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Novel milk–juice beverage with fermented sheep milk and strawberry (*Fragaria* × *ananassa*): Nutritional and functional characterization

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ABSTRACT

This study was aimed at developing a new functional fermented beverage manufactured with semi-skimmed sheep milk and strawberry pulp (*Fragaria* × *ananassa* Duch.) and commercial prebiotic ingredients. We also compared the performance of the yogurt starter cultures and a *Lactobacillus plantarum* strain (CECT_8328) with potential probiotic properties. We assessed the nutritional profile, bioactivity compounds, viability of lactic acid bacteria during storage, and survival of *L. plantarum* after in vitro simulated digestion during the storage period. The lactic acid bacteria were viable throughout the storage period, but only *L. plantarum* maintained good viability after simulated digestion. Nevertheless, neither inulin nor potato starch increased bacterial viability. The fermented semi-skimmed sheep milk strawberry beverages we developed are good sources of minerals and proteins.

Key words: milk–juice beverage, bioactive peptides, inulin, probiotic bacteria

INTRODUCTION

The popularity of dairy products fortified with prebiotics and probiotics continues to increase as consumers look for flavorful foods that fulfill their health needs (Allgeyer et al., 2010). In this environment, products from bovine milk play a leading role, but small ruminant milk has also proven to be a suitable raw material for fermented dairy products that are potentially capable of producing effects for human metabolism and health (Varga et al., 2014). Ready-to-drink products manufactured with fruit pulp and bioactive compounds are a new scientific and technological trend in the dairy sector in European and North American markets (Zulueta et al., 2013).

The small ruminant dairy sector consists of a large number of sheep and goats worldwide—approximately 2,200 million head—and 20.8% are intended for dairy production; dairy sheep and goat production systems are environmentally friendly and play a key role in developing rural communities (Pulina et al., 2018). As well, sheep milk has been reported as an adequate matrix for delivering prebiotic fibers and probiotic bacteria, and so it is promising for developing functional dairy products (Albenzio et al., 2016; Balthazar et al., 2018a). The processing of sheep milk generates products (e.g., fat and proteins) with an interesting nutritional profile and a good yield (higher total solids) compared with the milk of other domestic mammals, such as cows, goats, camels, donkeys, horses, and hinnies (Balthazar et al., 2017a).

To be consistent with and responsive to consumers' demands and expectations, milk-fruit beverages should be manufactured in a way that boosts their nutritional profile. Strawberry, the most consumed wild-harvested fruit in the world, is a good source of β-carotene, ascorbic acid and phenolic compounds (Ariza et al., 2016). Another important factor is that strawberry is the most common fruit added to flavored milk and dairy products because of its sensory acceptance (Li and Drake, 2015).

Moreover, in this expanding food sector, prebiotics and probiotics are key ingredients and successful examples of functional food classes that promote health and wellness. Prebiotics are "substrates that are particularly used by host microorganisms, which confer a health aid" (Gibson et al., 2017); probiotics are live microorganisms that, when administered in adequate amounts, grant a health benefit for the host (Hill et al., 2014). The probiotic effects are strain-specific and

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should be resistant to the digestive system and its acids; they should also be able to adhere to human enteric epithelial cells, conferring antimicrobial protection against harmful bacteria (Espitia et al., 2016). Among probiotics, *Lactobacillus plantarum* is an innate bacterium of the human gastrointestinal tract, with the potential to confer health benefits (Settachaimongkon et al., 2016).

Inulin is a prebiotic ingredient with recognized functional effects in humans. This fiber also has technological characteristics that make it ideal for fat replacement without changing sensory properties (Balthazar et al., 2017b). Resistant starch from potatoes is another interesting prebiotic fiber that has been studied for its technological applications (Zheng et al., 2016) and is easily found in the market.

Considering the health claims of the functional drink market and the opportunity to advance the technology of dairy foods based on sheep milk with functional characteristics, the present study was aimed at developing a new functional fermented sheep milk strawberry-based beverages and assessing the nutritional and functional properties of this novel product.

MATERIALS AND METHODS

Sheep Milk Strawberry Beverage Processing

We tested 5 different formulations of fermented semi-skimmed sheep milk strawberry beverages: a control beverage manufactured with yogurt culture (**SMB1**); a beverage manufactured with potential probiotic culture (**PPC**; **SMB2**); a beverage with PPC and inulin (**SMB3**); a beverage with PPC and potato starch (**SMB4**); and a beverage with PPC, inulin, and potato starch (**SMB5**).

