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Chapter

Comparision of Antioxidant Activity of Cow and Goat Milk During Fermentation with *Lactobacillus acidophilus* LA-5

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Abstract

This research aimed to evaluate the effects on the antioxidative capacity of cow and goat milk during fermentation with *Lactobacillus acidophilus* LA-5. The antioxidative capacity of milk samples during 28 days of storage was measured using a spectrophotometric decolorization assay by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. Also a principal component analysis (PCA) was used, to study the development of antioxidative activity during storage, and the connection to proteolysis and peptide concentration. The results of this study suggest that milk type (cow or goat) was a significant parameter for the proteolytic and antioxidative activity of fermented milk. Additionally, high degree positive and negative correlations were observed between the variables analyzed (0.511–0.787).

Keywords: probiotics, bioactive peptides, proteolysis, fermented milk, DPPH radical assay

1. Introduction

Milk and milk products are a source of vitamins, minerals, lipids, and proteins of high biological value [1]. For decades, fermented dairy products are considered beneficial foods for the health of human beings; this is due to a large part of the microorganisms involved in the fermentation and to the products released during this process [2]. The lactic acid bacteria (LAB), such as *Lactobacillus acidophilus,* are important in fermentation processes, which is why they are widely used in the food industry, due to their ability to acidify the food and preserve it from spores as well as to intervene in the texture, taste, and smell of fermented products [3]. The proteolytic activity of LAB in the milk fermentation process produces bioactive peptides that provide additional benefits in consumer health [2], such as those with antioxidant activity [4]. The composition of milk determines its nutritional quality and its properties in the manufacture of food products; goat milk has high nutritional values only surpassing human breast milk. Among the proteins of cow's milk and goat's milk, there are many differences in their composition [5], which is why fermented beverages based on cow's and goat's milk, inoculated with the probiotic *Lactobacillus acidophilus*, will be made in the present study. Additionally, the

correlation between proteolytic activities, peptide concentrations, and antioxidant activity were determined.

2. Background

2.1 Milk and its components

Milk is composed of water, carbohydrates, lipids and proteins, as well as enzymes, vitamins, and mineral salts [6]. Fat is the component that varies the most in milk and is the main determinant of its physical and organoleptic properties [7]. Lactose is the major carbohydrate in milk; it is formed by glucose and galactose, two simple sugars that the human body uses directly as an energy source [8]. It participates in the synthesis of cerebrosides and glycoproteins; also it acts to facilitate the absorption of calcium. Lactose and other sugars in milk also favor the growth of probiotics in the intestine [9]. In milk, two main classes of proteins are identified: the caseins, which represent 80% of the total proteins, comprised of several types (αs1, αs2, β, K, and γ), and serum proteins (α-lactalbumin, β lactoglobulin, and small amounts of serum albumin, immunoglobulins, and protease-peptone) [10]. These proteins are separated by the acidification of milk at pH 4.6, the isoelectric point of caseins, which produces its precipitation [6]. The biological value of casein in the diet is due to its content of essential amino acids [7]. The protein concentration, as well as the concentration and amino acid sequence of each of the milk proteins, depends on where the species comes from. Casein micelles are smaller in goat's milk (50 nm) than that in cow's milk (75 nm); these caseins present in goat's milk contain more glycine, less arginine, as well as sulfur-containing amino acids. Another difference between these types of milk is that cow's milk is slightly acidic and goat's milk is almost neutral (pH 6.7) because it has higher protein content and different combinations of phosphates [5]; also in cow's milk the largest fraction of protein is comprised of αS1-casein; however, in the case of goat's milk, the larger fractions include β-casein and αS2-casein [11]. Of the mineral elements, the milk contains sodium, potassium, magnesium, calcium, manganese, iron, cobalt, copper, fluorides, iodides, and phosphorus. Of which calcium, copper, iron, magnesium, manganese, phosphorus, and zinc are in the highest concentration in the membrane of the fat globules. In addition, the milk contains vitamins such as A, D, E, K, B1, B2, B6, B12, and C, carotenes, nicotinamide, biotin, and folic acid [7].

2.1.1 Proteolytic activity

Proteolysis is the process of degradation of proteins by the breakdown of their peptide bonds. From the abasement of milk proteins, peptides and free amino acids are formed, which can later present diverse bioactivities in the organism [12]. The proteolytic system of lactic acid bacteria is basically composed of proteinases that initially cleave the milk protein to peptides, and then these peptides are divided into peptides and smaller amino acids by intracellular peptidases. Subsequently, the amino acids are catabolized, producing a variety of low molecular weight compounds responsible for the formation of odor and taste compounds in fermented milks [13].

The initial step in the degradation process is carried out by the proteinase PrtP bound to the extracellular wall that decompose the proteins into peptides of 5–30 amino acids that are transported to the cells [2]. The action of the proteinases and peptidases provides the cells with peptides and free amino acids, which are

transported through the membrane by specific transport systems, where the peptides are hydrolyzed by cytoplasmic peptidases [13].

