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Microbiological and physicochemical characterization of probiotic fermented milk throughout the shelf life under different storage temperatures

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Abstract

Probiotics can be defined as viable or inviable microbial cell (vegetative/spore or intact/ruptured) that is potentially healthful to the host. Commonly linked to fermented milk, one of the most popular fermented beverage, due to the greater consumer acceptance of dairy products. However, technical aspects such as inappropriate storage conditions and transport facilities can influence the viability of the microorganisms in these products. Therefore, the aim of this study was to evaluate the microbiological and physicochemical quality of probiotic fermented milk sold in the city of Botucatu/SP during its shelf life, and to determine the effect of different storage temperatures (4 °C and 12 °C), comparing results with the standard limits. For this purpose, four brands of probiotic fermented milk (A, B, C, and D) were analyzed counting lactic acid bacteria and evaluating physicochemical parameters (pH, acidity, proteins, lipids, moisture, and ash). As a result, only brand D did not fit the parameters determined by the technical norms of the Brazilian legislation; the variation in storage temperature, did not show significant influence on the results of the tested parameters.

Keywords: functional foods; quality control; food safety; fermentation.

Practical Application: Microbiological and physicochemical parameters of probiotic fermented milk beverages.

1 Introduction

Human bodies possess several microorganisms that they encounter soon after birth. After the early years of life, the microbiota of the gastrointestinal tract is established and begins to transform into the microbiota that will be part of their adult life, becoming more diverse, and their maintenance depends on environmental factors, physiological and dietary components (Mendes, 2017). Thus, food significantly contributes to the health of human beings and the maintenance of their intestinal microbiota. As a result, there is an increased awareness toward the consumption of functional foods (Souza, 2015).

Functional foods have components that perform nutritional functions for humans and reduce the risk of diseases; because they act mainly in the modulation and activation of cellular components. Examples of functional foods include those that contain fatty acids, dietary fiber, probiotics, and phenolic compounds, including soy, fruits with peel, garlic, and yacon root (Carrara et al., 2009).

This study focused on probiotics. According to the definition of the Food and Agriculture Organization of the United Nations (2002), which was revised and maintained by the World Gastroenterology Organisation (2017), probiotics are living microorganisms that promote health benefits to the host when ingested in adequate amounts.

Although, the definition of the term probiotic has been changed and broadened over the years, according to Zendeboodi et al. (2020), probiotic can be defined as "viable or inviable microbial cell (veg91etative/spore or intact/ruptured) that is potentially healthful to the host".

These microorganisms mainly include lactic acid bacteria, which are gram-positive, catalase-negative, non-sporulating, mostly microaerophilic group, especially the genus *Lactobacillus* (Oliveira, 2007). Other microorganisms, such as the genus *Bifidobacterium,* and some species of yeast have probiotic characteristics (Mesquita et al., 2017). To be classified as probiotics, microorganisms need to possess the ability to survive the barriers of the gastrointestinal tract, such as a high pH, colonize the colon at least temporarily, be sensitive to antimicrobial agents and not pathogenic for humans (Brunser, 2017).

In recent decades, awareness about the effect of diet on health and tendency for consuming healthful food products directed manufactures to added probiotics to different types of foods and beverages (Ballus et al., 2010). Studies have shown several benefits of probiotics-containing food, including greater balance of the intestinal microbiota, modulation of constipation, anticarcinogenic effects (Nero et al., 2017), enhancing nutritional value of food products, controlling and reducing the serum cholesterol, improving the immune system, reducing lactose intolerance symptoms (Zendeboodi et al., 2020), and recently the effect of *Lactobacillus* on the gut–bone axis has been shown in researches, such as the one by Eor et al. (2020) and Lee et al. (2020).

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One of the most accepted sources by the consumer is probiotic dairy products (Marques, 2012); because milk is naturally rich in essential nutrients for humans and is an appropriate model for industrial manipulation (Ballus et al., 2010), the global market for functional dairy products is a very dynamic segment and was expected to reach a market value of 13.9 billion dollars by 2021 (Costa et al., 2020).