Whole raw sheep milk (Gentile di Puglia sheep) containing 6.3% (vol/vol) fat (about 11.3% wt/vol nonfat solids) was acquired in Foggia (Puglia, Italy). The sheep milk was skimmed to 1.6% (vol/vol) fat by centrifugation at 1,792 × *g* for 10 min at 4°C (Centrifuge 5810 R; Eppendorf AG, Hamburg, Germany). The semi-skimmed sheep milk was heat-treated in a stainless steel container equipped with an internal propeller (Casaro; Philips, Eindhoven, Netherlands) at 75°C for 15 s, immediately cooled using an ice bath and stored in a refrigerator at 4°C until use. Fresh ripe strawberries (*Fragaria* × *ananassa* Duch.) were acquired at a local market, blanched (100°C for 30 s), and stored in a refrigerator at 4°C until use. Long-branched inulin powder for food industry use (average degree of polymerization 60; inulin 90%; ACEF, Fiorenzuola D'Arda, Italy), potato starch (Paneangeli, Foggia, Italy), and sucrose (Belbake, Berlin, Germany) were acquired from a local market. Yogurt mix culture (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) was acquired from BMB s.r.l. Manufacturing and Trade (Bologna, Italy), and *Lactobacillus plantarum* (deposited at the Spanish Type Culture Collection under the Budapest Treatment with the code CECT_8328) was obtained from the Laboratory of Microbiology Collection, Department of the Sciences of Agriculture, Food and Environment, University of Foggia.

Lyophilized yogurt mix culture or PPC was added to semi-skimmed sheep milk at 9 log cfu/mL and incubated at 42 ± 1°C for 5 h or 31 ± 1°C for 72 h. The fermentation process was stopped when a pH of 4.6 was reached, and the content was refrigerated at 4°C. The formulations of the beverages (Table 1) were based on previous work conducted by Balthazar et al. (2018b). The ingredients were gently mixed in a double-jacketed stainless steel container with an internal propeller at 50°C for 10 min. Finally, the 5 beverage samples were packaged in propylene containers (200 mL) and stored at 4 ± 1°C for 30 d. The experiment was performed twice, and all analyses were conducted in triplicate (n = 6) during refrigerated storage (0, 15, and 30 d).

Activation Conditions for Potential Probiotic Strains. The *Lactobacillus plantarum* CECT_8328 strain (**Lp B2**), was inoculated using de Man, Rogosa and Sharpe (**MRS**) broth (CM0359, CM0275; Oxoid, Basingstoke, UK) and incubated at 31°C for 48 h. Afterward, cells were obtained by centrifugation (1,792 × *g* for 10 min at 4°C) and then suspended in 0.9% (wt/vol) NaCl solution (Sigma-Aldrich, Steinheim, Germany). This procedure was repeated until the supernatant was completely clean. The pellets were then lyophilized. The weight of each pellet was approximately 2.2 g and contained 9.8 log cfu/mL, as performed by plate count.

Viability Assay of Probiotics After In Vitro Digestion Simulation. The beverages were subjected to simulated in vitro digestion to test the viability of the bacteria through simulated gastrointestinal digestion conditions (Minekus et al., 2014). We evaluated lactic acid bacteria viability in beverages and digested beverages during refrigerated storage (0, 15, and 30 d), in triplicate, according to Codex standard 243–2003 for fermented milk (Codex Alimentarius, 2010). After the gastrointestinal digestion assay, aliquots of each beverage sample were frozen (−80°C) for further nutritional and functional analyses.

Physicochemical Analyses

We measured pH values using a digital pH meter (WTW 315i/SET portable pH meter; Wissenschaftlich GmbH & Co., Berlin, Germany). Protein content (N × 6.38) was determined using the Kjeldahl method, and

total nitrogen, nonprotein nitrogen and water-soluble nitrogen concentrations were calculated according to Albenzio et al. (2013).

Protein Profile by SDS-PAGE

The protein profile was acquired by SDS-PAGE according to Balthazar et al. (2019). Briefly, aliquots (100 μL) of beverages were mixed with 900 μL of buffer solution (0.125 *M* Tris HCl, 4% SDS, 2% glycerol, 2% 2-mercaptoethanol, 0.03 m*M* bromophenol blue, pH 6.8) and heated at 100°C for 5 min. The protein profile was identified on SDS-PAGE gels, using 15% acrylamide for the resolving gel and 4% for the stacking gel. Electrophoresis was performed at a constant voltage (200 V) for 50 min. As a molecular ladder, we used a low-molecular-weight standard kit (Amersham LMW calibration kit; GE Healthcare UK Ltd., Little Chalfont, UK); 3 μL of standard and 5 μL of samples were loaded into the gels. We used analysis software (Quantity One, Bio-Rad Laboratories Inc., Philadelphia, PA) to evaluate the SDS-PAGE gels for molecular weight and the saturation of each band.