2.1.2 Bioactive peptides

The biologically active peptides derived from milk are initially in inactive form within the sequence of the precursor molecules but can be released in different ways: by hydrolysis with digestive enzymes such as pepsin, trypsin, chymotrypsin, etc., proteolysis by enzymes derived from proteolytic microorganisms, and by fermentation of milk with proteolytic starter cultures [14]. During the fermentation of milk, LAB are able to produce bioactive peptides by the fermentation process; this is because they contain an active proteolytic system that allows the degradation and release of amino acids from milk proteins [2]. This system consists of a series of different intracellular peptidases, including endopeptidases, aminopeptidases, dipeptidases, and tri-peptidases. The production of various bioactive peptides, including antimicrobial peptides, immunomodulators, and antioxidants, has been demonstrated through microbial proteolysis [14]. Bioactive peptides derived from milk are generally composed of 2–20 amino acids and become reactive after the release of the precursor protein. Several lactic acid bacteria such as *Lactococcus lactis* and *Lactobacillus helveticus* have been reported to release bioactive peptides through the fermentation process [15].

2.1.2.1 Bioactive peptides with antioxidant activity

Oxidizing compounds can cause damage to proteins, lipids, or DNA. These damages are related to the development of various diseases and to aging. Antioxidant peptides present in dietary proteins can limit oxidative damage, both in food and in the oxidation of body cells when they are ingested in the diet [16]. There are dairy peptides with antioxidant activity, and caseins and whey proteins are considered as precursors of these peptides [4]. Antioxidant peptides derived from milk are formed from 5 to 11 hydrophobic amino acids, including proline, histidine, tyrosine, and tryptophan, in sequence, that are widely distributed among the caseins, which can work by eliminating or preventing the formation of radicals as well as inhibiting enzymatic and nonenzymatic lipid peroxidation [15].

1,1-Diphenyl-2-picrylhydrazyl (DPPH) is known as a stable free radical due to the delocalization of an unpaired electron over the entire molecule. The delocalization of the electron intensifies the violet color of the radical, which it absorbs in methanol at 517 nm. When the DPPH solution reacts with an antioxidant substrate that can donate a hydrogen atom, the violet color fades. The antioxidant activity cannot be measured directly, but it can be determined by the effects of the antioxidant compound in a controlled oxidation process [17].

2.2 Lactic acid bacteria

Lactic acid bacteria are microorganisms that have various applications, including the fermentation of foods such as milk, meat, and vegetables. These bacteria, in addition to contributing to the biopreservation of foods, help to improve taste, smell, texture, and nutritional quality [18]. In addition, beneficial effects on health are attributed to them through the direct effects of live microorganisms known as probiotics as well as indirect effects during fermentation, in which these microorganisms participate in the generation of secondary metabolites such as peptides with biological activities [19].

There are several genera of LAB, which are classified as homofermentative and heterofermentative based on the final product of their fermentation. The homofermentative produce lactic acid as a product of the fermentation of glucose. On the other hand, the heterofermentative produce lactic acid in addition to other products such as acetates, ethanol, and carbon dioxide as a product of its fermentation [18]. The LAB are characterized by Gram-positive cocci or bacilli, catalase and oxidase negative, facultative anaerobic, non-sporulated, and non-motile. In addition, they are tolerant acid, being able to grow some at pH values as low as 3.2 and others at values up to 9.6; however, most grow between pH of 4 and 4.5 [19].

2.2.1 Lactobacillus acidophilus

There are many probiotic bacteria that are used for human consumption, although the most used are *Lactobacillus* spp. These lactic acid bacteria have been used for food preservation through fermentation for hundreds of years, in addition to providing flavor and texture, and they increase the nutritional value and are also found in the gastro-intestinal tract of humans [20]. In addition, they are a key factor in the processes of competitive exclusion and immunomodulation carried out by commensal organisms. *Lactobacillus acidophilus* is a Gram-positive, non-spore-forming bacterium, homofermentative anaerobic, and catalase negative, 2–10 μm in diameter, which has an optimal growth temperature of 37°C and is a typical intestinal bacterium in humans [21]. This microorganism is not part of the natural flora of milk and acts on it very slowly, which is why it is essential to avoid contamination during the manufacture of a product [8].

Lactobacillus acidophilus uses the glycolysis or Embden-Meyerhof-Parnas route (EMP) to ferment hexoses and produce lactic acid. Lactic acid does not contribute to the aroma because it is odorless, but it helps the sour taste of dairy products [13]. *Lactobacillus acidophilus* LA-1/LA-5 is one of the main species of microorganisms that can potentially be used as probiotic cultures in dairy products. Some examples in the market of fermented milk products that include *Lactobacillus acidophilus* are Bioghurt, Aktifit, Actimel, Bifilac, Kaiku, and Kefir [22].

2.3 Fermented beverages

Today, the main function of fermented milk is to prolong shelf life, improve flavor and digestibility, and manufacture a wide range of dairy products. In fermented milk products, probiotic bacteria can act in the treatment of some infectious, atopic, and tumoral diseases, among others [23]. Fermented milks can be classified based on different criteria, among them are its fat content, the concentration of milk, separation of the whey, the use of milk from different species, and the type of fermentation process. Based on the type of fermentation, there are the products with a lactic fermentation such as ymer, langfil, viili, yogurt, and acidified milk. Among the products in which lactic fermentation is combined with the production of alcohol are kefir and koumiss [8]. Today, the main function of fermented milk is to prolong shelf life, improve flavor and digestibility, and manufacture a wide range of dairy products. In fermented milk products, probiotic bacteria can act in the treatment of some infectious, atopic, and tumoral diseases, among others [23]. Fermented milks can be classified based on different criteria, among them are its fat content, the concentration of milk, separation of the whey, the use of milk from different species, and the type of fermentation process. Based on the type of fermentation, there are products with a lactic fermentation such as ymer, langfil, viili, yogurt, and acidified milk. Among the products in which lactic fermentation is combined with the production of alcohol are kefir and koumiss [8].