Commonly linked to fermented milk, one of the most popular fermented products due to their particular sensory properties, health benefits and extended shelf life (Khorshidian et al., 2020).

Despite its widespread use, the food industry faces certain challenges in maintaining the viability of probiotic microorganisms (Rokka & Rantamäki, 2010). Cell viability is limited by the probiotic's ability to sustain the bile and gastric juices of the gastrointestinal tract; the bacteria's viability is also reduced during the manufacture, distribution and storage of food (Nazir et al., 2018).

The viability of probiotic microorganisms is a very important theme in the scientific community, than is possible to find studies related to the production of fermented milk and analysis of cell viability, such as the research by Pena et al. (2020) and Ozcan et al. (2020).

Brazil has a legislation that sets forth criteria for the appropriate production of probiotic fermented milk. According to the *Ministério da Agricultura, Pecuária e Abastecinmento* (Ministry of Agriculture, Livestock, and Food Supply), a minimum concentration of microorganisms is required in products containing probiotics to ensure the amount needed to maintain their action during its shelf life (Brasil, 2007). The indicated concentration is 6 log of colony-forming units (CFU) per gram of milliliter in the daily recommendation of microorganisms in the product ready for consumption, as described by Normative Instruction No. 46 (Brasil, 2007).

Moreover, Normative Instruction No. 46 (Brasil, 2007) also specifies that the production of good quality fermented milk requires it to have the following physicochemical characteristics: an acidity of 60 to 200 Dornic degrees (°D), milk fat content of no more than 0.5 (g/100 g) in non-fat dairy products, and milk protein of at least 2.9 (g/100 g).

The ideal storage temperature for fermented milk falls within the range of 2-4 °C; the activity of lactic acid bacteria is no longer reduced, and their metabolic activity is no longer controlled at temperatures above 10 °C (Nero et al., 2017).

Thus, the aim of this study was to analyze the quality and viability of various brands of probiotic fermented milk beverages, sold in the city of Botucatu, São Paulo, and verify if microbiological and physicochemical parameters are within the limits of the Brazilian legislation, that ensure their health benefits to the consumers.

Furthermore, investigate the impact of storage temperatures on the quality of probiotic-containing foods; the temperature of storage in one of the factors that influence probiotic growth and viability (Costa et al., 2020).

Therefore, our study evaluated parameters based on an ideal storage temperature and an elevated, which can occur in storage at supermarkets or distribution situations. These findings are relevance to be used to predict the fate of probiotic beverages after fluctuations in transport or handling temperatures, elucidate the interference of storage temperature on the quality of probiotic fermented milk during shelf life. The variation can compromise microbiological and physicochemical aspects, impairing sensory quality and the ability to provide benefits to the health.

2 Materials and methods

2.1 Collection and storage of probiotic products

Codes were used to refer to the incognito brands of probiotic fermented milk beverages analyzed in this stud. According, we used four brands and two batches from each brand for study.

The fermented milk beverages were first collected from supermarkets in the city of Botucatu, São Paulo, using an isothermal box to maintain their temperature, which should not exceed 10 °C, based on the literature (Brasil, 2015).

Additionally, the integrity of the packaging and the description of the presence of live microorganisms (only products with probiotic strains of *Lactobacillus* were collected), jointly formed the criteria for the selection of probiotic fermented milk beverages (Costa et al., 2013).

Table 1 below provides a description of the products obtained and their shelf life.

Over the course of the study, the products were incubated at different temperatures to evaluate the viability of the cultures and their physicochemical stability. Therefore, each product was stored at two storage temperatures and analyzed at three different time points. Furthermore, the intermediate date of analysis corresponds exactly to the intermediate day between the first and last days, as described below.

Temperatures:

- Appropriate (4 °C) and elevated (12 °C) temperatures for refrigeration.

