the lower proteolytic activity compared to *L. acidophilus* (Gandhi & Shah, 2014), and to the ability to produce exopolysaccharides, which show thickening properties (Folkenberg et al., 2006). Additionally, for this matrix, such thickening properties attributed to activity of *Streptococcus thermophilus* were correlated with BAs (Fig. 2 and Table S3). Finally, values of K (consistency coefficient), which gives an idea of the viscosity of the fluid, changed significantly only in GFM, decreasing until the 14th day (Table 1). Its reduction can be attributed to the same reasons previously discussed which lead to apparent viscosity reduction.

Differences in flow properties between the products (Table 1) can be attributed to the different nature of caprine and bovine casein structures and in the aggregation behavior of these proteins, whey retention capacity, and the gel mechanical properties (Vargas et al., 2008).

3.9. Effect of interaction of physicochemical and microbial variables on BAs accumulation in cow fermented milk

Results of PLS-VIP regression are shown in Figures 2S and 3S. The VIP method coupled to PLS regression was used to identify the parameters contributing to BAs accumulation. Thresholds for cutoff value in PLS-VIP were set as VIP >1.00 (Akarachantachote et al., 2014).

Putrescine, cadaverine and spermidine had significant correlation with the physicochemical and microbial parameters (Fig. 2S A, 2S B, and 2S C; Table S4). The production of theses BAs was enhanced in the presence of glucose, whereas lactose inhibited their production. Fermentable monosaccharides, such as glucose, stimulate both LAB growth and decarboxylase activity, possibly due to the provision of energy for enzyme activity (Lazárková et al., 2012). Regarding organic acids, formic acid appears to stimulate ornithine/agmatine and lysine decarboxylases, but citric acid seems to inhibit them. Citric acid as an inhibitor of some decarboxylases in LAB was previously described (Smit et al., 2008). *S. thermophilus* was responsible for the production of putrescine in CFM. Indeed, *S. thermophilus* has been described as a putrescine producer of dairy relevance (Benkerroum, 2016). On the other hand, *B. lactis* BB-12 produced cadaverine and spermidine. Consistently, most of the strains of *Bifidobacterium* genus isolated from dairy products are specially producers of cadaverine (Lorencová et al., 2012). Finally, acidity was a relevant factor in stimulating ornithine/agmatine decarboxylase, because acidic pH was associated with putrescine accumulation.

S. thermophilus and *L. acidophilus* LA-5 were significant producers of spermine and tyramine (Fig. 2S D and 2S E). Both are described as tyramine producers of dairy relevance (Benkerroum, 2016). In addition, *S. thermophilus* was reported to be able to produce spermine (Gezginc et al., 2013). Enhanced proteolysis with consequent reduction of texture and viscosity parameters were related to the accumulation of these amines. This can be attributed to *L. acidophilus*, which shows high proteolytic activity, releasing free amino acids which are substrates for BAs production (Gandhi & Shah, 2014). Additionally, acidity leads to the accumulation of these BAs. Indeed, it was reported that the optimum conditions for growth of *Lactobacillus* strain and tyramine production was anaerobic incubation at acidic pH (4.4) (Smit et al., 2008). Galactose correlated with the accumulation of spermine and tyramine. In addition, the increase on color intensity (b^* values) was also associated with the accumulation of these BAs. This suggests that Maillard reaction involving galactose is a relevant factor in the accumulation of these amines in CFM. Galactose reacts with milk proteins leading to the formation of browning compounds, which increase color intensity; these Maillard conjugates have an increased susceptibility to proteolysis (Joubran et al., 2017).

S. thermophilus and *L. acidophilus* LA-5 significantly contributed to total BAs accumulation (Fig. 2S F). The increase on galactose, glucose, formic acid, acidity and color intensity (a^* values) were associated with total BAs accumulation. In this context, treatments which delay the post acidification of fermented milk during storage, such as the use of cinnamon extract (Choi et al., 2016) can be useful to reduce total BAs during storage. Additionally, means to reduce Maillard reaction during storage of CFM, as the selection and use of galactose-positive *S. thermophilus* strains, can also be useful to reduce them, because it will prevent the formation of residual galactose.