3. Materials and methods

3.1 Treatment of fermented beverages

Cow's milk (10 L) and goat's milk (10 L) were separately subjected to a heat treatment at 95°C for 20 minutes and then cooled down to 37°C. Four treatments were prepared by making three batches of each of them, all incubated at 37°C until a pH of 4.5. Two of the treatments were fermented with *Lactobacillus acidophilus* LA-5 (Chr. Hansen) at 1%, only one of them with goat's milk and the other with cow's milk; the other two treatments that were the controls were not added with probiotics, and their fermentation occurred due to thermodynamic microorganisms (persistent after pasteurization). The beverages were kept refrigerated at 8°C during their shelf life.

3.2 Physicochemical analysis

The physicochemical analysis was formed by fat (%), nonfat solids (%), density (kg/m^3), lactose (%), protein (%), total solids (%), added water (%), and freezing point (°C), and electrical conductivity (Ms/cm) was performed in triplicate of the raw material (cow's and goat's milk) in the Lactoscan Milk Analyzer (Lactoscan SA, Milkotronic Ltd., Bulgaria).

3.3 Determination of titratable acidity

It was determined based on the norm NOM-155-SCFI-2012 [24], taking 10 mL of sample, 20 mL of distilled water, and two drops of phenolphthalein, carrying out the titration with 0.1 N NaOH. The calculation of titratable acidity was carried out using the following equation:

$$\text{Acidity}\left(^{g}/_{L}\right) = \frac{(V)(N)(90)}{M} \quad (1)$$

where V = milliliters of 0.1 N NaOH solution, spent in the titration; N = normality of the NaOH solution; M = volume of the sample in mL; 90 = lactic acid equivalent.

3.4 Preparation of the filtrates

The samples were treated as described by Donkor [25], in which 5 mL of each was taken and mixed with 10 mL of 0.75% trichloroacetic acid (TCA), passing the mixture through filter paper (Whatman No. 1 of 150 mm), obtaining the filtered beverages (FB), which were frozen (−20°C) until analysis. The filtrates were carried out at 0, 7, 14, 21, and 28 days to determine the proteolytic activity, the total peptide concentration, and the antioxidant activity.

3.5 Proteolytic activity

The proteolysis of each of the FB was determined in triplicate based on the reaction of the free primary amines (NH3) with O-phthaldialdehyde (OPA) and b-mercaptoethanol, according to the Church method [26]. The OPA reagent was prepared as follows: 25 mL of 100 mM sodium tetraborate, 2.5 mL of 20% sodium dodecyl sulfate (SDS), 40 mg of OPA in 1 mL of methanol, 100 mL of b-mercaptoethanol, and capacity to 50 mL with tridestilated water. For the readings, 100 mL of each sample was taken and mixed with 2 mL of the OPA reagent by inversion of the

quartz cell, with 2 minutes of incubation at room temperature and inside the equipment to avoid exposure to light; the absorbance in a spectrophotometer (Genesys 10S UV-Visible, Thermo, USA) at a wavelength of 340 nm was read. The degree of proteolysis was determined as the difference between the proteolytic activity in the treatments (beverages fermented with probiotics) and the control samples (fermented beverages without probiotics).

3.6 Total peptide concentration

The concentration of the peptides contained in each of the FB was determined in triplicate using the Bradford method [27]. This is based on the reaction of the proteins with the bright blue dye of Comassie G-250, to form a colorful compound that absorbs strongly at 595 nm. For which, a calibration curve was made using eight bovine serum albumin (BSA) standards; at concentrations of 0.1–0.01 mg/mL, the standards were prepared using 0.15 M saline solution. The absorbance reading was performed in the spectrophotometer (Genesys 10S UV-Visible, Thermo, USA), and a calibration curve was made. A linear regression was made from the given curve, obtaining the following equation:

$$Y = 0.3123X - 0.1007, R^2 = 0.9977 \quad (2)$$

Based on the equation, the peptide concentration of each one of the filtrates during its shelf life could be determined from the given absorbance reading.

3.7 Antioxidant activity

This activity was evaluated by means of the spectrophotometric technique described by Pritchard [28], which determines antioxidant activities with the DPPH radical (1,1-diphenyl-2-picrylhydrazyl) in the presence of an antioxidant substance (in this case the content of FB), measuring the inactivation potential of said radical in aqueous medium. For this, we started from an initial concentration of free radical at 0.1 mM DPPH in ethanol, respectively, diluting 1500, 1000, and 750 μL plus 500 μL of the FB adjusting to a volume of 2 mL with water HPLC grade, which generated three concentrations of the radical (0.075, 0.05 and 0.0375 mM). Water HPLC grade dissolved in DPPH was used as control, according to the concentration used. Subsequently, the samples were subjected to centrifugation at 9470 g (Spectrafuge 16 M, Labnet, USA) for 2 minutes, and the absorbance at 517 nm was measured in the spectrophotometer (Genesys 10S UV-Visible, Thermo, USA). The percentages of inhibition were calculated by the following equation:

$$\%inibición = \frac{A^{control} - A^{extracto}}{A^{control}} x\ 100 \quad (3)$$