Time points for analysis:

- P0 (initial) – immediately after collection;

Table 1. Description of the products used in this study.

Brand	Expiration Date	Manufacturing Date
Brand A Batch 1	July 24, 2020	June 23, 2020
Brand A Batch 2	July 30, 2020	June 29, 2020
Brand B Batch 1	August 3, 2020	June 19, 2020
Brand B Batch 2	August 6, 2020	June 22, 2020
Brand C Batch 1	July 31, 2020	June 13, 2020
Brand C Batch 2	August 14, 2020	June 27, 2020
Brand D Batch 1	August 3, 2020	June 24, 2020
Brand D Batch 2	August 16, 2020	July 7, 2020

- P1 (intermediate) – intermediate date;
- P2 (final) – expiration date.

2.2 Microbiological analysis: lactic acid bacteria count

The samples were processed using the adapted methodology described by Oliveira et al. (2018). We performed serial dilutions with 0.1% peptone salt solution and plated with Man Rogosa and Sharpe culture medium, using the Pour Plate technique. In accordance with the literature, the plates were incubated at 35 °C for 48 h (Oliveira et al., 2018). After the incubation period, the typical colonies were counted, and the results were expressed as log CFU/mL (Guerra, 2016).

2.3 Physicochemical characterization of the probiotic fermented milk beverages

The following physicochemical parameters were examined in duplicates, using the conventional methods recommended by Instituto Adolfo Lutz (2008): pH by the potentiometric method, titratable acidity in Dornic degrees, fats by the Gerber method, proteins by the micro-Kjeldahl method, moisture content by drying oven and ash content by muffle furnace incineration.

2.4 Statistical analysis

Analysis of variance, along with Tukey's test, was used to analyze the assay values to compare the means at 5% significance with the Origin 9.1 Professional Single License – Academic Program. The graph was constructed using Microsoft Excel (2010).

3 Results and discussion

3.1 Results of microbiological analysis: lactic acid bacteria count

As for the lactic acid bacteria count, the statistical analysis showed no significant variation in the values ($p>0.05$) during the three storage periods at 4°C for all brands. However, when we analyzed the products stored at 12 °C, there was a significant increase ($p<0.05$) in CFU counts only for brand A products at the intermediate time point.

For the bacterial count, there was no significant difference at the ideal (4 °C) and elevated (12 °C) storage temperatures in the same period. Thus, the variation in temperature did not increase or decrease the CFU count of lactic acid bacteria for all brands (Figure 1).

In relation to the Brazilian legal requirements, the products of brands A, B, and C demonstrated a minimum of 6 log CFU/mL during their entire shelf life at both storage temperatures. Brands A and C even presented values above the lower limit prescribed by the legislation. Brand D products had a lower bacterial load in the P2 stage at 4 °C and in the P1 and P2 stage at 12 °C, thus not fulfilling the minimum number of bacteria per milliliter of fermented milk needed to be considered healthy for those who consume it. In addition to the health benefits, the lactic acid bacteria present in these beverages are also related to the development of organoleptic properties of fermented milk and