3.10. Effect of interaction of physicochemical and microbial variables on BAs accumulation in goat fermented milk

Results of PLS-VIP regression in GFM are reported in Figure 3S. For the production of putrescine, spermidine and spermine in GFM, proteolysis was the most relevant physicochemical variable, followed by alkalinity (Figs. 3S A, 3S B, and 3S C; Table S4). Production of higher putrescine contents in neutral and alkaline compared to the acidic medium was previously reported, demonstrating that BAs formation is not only dependent on the optimum pH (acidic) for decarboxylase, but also on bacteria growth (Lazárková et al., 2012). In addition, monosaccharides, in general, affected negatively the production of these BAs. Lower levels of BAs were reported in fermented food with sugar (Bover-Cid et al., 2001). The production of BAs by LAB can be enhanced under poor growth conditions, as when the medium has a shortage of fermentable sugars. However, the presence of sugar can have a stimulating effect on decarboxylation (Smit et al., 2008) as seen here for CFM. Moreover, the reduction on viscosity and consistency coefficient (K) lead to the accumulation of spermine (Fig. 3S C). This can be attributed to the contribution of *L. acidophilus* to the production of spermine, because this bacterial specie has high proteolytic ability (Gandhi & Shah, 2014). On

the other hand, *B. lactis* BB-12 and *S. thermophilus* favored significantly the accumulation of putrescine, while only *B. lactis* BB-12 was relevant for the accumulation of spermidine.

S. thermophilus and *L. acidophilus* LA-5 were responsible for the production of tyramine (Fig. 3S D). Consistently, reductions on viscosity and consistency coefficient were associated with the production of tyramine. Furthermore, acidity also contributed to tyramine production, as reported for CFM. Acidic pH stimulates tyrosine decarboxylase activity (Smit et al., 2008).

S. thermophilus and *L. acidophilus* LA-5 lead to increased levels of total BAs. Similar to CFM, *B. lactis* BB-12 was not relevant for bioactive amines build up in milk. Accumulation of total BAs in GFM was mainly affected by reduction of both viscosity and consistency coefficient, as well as by the decrease of glucose. Low viscosities can also intensify proteolysis facilitating the access of proteolytic enzymes to the protein network. Therefore, the use of milk proteins cross-linking agents during fermentation, as transglutaminase, which increase gel strength, thus reducing proteolysis during storage (Lorenzen et al., 2002) can contribute to the reduction of total BAs in GFM.

4. Conclusions

S. thermophilus and *L. acidophilus* LA-5, but not *B. lactis* BB-12, were responsible for the accumulation of tyramine and increased levels of total bioactive amines both in cow and goat fermented milk. Post acidification and Maillard reaction involving galactose were the most relevant phenomena for an increase on total bioactive amines in fermented cow milk. Thus, this study shows that a decrease in residual milk galactose during storage and a delay in post acidification can play a key role in the mitigation of bioactive amines formation in fermented cow milk. Regarding fermented goat milk, a loss in viscosity and consistency coefficient was the most relevant phenomenon for the increase on total bioactive amines. Thus, by controlling viscosity parameters, the accumulation of bioactive amines in fermented goat milk during storage can be reduced.

Acknowledgements

The authors thank Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (process no. E-26/201.185/2014, E-26/010.001.911/2015 and E-26/203.049/2017, FAPERJ, Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (process no. 311422/2016–0, 439731/2016–0 and 150200/2017–0 CNPq, Brazil), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (process no. 125, CAPES/Embrapa 2014; CAPES, Brazil) for the financial support. C.P. Vieira was supported by CAPES.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2020.110700.

Author contributions

Marion P. da Costa, Carla P. Vieira and Carlos A. Conte-Junior: conceived and designed the experiments; Carla P. Vieira, Vitor L. de Melo Silva, Beatriz da S. Frasao and Karina F. Delgado: performed the experiments; Carla P. Vieira and Yves Eduardo C. O. Nunes: analyzed the data; Marion P. da Costa and Carlos A. Conte-Junior: contributed reagents/materials/analysis tools; Carla P. Vieira, Marion P. da Costa; Maria Beatriz A. Gloria, Thaisa A. Elias and Carlos A. Conte-Junior: wrote the paper.

Declarations of interest

The authors declare none competing interests.

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