Free AA Profile

Free amino acids (FAA) were determined by HPLC according to Balthazar et al. (2019). The HPLC system (1260 Infinity series; Agilent Technologies, Waldbronn, Germany) was equipped with a micro-vacuum degasser, thermostat-controlled autosampler, column compartment with a detector (model G1321A), and array detector (model G1315A). We conducted the analyses using a Zorbax Eclipse AAA column (150 × 4.6 mm i.d., prepacked with 3.5-μm particles; Agilent, Santa Clara, CA); the column temperature was stabilized at 40°C. Fluorescence detection was monitored at 340 nm excitation and 450 nm emission. To determine the Cystine UV diode array, the detector was set at 338 nm. The amino acid peaks (Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val) were identified by comparing their retention times to their respective standards (Sigma-Aldrich, St. Louis, MO).

Ascorbic Acid Content

Ascorbic acid content was determined using the titration method with 2,6-dichlorophenol-indophenol (Cappato et al., 2018). For the extraction, a 5-mL sample was mixed in 25 mL of metaphosphoric acid solution and centrifuged at 13,715 × *g* for 10 min at room temperature. Then ascorbic acid was quantified using an aliquot of the supernatant (approximately 5 mL), which was diluted in 15 mL of extraction solution and titrated with the indophenol solution and the samples in the absence of light. The ascorbic acid content was expressed in milligrams per gram.

Bioactive Compounds

To quantify the bioactive compounds in the beverages, samples were initially extracted with acidified water (pH 4.6, 5 min under stirring) to precipitate casein and

Table 1. Semi-skimmed (1.6% vol/vol fat) fermented sheep milk strawberry beverage formulation, nutritional content, and pH at d 0 of refrigerated storage (4 ± 0.5°C)[1]

Item	SMB1	SMB2	SMB3	SMB4	SMB5
Ingredients					
Sheep milk (g/100 g)	56.50	55.45	53.95	53.95	53.95
Strawberry pulp (g/100 g)	38.50	37.45	35.95	35.95	35.95
Sucrose (g/100 g)	4.90	4.90	4.90	4.90	4.90
Inulin (g/100 g)	—	—	3.00	—	1.50
Potato starch (g/100 g)	—	—	—	3.00	1.50
Yogurt mix culture[2] (g/100 g)	0.1	—	—	—	—
Probiotic culture[3] (g/100 g)	—	2.20	2.20	2.20	2.20
Total	100.00	100.00	100.00	100.00	100.00
Nutritional content					
Casein (g/100 g)	$3.60^{a} \pm 0.1$	$3.27^{b} \pm 0.1$	$3.07^{c} \pm 0.1$	$3.02^{c} \pm 0.1$	$3.04^{c} \pm 0.1$
Whey (g/100 g)	$0.39^{a} \pm 0.1$	$0.30^{b} \pm 0.1$	$0.28^{b} \pm 0.1$	$0.28^{b} \pm 0.1$	$0.26^{b} \pm 0.1$
Fat (g/100 g)	$0.90^{a} \pm 0.1$	$0.89^{a} \pm 0.1$	$0.86^{a} \pm 0.1$	$0.86^{a} \pm 0.1$	$0.86^{a} \pm 0.1$
Vitamin C (mg/g)	$4.82^{a} \pm 0.1$	$4.81^{a} \pm 0.1$	$4.83^{a} \pm 0.1$	$4.84^{a} \pm 0.1$	$4.82^{a} \pm 0.1$
pH	$4.45^{a} \pm 0.1$	$3.99^{b} \pm 0.1$	$3.97^{b} \pm 0.1$	$4.01^{b} \pm 0.1$	$3.98^{b} \pm 0.1$

[a–c]Different superscript letters in the same row indicate a significant difference ($P < 0.05$).

[1]Values are expressed as mean ± SD. SMB1 = yogurt beverage; SMB2 = potential probiotic culture beverage; SMB3 = potential probiotic culture inulin beverage; SMB4 = potential probiotic culture potato starch beverage; SMB5 = potential probiotic culture inulin + potato starch beverage.

[2]*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*.

[3]*Lactobacillus plantarum* CECT_8328.

then centrifuged at 10,000 × *g* for 15 min (4°C). Then, the supernatants were separated by filtration using a 0.45-μm syringe filter (Mixed Cellulose Esters; EMD Millipore Corp., Burlington, MA) and stored at −20°C for bioactive compound analyses.