3.8 Statistical analysis

The analysis will be carried out using the SAS statistical package [29], in which an analysis of variance was carried out with the GLM procedure; considering a block design (three lots), treatments were used as qualifying variables and as variables of response (proteolysis, peptide concentration, and antioxidant activity). Considering the following model:

$$yijkl = \mu + \tau i + Dj + (\tau D)ij + \beta k + \theta(ij) + \varepsilon ijkl \quad (4)$$

where yijkl = response variable measured over time; μ = general average; ti = fixed effect of the i-th treatment; Dj = effect of the j-th day (0, 7, 14, 21, 28); (tD) ij = fixed effect of the interaction between the i-th treatment and the j-th day; βk = random effect of the k-th block; Θ (ij) = random effect of the j-th experimental unit, nested in the i-th treatment; eijkl = random error distributed in normal form with zero mean and variance; and eij = N (0, s2).

A principal component analysis was also performed using the PRINCOMP procedure, and it was determined as response variables (proteolysis, peptide concentration, and antioxidant activity) within which its correlations will be determined.

4. Results and discussion

4.1 Treatment of fermented beverages

The time elapsed after the pasteurization of the cow's milk until it reached a pH of 4.5 for the beverages inoculated with *Lactobacillus acidophilus* was 16 hours, while for the controls the necessary time was 27 hours. On the other hand, in goat's milk, the beverages inoculated with *Lactobacillus acidophilus* needed a time of 11 hours, whereas the controls 19 hours. In both types of milk for the controls, a longer time lapse is observed to reach the ideal pH; this because the fermentation of the milk in these treatments was carried out by the thermoduric microorganisms, which tolerate the thermal treatments applied to the milk. In the pasteurization process, it has also been observed that as the incubation temperature of the milk increases, there is evidence of greater microbial development of thermoduric species [30].

4.2 Physicochemical analysis

A physicochemical analysis was carried out in triplicate in cow and goat milk, as shown in **Table 1**. Between each parameter analyzed by the type of milk, a significant difference ($p \leq 0.05$) occurred, because milk differs in its composition depending on the species where it comes from. For cow's milk, the average percentage of total solids that it must contain is 12.7 [31], fat 4.2, protein 3.3, lactose 4.7, and nonfat solids 8.8%, while in goat's milk, its fat content should be 4.5, protein 2.9–4.60, lactose 4.1, nonfat solids 8.9%, and total solids from 11.70 to 15.21%; however, all these values depend on several factors such as the breed of the animal, its age, the period of lactation, and feeding, among others [32]. For cow milk analyzed, the percentage of protein and total solids that was obtained is within the reported parameters, although a smaller amount was registered in the parameter corresponding to fat and a slight increase in the percentage of lactose and nonfat solids. On the other hand, in goat's milk the percentages of total solids and protein are within the established ranges; there was a slight increase in both fat and lactose and a lower percentage in nonfat solids. However the percentage of protein in goat's milk is within the parameters reported for a good quality milk compared to the percentage obtained in cow's milk that present a significant difference ($p \leq 0.05$), surpassing the milk of cow.

Regarding the physical properties, at 20°C the density of the milk is approximately 1030 kg/m^3, but this depends on its composition [8]. Cow's milk showed an optimum density, while a lower density was registered in goat's milk (1027.5 kg/m^3). Based on the freezing point, this is relatively constant and is between −0.510 and −0.560°C due to the natural fluctuations of the composition of the milk [32], the freezing point recorded in the sample of cow's milk was −0.580°C, so it may be that an balance in

Parameter	Parameter reading	
	Cow milk	Goat milk
Fat	3.66% ± 0.01[b]	5.57% ± 0.02[a]
NFS	9.13% ± 0.01[a]	8.52% ± 0.02[b]
Lactose	5.01% ± 0.00[a]	4.68% ± 0.01[b]
Protein	3.33% ± 0.00[a]	3.09% ± 0.00[b]
Total solids	12.79% ± 0.01[b]	14.09% ± 0.01[a]
Added water	0% ± 0[a]	0% ± 0[a]
Density	1031.39 kg/m^3 ± 0.04[a]	1027.5 kg/m^3 ± 0.09[b]
Freezing point	−0.58°C ± 0.00[b]	−0.55°C ± 0.00[a]
Electric conductivity	4.64 mS/cm ± 0.01[b]	5.49 mS/cm ± 0.02[a]

NFS: non-fatty solids.
[a, b]Different literals indicate significant differences ($p \leq 0.05$) between parameters by type of milk.

Table 1.
Physicochemical parameters of cow's and goat's milk (raw material).

the salt-lactose ratio has occurred in the cow's milk. In the goat's milk there was a freezing point of −0.550°C, being within the acceptable range. On the other hand, the electrical conductivity of milk is given by the presence of ions such as chlorides, phosphates, calcium, as well as sodium, and its value is between 4.0 and 6.0 mS/cm for a good quality milk. Mastitis is part of the risk conditions in the process of milk production, but through electrical conductivity it is possible to identify the beginning of this disease, because mastitis causes an increase in the concentration of sodium and chloride in the milk, increasing the conductivity values [33]. The conductivity of cow and goat milk was 4.64 and 5.49 mS/cm, respectively, so the animals from which the milk came were free from mastitis.