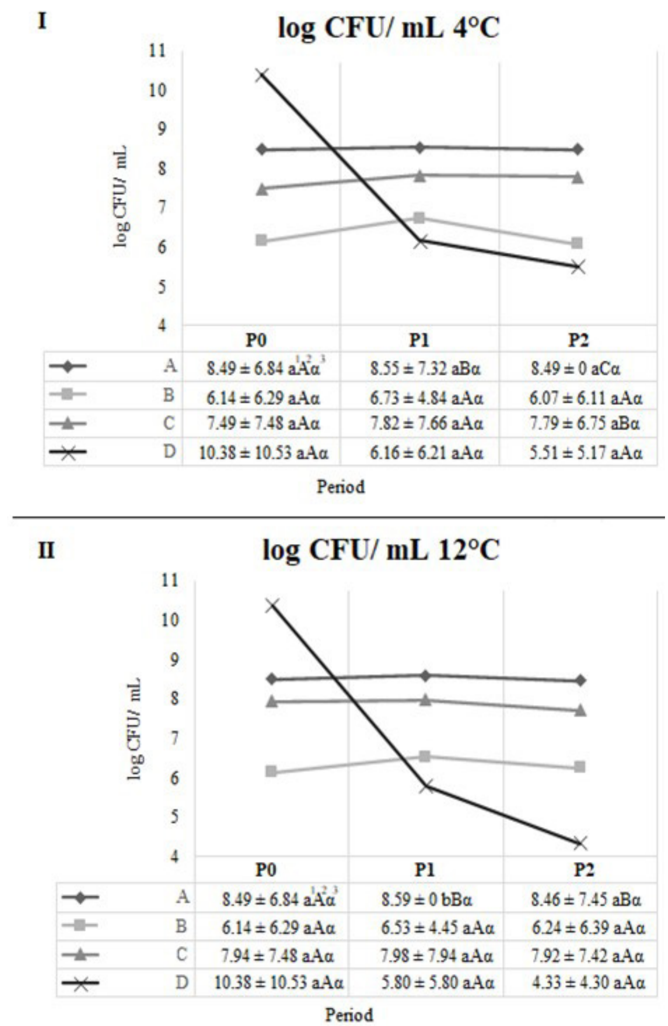

Figure 1. Mean ± standard deviation of the lactic acid bacteria count (log CFU) for different brands of fermented milk (A, B, C, and D). Statistical analysis ANOVA complemented with Tukey's test at 5% (I - log CFU/mL at 4 °C/II - log CFU/mL at 12 °C). [1]Lowercase letter: sets the brand, evaluation based on the periods (at the same temperature); [2]Uppercase letter: sets the period, comparative evaluation between the brands (at the same temperature); [3]Greek letter: sets the brand, comparative evaluation for each period at 4 °C and 12 °C.

acidification of the medium, thereby preventing the growth of other microorganisms that could alter the quality of the beverage (Tebaldi et al., 2007).

With respect to similar studies in the literature, Bressan et al. (2014) observed that 66.7% (n=10) of the samples were in compliance with the Brazilian legislation. Tebaldi et al. (2007), however, found that only one of the five brands tested presented values within the established counting standards.

3.2 Results of physicochemical characterization of the probiotic fermented milk beverages

In relation to the physicochemical analyses, the pH values decreased significantly as the study progressed for brands A, C, and D at both temperatures (4 °C and 12 °C). However, at both temperatures, brand B presented a decrease in pH values from the initial time point to the intermediate time point and an increase thereafter up to the final time point. The difference between the storage temperatures did not lead to significant variations in pH values, as shown in Table 2.

It was not possible to compare the pH values with those provided by the Brazilian legislation, since it does not specify a suitable pH range to follow in the *Regulamento Técnico de Identidade e Qualidade* (Technical Standards for Identity and Quality - RTIQ).

At the beginning of the shelf life of fermented milk beverages, Gallina et al. (2011) obtained an average pH value of 4.42, which fell to 4.29 and finally to 4.19. This gradual decline over the course of the product's shelf life, similar to that observed in our study, is possibly due to the metabolic action of the fermentation of the lactic acid bacteria present in the samples. Thus, over the course of the storage period, acid production by microorganisms, especially lactic acid, leads to a decrease in pH (Barboza & Belo, 2017).

The increase in pH observed at both the temperatures for brand B products, from the intermediate to final time point, may be linked to the lack of nutrients in the medium. Consequently, the microorganisms present in the sample consume other substances, such as casein protein, as a source of energy, resulting in the formation of NH_3, and a subsequent increase in pH (Gram et al., 2002).

However, there was no significant change in acidity (°D) for brands A and D at either temperature throughout the shelf life. For brands B and C, changes occurred during storage at 12 °C. Brand B exhibited a significant increase in acidity, while brand C presented a significant decrease at the same temperature at the final time point. The difference between the storage temperatures did not lead to significant variations in acidity values, as shown in Table 3.