We determined angiotensin I converting enzyme inhibitor (**ACEI**) activity using a spectrophotometric assay (Balthazar et al., 2018a). We calculated ACEI activity using Equation [1], where A was the absorbance with ACEI and the ACEI component, B was the absorbance without the ACEI component, and C was the absorbance with no ACEI:

$$\text{ACEI } (\%) = \left(\frac{\text{B} - \text{A}}{\text{B} - \text{C}}\right) \times 100. \quad [1]$$

Antioxidant activity was analyzed by the 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) radical scavenging method (Balthazar et al., 2018a). We calculated DPPH radical scavenging activity (in % inhibition) using the following formula:

$$\text{DPPH } (\%) = \left[1 - \left(\frac{\text{sample absorbance at 517 nm}}{\text{control absorbance at 517 nm}}\right)\right] \times 100. \quad [2]$$

The α-amylase and α-glucosidase enzyme inhibition assay was performed according to Ayyash et al. (2018). We determined α-amylase activity by measuring absorbance (Abs) at 540 nm and α-glucosidase activity by measuring the release of *p*-nitrophenol at 400 nm. We used solutions without the water-soluble extract and substrate sample as a control and a blank, respectively. The inhibition percentage was calculated as follows:

$$\text{Inhibition } (\%) = \left(1 - \frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control}}\right) \times 100. \quad [3]$$

Mineral Content and Bioaccessibility

The mineral content analysis was performed according to Silva et al. (2018b), where the Ca, P, K, Mg, and Zn contents of the fermented semi-skimmed sheep milk strawberry beverage samples were analyzed by atomic absorption spectrometry using air-acetylene flame in an iCE 3000 Series Atomic Absorption Spectrometer (Thermo-Scientific, Hemel Hempstead, UK). The spectrometer was equipped with a deuterium lamp (SMI-LabhutLtd., Churcham, UK) for correction and cathode lamps (SMI-Labhut Ltd.) to quantify each element. We quantified the calcium concentration by mixing the samples to obtain La^{3+} concentrations of 0.5%. The phosphorous content was determined by the molybdenum method with hydroquinone and sulfate using spectrophotometer Helios β at λ = 460–480 nm (PN-ISO 13730).

The in vitro availability of minerals was assayed using an enzymatic hydrolysis system according to Silva et al. (2018b), which simulates the conditions of the human gastrointestinal tract. We calculated the availability of minerals in the beverage formulations using the following equation:

$$\text{Availability}(\%) = \frac{\text{mineral content after filtration} \times 100}{\text{total mineral content in sample}}. \quad [4]$$

Statistical Analysis

Data were analyzed using 2-way ANOVA, sample and storage time interaction, and a Tukey test at a 95% confidence level. We also used Pearson's correlation coefficient to explore possible associations between the responses. The FAA data were organized and autoscaled before principal component analysis according to Balthazar et al. (2019), where matrix data were composed of 5 rows (beverage samples) and 17 columns (FAA). The statistical analyses were conducted using the XLSTAT software (version 2018.4; Adinsoft, Paris, France).

RESULTS AND DISCUSSION

Lactic and Probiotic Bacteria Count

The viability of the lactic acid bacteria in the beverages is shown in Figure 1, which reports the microbial counts (Figure 1A) in different beverage formulations during refrigerated storage at d 0, 15, 30 and after simulated digestion (Figure 1B) at the same time points. In the yogurt control beverage (SMB1), we found a total of 8.60 log cfu/mL at d 0 for both *S. thermophilus* and *L. bulgaricus*. The rod-shaped bacteria dropped significantly 2 log cycles (6.70 log cfu/mL) at d 30, and the cocci-shaped bacteria decreased 0.7 log cycle (7.97 log cfu/mL). However, as expected, *S. thermophilus* did not survive the simulated digestion assay (Figure 1B). After in vitro gastrointestinal digestion at d 0 and 15, *L. bulgaricus* survived at 4.3 ± 0.1 log cfu/mL. The extended storage period may have been responsible for the decrease in *L. bulgaricus* counts. Conversely, *Lactobacillus plantarum* CECT_8328 (Lp B2) maintained a considerable count (over 9 log cfu/mL) during 30 d of storage in samples SMB2, SMB3, SMB4, and SMB5 (Figure 1A). Beverage SMB2 had the highest

Lp B2 count (9.40 log cfu/mL at d 30), implying that the addition of potato starch, inulin, or both did not positively affect Lp B2 survival (Figure 1A). The Lp B2 counts decreased almost 3 log cycles after in vitro simulated digestion in all probiotic beverages (SMB2 to SMB5), without significant differences during the storage period (Figure 1B). The counts ranged from 6.47 to 6.92 log cfu/mL, above the probiotic critical threshold of 6 log cfu/mL (Fazilah et al., 2018).