4.3 Determination of titratable acidity

After the incubation of the beverages, the titratable acidity of each of the treatments was measured, which is shown in **Table 2**. The predominant acid in the fermented beverages is lactic acid, although bacterial fermentation can determine the production of other acids other than lactic acid, such as acetic acid [8]. A significant difference ($p \leq 0.05$) was observed between the two treatments, where the beverages inoculated with *Lactobacillus acidophilus* showed higher values of titratable acidity; this may be due to the fact that the probiotic favored the production of lactic acid. Regarding the type of milk, there was no significant difference ($p \leq 0.05$).

Mexican standard NOM-181-SCFI-2010 [34] for yogurt indicates a minimum acidity of 0.5% lactic acid, equivalent to 5 g/L of lactic acid, while CODEX STAN [35] establishes a minimum acidity of 0.6% lactic acid; therefore, both controls and treatments inoculated with *Lactobacillus acidophilus* in the two types of beverages presented higher values than those established as minimum required acidity.

4.4 Proteolytic activity

The production of fermented beverages is a process that involves many physical and chemical changes during its production and shelf life. One of these changes is proteolysis, which consists in the progressive hydrolysis of milk caseins to

Fermented beverage type	Lot number	Titratable acid (g/L)	
		LA-5	Control
Cow milk	1	9.67 ± 0.31[a]	7.15 ± 1.97[b]
	2	6.34 ± 0.57[a]	7.42 ± 0.31[b]
	3	7.56 ± 0.38[a]	5.31 ± 0.12[b]
Goat milk	1	8.05 ± 0.06[a]	5.94 ± 0.38[b]
	2	8.01 ± 0.12[a]	6.70 ± 0.31[b]
	3	7.38 ± 0.12[a]	6.79 ± 0.19[b]

LA-5: fermented beverage with Lactobacillus acidophilus.
[a, b]Different literals indicate significant differences ($p \leq 0.05$) between treatments.

Table 2.
Titratable acid values (g/L lactic acid) for each treatment of the fermented beverages.

smaller polypeptides, peptides, and amino acids by intracellular peptidases [25]. In **Figure 1**, the percentages of proteolytic activity of each type of fermented beverage are shown, which were compared with their respective control, taking it as 0%, to observe the percentage of proteolytic activity obtained in each type of beverage by the effect of the addition of the probiotic *Lactobacillus acidophilus.* The proteolytic activity of fermented beverages based on cow's milk ranged from 17.0 to 49.9% during their shelf life, while beverages based on goat milk ranged from 15.8 to 58.8%. For the two types of fermented beverages, a significant difference ($p \leq 0.05$) occurred during their shelf life, showing a tendency to increase the percentage of proteolysis over time.

Based on the type of beverage, there was also a significant difference ($p \leq 0.05$), where from day 0 to 7 the cow milk-based beverages had the highest percentage of proteolysis, while from 14 to 28 beverages, fermented milk-based goats presented the highest percentages; this may be due to the fact that the casein concentration is higher in goat's milk [10], which generates a greater proteolytic activity in the beverages.

Considering the absorbance at 340 (**Figure 2**), the proteolytic activity was estimated by determining the free amino groups using the OPA method. There was a significant difference ($p \leq 0.05$) between the days of monitoring, the treatments, and the type of fermented beverage. The absorbance of the controls was lower compared to the absorbance of the beverages inoculated with *Lactobacillus acidophilus* throughout their shelf life; these values are influenced by the effect of the probiotic in the milk. However, species and strains of lactic acid bacteria differ in their ability to hydrolyze proteins, due in part to the organization of proteolytic enzymes [8]. It is observed that cow's milk-based beverages have greater absorbance throughout their shelf life. However, in **Figure 1**, these beverages only show higher proteolytic activity on days 0 and 7; this is because the beverages are based on goat's milk; although they have less absorbency, from day 14 they have greater absorbance than their control, unlike cow milk-based beverages, which is why their percentage of proteolytic activity is higher. On the other hand, for beverages based on cow's milk, their absorbance is in a range of 0.96–1.49 during their shelf life, values higher than those reported by Donkor [25], who estimated values of 0.80–1.03 during the same days of monitoring; these differences may be due to the fact that in their research they also used the probiotics *Lactobacillus delbrueckii* and *Streptococcus thermophilus.* The ability of LAB to grow at high cell densities in milk depends on a proteolytic system that can release essential amino acids from