With respect to acidity (in °D), Cunha et al. (2008) obtained an average acidity of 70.33 ± 0.58 °D, which is lesser than our findings, while Thamer & Penna (2006) reported an average acidity of 50.39 ± 0.11 °D, which is below the legally acceptable range of acidity.

According to the Brazilian legislation, fermented milk should have an acidity value between 60 °D and 200 °D, thus the products of all the brands demonstrated values in accordance with the legislation, during their entire shelf life at both storage temperatures.

As for total protein, brands A, B, and C did not exhibit significant variation throughout their shelf life at either temperature. At both temperatures, brand D presented a significant increase in total protein in the intermediate period of its shelf life, as shown in Table 4.

In previous studies, Gallina et al. (2011) found an average protein content of 4.2065 g/100 g for milk protein, and Cunha et al. (2008) reported an average value of 2.80 ± 0.23 g/100 g. In this study, we found a minimum protein content of 1.83 ± 0.2 g/100 g

Table 2. Mean ± standard deviation of pH for different brands of fermented milk (A, B, C, and D). Statistical analysis ANOVA complemented with Tukey's test at 5%.

Temperature	Period	Brands			
		A	B	C	D
4 °C	**P0**	3.63 ± 0.06 b[1]A[2]α[3]	3.80 ± 0.01 bBα	3.69 ± 0.01 bAα	3.76 ± 0.01cBα
4 °C	**P1**	3.37 ± 0.19 abAα	3.52 ± 0.08 aAα	3.34 ± 0.16 aAα	3.37 ± 0.05 aAα
4 °C	**P2**	3.31 ± 0.11 aAα	3.74 ± 0.08 bCα	3.46 ± 0.11 aABα	3.58 ± 0.05 bBα
12 °C	**P0**	3.63 ± 0.06 bAα	3.80 ± 0.01 bBα	3.69 ± 0.01 bAα	3.76 ± 0.01 cBα
12 °C	**P1**	3.36 ± 0.19 aAα	3.54 ± 0.10 aBα	3.34 ± 0.12 aAα	3.35 ± 0.02 aAα
12 °C	**P2**	3.29 ± 0.07 aAα	3.75 ± 0.06 bDα	3.46 ± 0.13 aBα	3.58 ± 0.03 bCα

[1]Lowercase letter: sets the brand, evaluation based on the periods (at the same temperature); [2]Uppercase letter: sets the period, comparative evaluation between the brands (at the same temperature); [3]Greek letter: sets the brand, comparative evaluation for each period at 4 °C and 12 °C.

Table 3. Mean ± standard deviation of acidity (°D) for different brands of fermented milk (A, B, C, and D). Statistical analysis ANOVA complemented with Tukey's test at 5%.

Temperature	Period	Brands			
		A	B	C	D
4 °C	**P0**	153.75 ± 11.09 a[1]B[2]α[3]	125 ± 7.07 aAα	135 ± 10 aAα	142.5 ±2.89 aABα
4 °C	**P1**	152.5 ± 2.89 aAα	131.25 ± 8.54 aAα	131.25 ± 16.52 aAα	137.5 ± 11.90 aAα
4 °C	**P2**	138.75 ± 10.31 aAα	128.75 ± 23.23 aAα	121.25 ± 10.31 aAα	142.5 ± 11.90 aAα
12 °C	**P0**	153.75 ± 11.09 aBα	125 ± 7.07 aAα	135 ± 10 aAα	142.5 ±2.89 aABα
12 °C	**P1**	153.75 ± 4.79 aBα	133.75 ± 7.5 abAα	131.25 ± 2.5 aAα	137.5 ± 5 aAα
12 °C	**P2**	145 ± 10 aBα	143.75 ± 4.79 bBα	116.25 ± 2.5 bAα	137.5 ± 14.43 aBα

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and a maximum content of 9.35 ± 0.22 g/100 g (brand D). The minimum value observed is lesser than the lowest acceptable limit for total protein provided by law, which is 2.9 g/100 g. Rossetto (2015) also obtained protein values lower than those stipulated by law throughout the shelf life during 16 days of refrigerated storage between 8 °C and 10 °C. The different values found among the products are mainly linked to the initial formulation of the raw material used to produce the fermented milk and to the addition (or not) of whey in its formulation (Thamer & Penna, 2006).