Yogurt bacteria showed satisfactory growth (d 0, 8.6 log cfu/mL for both bacteria), in accordance with the recommendations of Codex Alimentarius (2010), which stipulates a total bacteria count above 6 log cfu/mL. *S. thermophilus* did not survive the in vitro digestion assay, and the *L. bulgaricus* counts decreased during storage ($P < 0.05$). These bacteria can barely survive the gastrointestinal tract passage and have no ability to colonize the colon (Fazilah et al., 2018). The stomach is considered a biological and chemical barrier because of its low pH and the presence of proteolytic enzymes that promote an unfavorable environment to microorganisms. The pH difference in the intestine, combined with pancreatic fluids and bile salts, affects the chemical stability of cell membranes (proteins and phospholipids), which can cause cell disruption and homeostase malfunction (Uriot et al., 2016).

Many studies have shown that different strains of probiotic *Lactobacillus* spp. added to dairy products can survive digestion (Ranadheera et al., 2018). Indeed, Lp B2 is a riboflavin-overproducing strain that has been proposed for the formulation of in situ biofortified functional fermented foods (Russo et al., 2016.) Moreover, this strain has been well characterized for its probiotic potential using in vitro and in vivo models, suggesting that it could further increase riboflavin supply and may enhance immunomodulation capacity (Arena et al., 2016).

According to Lee et al. (2018), native potato starch has a high resistant starch content (86.03%) in its uncooked state, making this ingredient a potential prebiotic ingredient. However, prebiotic fiber, such as inulin and resistant starch in potato starch, showed no effect on Lp B2 viability throughout the simulated gastrointestinal passage.

Evaluation of pH

We assessed the effect of post-acidification during refrigerated storage (Figure 1C), and the pH values of beverages at d 0 are presented in Table 1. The yogurt mix culture of SMB1 showed a pH drop of almost 0.4 units during storage ($P < 0.05$), and beverages containing Lp B2 (SMB2 to SMB5) dropped 0.2 units ($P > 0.05$) at d 30, showing that the post-acidification effect occurred only in the beverage fermented with yogurt bacteria ($pH_{30d} = 4.06$). Sheep milk has a great potential for acidification and formation of yogurt with a low pH (Balthazar et al., 2017a); however, inulin can delay post-acidification in sheep milk yogurt, making it bet-