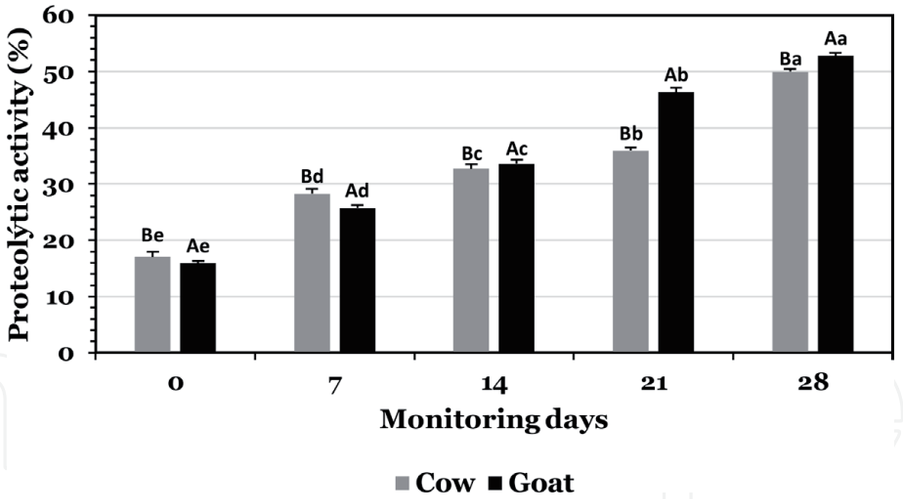


Figure 1.
Percentage of proteolytic activity of each type of beverage fermented during its shelf life compared to its respective control. $^{A, B}$*Different literals in uppercase indicate significant difference ($p \leq 0.05$) per type of fermented drink.* $^{a, b, c, d, e}$*Different literals in lowercase indicate significant difference ($p \leq 0.05$) due to the effect of monitoring day.*

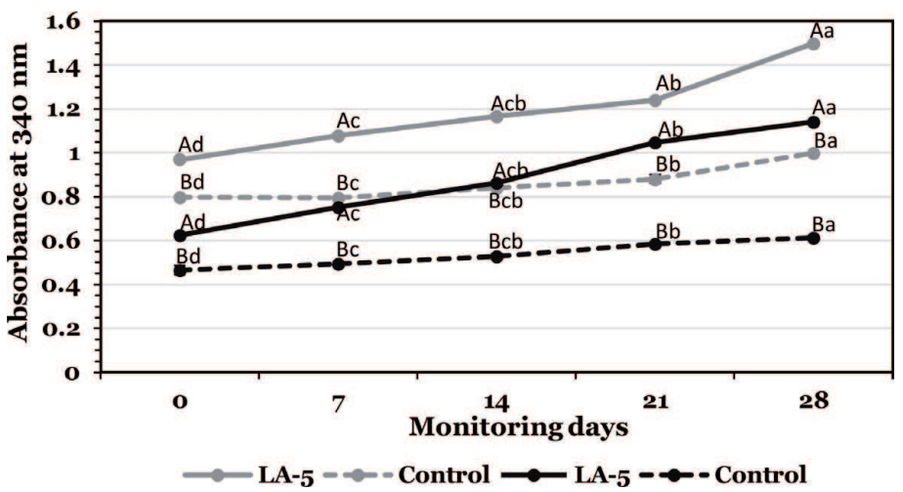


Figure 2.
Proteolytic activity of fermented beverages during their shelf life. Gray color, drinks based on cow's milk; black color, drinks based on goat's milk. LA-5: fermented beverages with Lactobacillus acidophilus. With significant difference ($p \leq 0.05$) between milk types. $^{A, B}$*Different literals in uppercase indicate significant difference ($p \leq 0.05$) between treatments by the type of milk.* $^{a, b, c, d}$*Different literals in lowercase indicate significant difference ($p \leq 0.05$) due to the effect of monitoring day.*

casein-derived peptides; ultimately these amino acids are catabolized producing many low molecular weight compounds such as aldehydes, alcohols, carboxylic acids, esters, and sulfur compounds [13]. That is why as they lived their shelf life, a more intense aroma in the drinks was perceived, due to the compounds that were forming.

4.5 Total peptide concentration

To determine the concentration of the peptides contained in each of the filtrates, a standard calibration curve was first performed (**Figure 3**), which is used to determine the protein concentration in unknown samples. The Bradford method [27] is based on the specific binding of the Coomassie G-250 bright blue dye (GBB) to the Arg, Trp, Tyr, His, and Phe residues of the proteins, producing a maximum absorbance at 595 nm, whereas the free dye has an absorbance at 470 nm.

Regarding the total peptide concentration (**Figure 4**), the highest value recorded was 0.105 mg/mL, which corresponds to the zero day control of goat milk-based beverages, and the lowest value was of 0.018 mg/mL corresponding to the LA-5 of day 28 of the drinks based on cow's milk. Based on the monitoring day, there was a significant difference ($p \leq 0.05$), observing that as time went by the peptide concentration was decreased, both in the controls and in the beverages fermented with *Lactobacillus acidophilus.* Considering the type of fermented beverage, beverages based on goat's milk always had the highest peptide concentration; this is due to the fact that goat's milk contains a higher concentration of caseins [10]. Added to this, in the controls there was a higher peptide concentration; this was because beverages fermented with *Lactobacillus acidophilus* had more microorganisms than degraded milk proteins, since LAB proteinases are able to hydrolyze more than 40% of the peptide bonds of the caseins, which generates a large amount of peptides, which can be degraded by peptidases to generate various volatile compounds [2]. This could be observed in the decrease of the peptide concentration along the length of the fermentation time during the shelf life.

4.6 Antioxidant activity

The two types of fermented beverages were analyzed for the presence of antioxidant activity by determining whether the generated peptides inhibit 1,1-diphenyl-2-picrylhydrazyl (DPPH), a free radical. The antioxidant activity of the peptides is due to the unique physicochemical properties conferred by their amino acid sequences. The fermented beverages presented antioxidant activity as shown in **Table 3**.