With regard to the parameters of moisture and volatiles, our results reveal that brands A, B, and D did not change significantly ($p>0.05$) during the product's shelf life at both temperatures, However, brand C products showed a significant increase ($p<0.05$) in moisture content at the final time point at both temperatures, as illustrated in Table 5.

As for the content of the fixed mineral residue (ash) present in the samples, brands A and D did not undergo significant changes throughout the shelf life at the temperatures tested, while brands B and C showed a significant decrease in the ash content at the intermediate time point at both temperatures, as shown in Table 6.

Similar to pH, the results for moisture and ash content cannot be compared to the standard value provided by the RTIQ. The variation in moisture content between the samples during the test period can be explained by the difference in the composition of the packaging of the fermented milk. According to Silva et al. (2017), some types of packaging generate greater moisture than others and can even influence food perishability.

The ash present in the dairy products is composed mainly of oxides of potassium, sodium, calcium, magnesium, phosphorus,

Table 4. Mean ± standard deviation of total protein (g/100 g) for different brands of fermented milk (A, B, C, and D). Statistical analysis ANOVA complemented with Tukey's test at 5%.

Temperature	Period	Brands			
		A	B	C	D
4 °C	**P0**	2.44 ± 1.39 a[1]A[2]α[3]	2.10 ± 0.85 aAα	2.61 ± 0.49 aAα	1.83 ± 0.2 aAα
4 °C	**P1**	2.51 ± 2.24 aAα	3.49 ± 3.19 aAα	5.99 ± 3.17 abAα	5.39 ± 0.68 bAα
4 °C	**P2**	3.80 ± 2.40 aAα	5.72 ± 1.37 aABα	8.36 ± 0.78 bBα	6.75 ± 1.28 bABα
12 °C	**P0**	2.44 ± 1.39 aAα	2.10 ± 0.85 aAα	2.61 ± 0.49 aAα	1.83 ± 0.2 aAα
12 °C	**P1**	3.25 ± 1.95 aAα	4.16 ± 2.38 aAα	6.22 ± 4.46 aAα	6.59 ± 1.75 bAα
12 °C	**P2**	4.30 ± 1.87 aAα	6.18 ± 3.04 aABα	7.28 ± 1.17 aABα	9.35 ± 0.22 cBβ

[1]Lowercase letter: sets the brand, evaluation based on the periods (at the same temperature); [2]Uppercase letter: sets the period, comparative evaluation between the brands (at the same temperature); [3]Greek letter: sets the brand, comparative evaluation for each period at 4 °C and 12 °C.

Table 5. Mean ± standard deviation of moisture and volatiles (g/100 g) for different brands of fermented milk (A, B, C, and D). Statistical analysis ANOVA complemented with Tukey's test at 5%.

Temperature	Period	Brands			
		A	B	C	D
4 °C	**P0**	82.21 ± 0.26 a[1]B[2]α[3]	85.97 ± 0.53 aCα	81.68 ± 0.12 aABα	81.27 ± 0.65 aAα
4 °C	**P1**	71.66 ± 19.43 aAα	85.98 ± 0.38 aAα	81.67 ± 0.25 aAα	61.89 ± 39.69 aAα
4 °C	**P2**	82.05 ± 1.01 aAα	83.70 ± 4.99 aAα	82.55 ± 0.21 bAα	81.53 ± 0.39 aAα
12 °C	**P0**	82.21 ± 0.26 aBα	85.97 ± 0.53 aCα	81.68 ± 0.12 aABα	81.27 ± 0.65 aAα
12 °C	**P1**	77.92 ± 8.45 aAα	86.47 ± 0.19 aAα	82.12 ± 0.37 abAα	80.89 ± 0.40 aAα
12 °C	**P2**	81.99 ± 0.64 aBα	86.47 ± 0.25 aCα	82.44 ± 0.43 bBα	81.06 ± 0.31 aAα