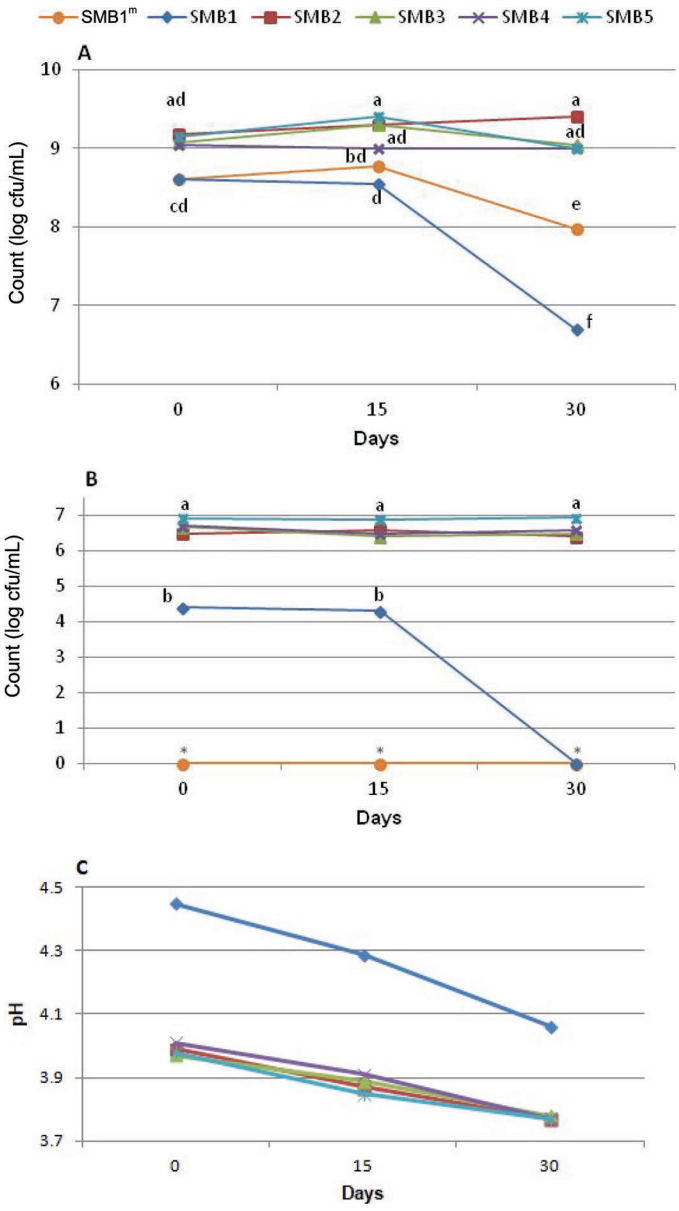

Figure 1. Yogurt mix and potential probiotic bacteria (A) viability and (C) pH in fermented semi-skimmed sheep milk strawberry beverages during storage (4°C ± 0.5) and (B) survival through an in vitro digestion assay. SMB1 (panels A and B) = bacteriological count in M17 agar medium (*Streptococcus thermophilus*); all other counts were performed in de Man, Rogosa, and Sharpe agar (*Lactobacillus* spp.). Letters (a–f) indicate a significant difference ($P < 0.05$); *not detected. SMB1 = yogurt beverage; SMB2 = potential probiotic culture beverage; SMB3 = potential probiotic culture inulin beverage; SMB4 = potential probiotic culture potato starch beverage; SMB5 = potential probiotic culture inulin + potato starch beverage.

ter accepted by consumers (Balthazar et al., 2015). In addition, the lower acidic environment is an ideal condition for adding probiotic bacteria, which are sensitive to the post-acidification of yogurt (Fazilah et al., 2018).

Protein and AA Profile

The content of proteins in fermented semi-skimmed sheep milk strawberry beverages is presented in Table 1. Samples SMB1 and SMB2 had significantly higher casein and whey protein content than the other beverages because higher concentrations of semi-skimmed sheep milk were used in the beverage formulations. However, from a nutritional standpoint, these different protein contents were not significant with respect to the recommended daily intake (Institute of Medicine of the National Academies, 2017).

Figure 2 shows a representative scheme of the SDS-PAGE electrophoresis gel protein profile of the fermented semi-skimmed sheep milk strawberry beverages. We observed no changes in protein profile during storage. Furthermore, after in vitro digestion, beverages did not show any protein band on the electrophoresis gel due to hydrolysis from gastrointestinal enzymes.

We compared the sheep milk fractions from SDS-PAGE with those present in the milk protein, to ob-