The highest percentage of inhibition occurred in the concentration of 0.05 mM DPPH on day 0 for the drink based on cow's milk fermented with *Lactobacillus acidophilus* with a value of 73.30%, while the lowest percentage of inhibition was obtained by the control of the drink based on cow's milk on day 28 of monitoring at the same concentration of DPPH, with a value of 23.71%. There was a significant difference ($p \leq 0.05$) between the concentrations of the DPPH radical used, where the treatments containing a concentration of 0.075 mM DPPH obtained on average the highest percentages of radical inhibition. Regarding shelf life, there was also significant difference ($p \leq 0.05$), where on day 7 the highest percentages of inhibition were presented, followed by day 0; however, from day 14 the percentages of inhibition were decreasing considerably for all the concentrations of the radical; this is related to the peptide concentration that was obtained, where at a lower concentration of peptides in the product, the percentage of inhibition is also lower.

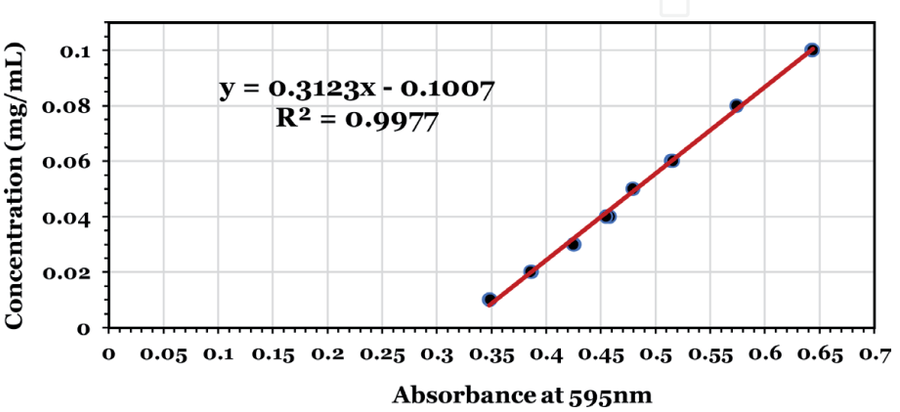


Figure 3.
Calibration curve for the determination of protein concentration (serum bovine albumin).

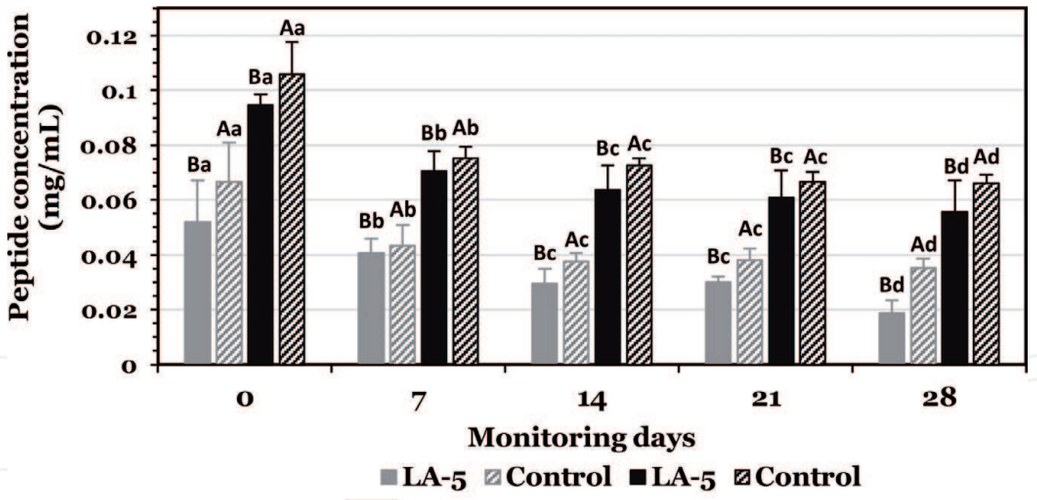


Figure 4.
Total peptide concentration of the fermented beverages during their shelf life. Gray color, drinks based on cow's milk; black color, drinks based on goat's milk. LA-5: fermented beverages with Lactobacillus acidophilus. With significant difference ($p \leq 0.05$) between mil types. $^{A, B}$Different literals in uppercase indicate a significant difference ($p \leq 0.05$) between treatments. $^{a, b, c, d}$Different literals in lowercase indicate significant difference ($p \leq 0.05$) due to the effect of monitoring day.