[1]Lowercase letter: sets the brand, evaluation based on the periods (at the same temperature); [2]Uppercase letter: sets the period, comparative evaluation between the brands (at the same temperature); [3]Greek letter: sets the brand, comparative evaluation for each period at 4 °C and 12 °C.

Table 6. Mean ± standard deviation of ash (g/100 g) for different brands of fermented milk (A, B, C, and D). Statistical analysis ANOVA complemented with Tukey's test at 5%.

Temperature	Period	Brands			
		A	B	C	D
4 °C	**P0**	1.6 ± 1.17 a[1]A[2]α[3]	0.72 ± 0.33 bAα	0.56 ± 0.05 bAα	0.61 ± 0.05 aAα
4 °C	**P1**	0.47 ± 0.09 aAα	0.52 ± 0.05 aAα	0.44 ± 0.06 aAα	0.55 ± 0.04 aAα
4 °C	**P2**	0.55 ± 0.08 aAα	0.53 ± 0.02 aAα	0.46 ± 0.03 abAα	0.53 ± 0.03 aAα
12 °C	**P0**	1.6 ± 1.17 aAα	0.72 ± 0.33 bAα	0.56 ± 0.05 bAα	0.61 ± 0.05 aAα
12 °C	**P1**	0.53 ± 0.04 aAα	0.52 ± 0.05 aAα	0.42 ± 0.06 aAα	0.86 ± 0.76 aAα
12 °C	**P2**	0.48 ± 0.23 aAα	0.65 ± 0.32 aAα	0.47 ± 0.06 abAα	0.53 ± 0.07 aAα

[1]Lowercase letter: sets the brand, evaluation based on the periods (at the same temperature); [2]Uppercase letter: sets the period, comparative evaluation between the brands (at the same temperature); [3]Greek letter: sets the brand, comparative evaluation for each period at 4 °C and 12 °C.

and chlorides (Venturini, 2018). The difference in ash content between the brands may be due to the possible addition of cheese whey in the manufacture of some commercialized fermented milk products (Venturini, 2018). Moreover, the different storage temperatures (4 °C and 12 °C) did not result in significant changes between the values of moisture and ash.

Finally, relative to lipid composition (g/100 g), the four different brands of fermented milk unanimously presented values under 0.01 g/100 g at both storage temperatures during the three time points of analysis. This implies that the products had an insignificant amount of fat, which corresponded to the amount conveyed on the nutrition facts label of the fermented milk beverages under analysis. Moreover, this composition complies with the specifications of the Brazilian legislation, which specifies that fermented nonfat milk should contain up to 0.05 g/100 g of milk fat content. Rossetto (2015) and Di Cicco (2012) also found the same lipid composition in their respective studies.

4 Conclusion

With regard to the use of different storage temperatures during the shelf life of the beverages, it was not possible to observe significant changes in the comparison of the results of the tested parameters for the products stored at the appropriate (4 °C) and elevated temperatures (12 °C). Although no changes occurred, exposure to elevated temperatures is not allowed by health regulations because it can lead to the growth of bacteria involved in the deterioration of fermented milk.

When we compared the products' compliance with the standard requirements, only brand D demonstrated a greater number of parameters outside the standard specifications (lower values of protein g/100g and CFU/mL). Therefore, brand D may not confer the expected health benefits to those who consume it, since it does not ensure thar viable microorganisms reach the human gastrointestinal tract.

Moreover, as a scope for future research, a higher temperature range could be used to verify if elevate storage temperatures during the shelf life of fermented milk beverages interferer in the quality and viability.

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