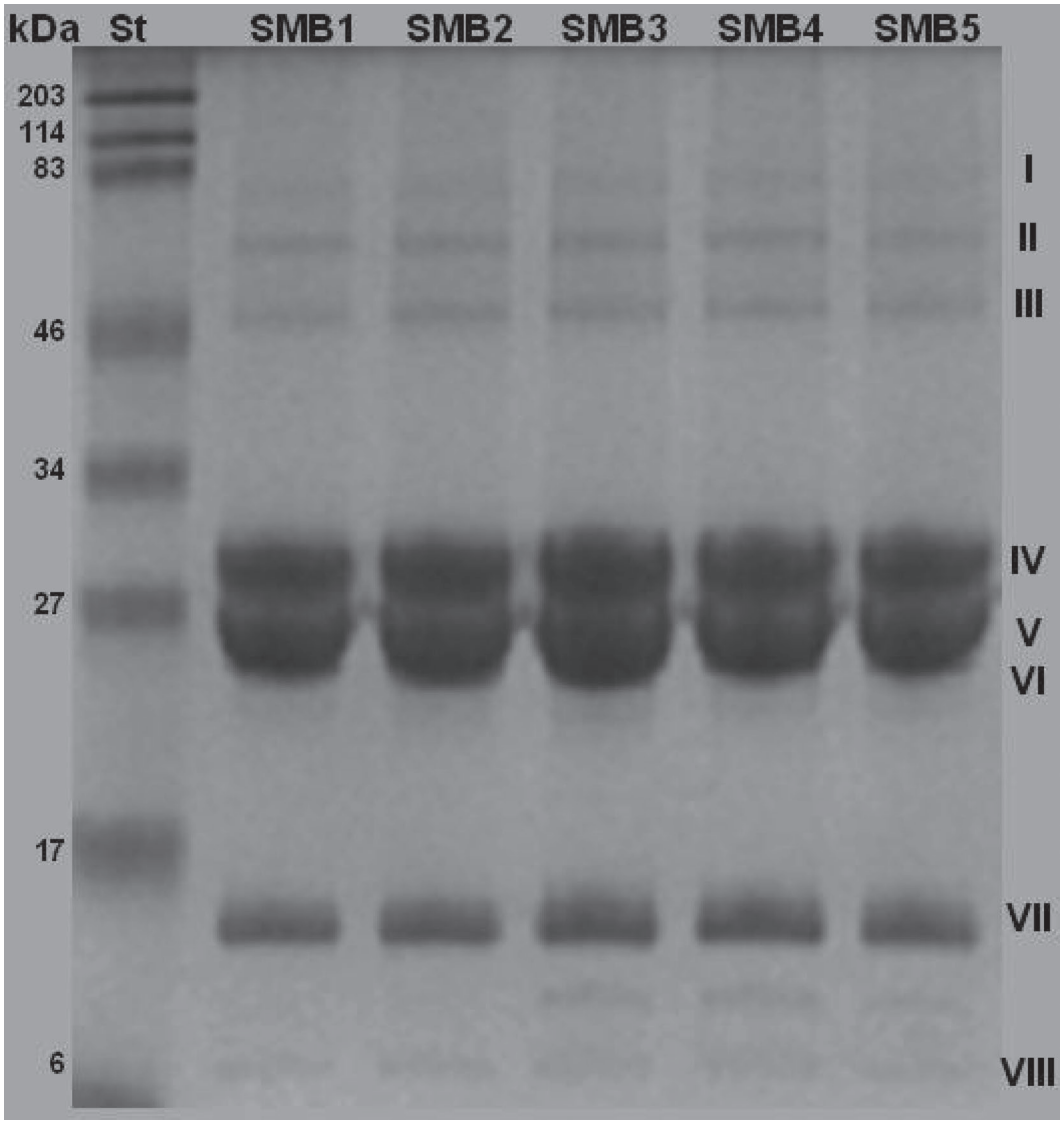

Figure 2. Representative scheme of SDS-PAGE electrophoresis gel protein profile of fermented semi-skimmed sheep milk strawberry beverages. St = molecular weight standard; SMB1 = yogurt beverage; SMB2 = potential probiotic culture beverage; SMB3 = potential probiotic culture inulin beverage; SMB4 = potential probiotic culture potato starch beverage; SMB5 = potential probiotic culture inulin + potato starch beverage. I = immunoglobulin; II = lactoferrin; III = serum albumin; IV = α-CN; V = β-CN; VI = κ-CN; VII = β-LG; VIII = α-LA.

serve the difference between both materials (Grappin et al., 2003). Immunoglobulin (average = 100.2 kDa), lactoferrin (average = 86.2 kDa), and serum albumin (average = 71.2 kDa) appeared at the top of electrophoresis gel; below them appeared the casein fraction: α (average = 36 kDa), and β and κ (average = 28.5 kDa; Figure 2). Bands ascribed to the latter casein fractions were not resolved because they had a similar molecular weight range and there is low κ-casein content in sheep milk (Balthazar et al., 2017a, 2019). The β-LG (average = 19.1 kDa) and α-LA (average = 15.7 kDa) appeared in the lower part of the gel as well, a band characterized by a molecular weight of 17.3 kDa was highlighted in samples SMB3 to SMB5. This finding was probably associated with the presence of both probiotics and fiber as inulin, potato starch, or both. Uriot et al. (2016) reported that small heat shock proteins (12 to 43 kDa) are considered the first line of defense against cellular damage induced by stress.

In evaluating the FAA profile during storage time, we found that almost all FAA increased over storage due to proteolysis and the metabolic activity of the lactic acid bacteria (Table 2). However, we observed significant differences in FAA levels only between 0 and 30 d. The total FAA in these beverages ranged from 15.46 to 30.11 mg/100 g. The semi-skimmed sheep milk strawberry beverage contained most of the essential amino acids (His, Ile, Leu, Lys, Met, Phe, Thr and Val), as well as nonessential amino acids available for absorption by humans. The Glu content was rather low; glutamic acid is a specific precursor of other amino acids, such as Arg and Pro, and other bioactive molecules, such as γ-aminobutyric acid and glutathione (Zareian et al., 2012). Another interesting finding of our FAA data evaluation was the low amount ($P < 0.05$) of Gln in beverages SMB3 and SMB5 (0.58 and 0.87 mg/100 g, respectively).

We performed principal component analysis using the FAA data before digestion assay (Figure 3): a 2-dimensional projection explained 62.77% of the variability in FAA using 2 components, in which the first dimension accounted for 38.65% and the second dimension (D2) accounted for 24.12%. The different samples were allocated in distinct quadrants of the principal component analysis: SMB4 in the first quadrant, SMB1 and SMB2 in the second, and SMB3 and SMB5 in the fourth. Beverages SMB3 to SMB5 were located in a zone of the plot denoting higher FAA content.