Day	Treatment	DPPH0.075mM		DPPH 0.05mM		DPPH 0.037mM	
		Fermented beverage		Fermented beverage		Fermented beverage	
		Cow milk[a]	Goat milk[b]	Cow milk[a]	Goat milk[b]	Cow milk[a]	Goat milk[b]
0	LA-5[a]	69.57±1.07^{Aab}	43.62±1.20^{Aab}	73.30±0.75^{Bab}	38.14±2.01^{Bab}	50.58±1.45^{Bab}	50.58±1.45^{Bab}
	Control[b]	37.80±0.51^{Aab}	43.06±0.69^{Aab}	56.88±3.76^{Bab}	35.65±0.75^{Bab}	39.39±1.21^{Bab}	51.51±0.80^{Bab}
7	LA-5[a]	75.05±0.19^{Aa}	48.21±1.35^{Aa}	69.48±1.25^{Ba}	47.42±0.75^{Ba}	69.69±1.75^{Ba}	45.92±2.45^{Ba}
	Control[b]	73.71±0.69^{Aa}	39.03±1.27^{Aa}	51.24±3.58^{Ba}	43.44±2.35^{Ba}	33.79±3.15^{Ba}	49.41±2.45^{Ba}
14	LA-5[a]	73.26±1.35^{Abc}	40.60±1.00^{Abc}	59.70±0.86^{Bbc}	40.13±2.07^{Bbc}	40.32±0.80^{Bbc}	45.22±1.06^{Bbc}
	Control[b]	72.14±1.20^{Abc}	34.34±2.60^{Abc}	48.59±2.01^{Bbc}	33.00±2.24^{Bbc}	27.73±2.82^{Bbc}	39.16±1.85^{Bbc}
21	LA-5[a]	65.65±0.96^{Acd}	40.38±1.07^{Acd}	54.72±1.79^{Bcd}	37.97±2.24^{Bcd}	37.06±2.52^{Bcd}	39.86±3.04^{Bcd}
	Control[b]	50.22±0.38^{Acd}	34.67±0.84^{Acd}	29.51±3.19^{Bcd}	32.17±1.74^{Bcd}	26.57±3.20^{Bcd}	36.13±1.75^{Bcd}
28	LA-5[a]	55.59±1.17^{Ad}	40.26±0.58^{Ad}	37.97±2.01^{Bd}	34.82±0.99^{Bd}	32.86±1.85^{Bd}	35.89±2.13^{Bd}
	Control[b]	48.21±1.17^{Ad}	29.86±0.67^{Ad}	23.71±1.74^{Bd}	28.02±2.55^{Bd}	25.87±1.21^{Bd}	24.70±1.75^{Bd}

LA-5: drink fermented with Lactobacillus acidophilus.
$^{A, B}$Different literals in uppercase indicate a significant difference ($p \leq 0.05$) between concentrations of DPPH.
$^{a, b, c, d}$Different literals in lowercase indicate significant difference ($p \leq 0.05$) due to the effect of monitoring day, with a significant difference ($p \leq 0.05$) between the type of milk and treatment.

Table 3.
Percentage (%) of inhibition of DPPH radical in three different concentrations.

The levels of antioxidant activity determined in the present study are higher than those reported by Amirdivani and Salihin [36], who reported values of 28–34% inhibition of the radical during the shelf life of their drinks; these differences may be due to the concentration of DPPH used as well as the LAB used in the process of making the drink, since they used *Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, and *Bifidobacterium bifidum* as probiotics.

Likewise, Amirdivani and Salihin [36] also reported the highest percentages of antioxidant activity on day 7 of refrigeration, which can be attributed to metabolically active BAL even at low temperature. In this sense, the consumption of fermented beverages is highly recommended within 7 days after its preparation to

Variables	Proteolytic activity	Total peptide concentration	Antioxidant activity
Proteolytic activity	1		
Total peptide concentration	−0.787	1	
Antioxidant activity	0.511	−0.606	1

Table 4.
Pearson correlation coefficients (R) given by the principal component analysis determined for different variables obtained in fermented beverages.

benefit from the high content of biopeptides and high antioxidant activities useful for consumer health. Free amino acids are generally not effective as antioxidants in food, so extensive proteolysis of proteins results in a decrease in antioxidant activity [37], which is reflected in the decrease in the percentage of inhibition of the radical when proteolysis increases during shelf life.

4.7 Principal component analysis

The antioxidant activity is given for 0.075 μM DPPH. Level of significance of the correlations ($p < 0.01$).

For the variables analyzed (proteolytic activity, total peptide concentration, and antioxidant activity) of the fermented beverages during their shelf life, a correlation coefficient was performed as shown in **Table 4**. The Pearson correlation coefficient is an index that measures the degree of covariation between different linearly related variables, where the correlation between directly proportional variables is positive and inversely proportional negative [38].

In this analysis, it is observed that the correlation between equal variables is positive, because exactly as one variable increases, the other increases [39] because the same data is analyzed in the two axes. The proteolytic activity and the total peptide concentration showed a negative correlation with a value of −0.787, since there is a tendency between the increase in proteolysis and the decrease in the peptide concentration.

5. Conclusion

The analyzed physicochemical parameters of cow and goat milk showed values of a good quality product. The beverages fermented with *Lactobacillus acidophilus*, as well as the controls, presented an acidity higher than the minimum required for commercial yogurts; in addition, no significant variations were observed in the titratable acidity for the two types of milk. Regarding the proteolytic activity, this was significantly augmented during the shelf life of the beverages compared to the antioxidant activity and the peptide concentration, which were decreasing. For the proteolytic activity and the peptide concentration, goat's milk-based beverages had the highest values; however, in the antioxidant activity, cow's milk beverages had the highest percentages of radical inhibition. In the peptide concentration, the controls showed the highest concentration, confirming the effect of the addition of lactobacilli to transform the proteins into different compounds, for which a continuation study is suggested where the volatile compounds that are suggested are quantified they have formed.

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