Compared with sheep milk (Balthazar et al., 2019; Rafiq et al., 2016), some FAA (Ala, Arg, Cys, His, Met, Phe, Pro, Ser, and Val) presented in higher concentrations in all beverages, meaning that proteolysis occurred in response to bacterial activity during fermentation of the semi-skimmed sheep milk or in the beverage during storage.

Evaluation of Ascorbic Acid

The ascorbic acid content was approximately 4.83 mg/g in all beverages at d 0 (Table 1); the strawberry pulp was responsible for providing this compound to the beverages (Forbes-Hernandez et al., 2016). We also found a significant decrease (around 4%) of ascorbic acid content in all samples (SMB1 4.63 mg/g; SMB2 4.66 mg/g; SMB3 4.61 mg/g; SMB4 4.62 mg/g; SMB5 4.65 mg/g) at the end of refrigerated storage, but we observed no significant differences among samples.

Bioactive Compounds

Yogurt starter bacteria (SMB1) and Lp B2 (SMB2 to SMB5) showed chemical antioxidant activity (Figure 3A). In general, beverages fermented with Lp B2 had higher ($P < 0.05$) antioxidant activity. Moreover, fiber addition to beverages (SMB3 and SMB4) significantly increased the antioxidant activity during storage compared with SMB1 and SMB2 (without fiber addition), showing a possible synergism between Lp B2 and fibers. It is likely that the inulin and potato starch together enhanced the proteolytic activity of Lp B2, producing bioactive peptides.

Antioxidant activity inhibits the oxidation of molecules caused by free radicals and is important for the shelf life of dairy foods and to protect the human body against oxidative damage upon consumption. Bioactive compounds in foods, especially fermented dairy products, play a crucial role in elevating the effect of reactive oxygen species such as superoxide, hydroxyl, and peroxyl radicals formed by cells under oxidative stress. These bioactive compounds, especially protein-derived peptides, can donate electrons to neutralize free radicals. Moreover, the presence of several amino acid residues in the peptide chains can enhance antioxidant properties. Bioactive peptides as antioxidant agents in fermented milk prevent enzymatic and non-enzymatic peroxidation of essential fatty acids. The DPPH free radical is commonly used to estimate the antioxidant activity in certain particular compounds due to the utilization of its feature to be scavenged by electron-donating substances such as antioxidants (Alenisan et al., 2017).

Figure 4B illustrates the ACEI activity provided by the beverages. The ACEI activity ranged from 36 to 60% in the beverage formulations manufactured with fiber ($P < 0.05$), in which beverage SMB5 presented the highest antihypertensive activity. Overall, compared

Table 2. Free AA profile (mg/100 g) in semi-skimmed (1.6% vol/vol fat) fermented sheep milk strawberry beverages and in digested beverage at d 0 and 30 of refrigerated storage (4 ± 0.5°C)[1]

Beverage[2]	Day	Ala	Arg	Asp	Cys	Glu	Gln	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Tyr	Val	Total
Storage																				
SMB1	1	1.62^{ef}	4.84^{de}	0.15^{i}	0.73^{e}	0.43^{g}	2.45^{b}	0.50^{a}	3.29^{a}	0.44^{b}	0.24^{e}	0.88^{a}	0.77^{c}	0.70^{bc}	0.12^{e}	2.47^{e}	—	0.40^{c}	0.94^{e}	20.98^{d}
	30	1.88^{d}	5.41^{cd}	0.22^{f}	0.96^{de}	0.70^{c}	2.43^{b}	0.40^{b}	1.70^{d}	0.24^{e}	0.23^{e}	0.83^{b}	0.93^{b}	0.79^{b}	0.23^{a}	2.99^{a}	—	0.50^{b}	1.50^{b}	21.94^{c}
SMB2	1	1.92^{d}	5.75^{c}	0.25^{e}	1.20^{cd}	0.40^{g}	2.18^{c}	0.15^{d}	1.64^{d}	0.76^{a}	0.10^{f}	0.29^{f}	0.53^{e}	0.52^{d}	0.17^{cd}	2.64^{bc}	—	0.51^{b}	0.64^{f}	19.66^{e}
	30	1.75^{de}	7.46^{b}	0.44^{b}	1.96^{a}	0.64^{d}	2.05^{d}	0.40^{b}	2.27^{c}	0.29^{de}	0.29^{cd}	0.84^{ab}	0.72^{cd}	0.57^{cd}	0.18^{bc}	2.51^{de}	—	0.57^{a}	0.89^{e}	23.84^{b}
SMB3	1	1.56^{f}	4.59^{e}	0.17^{h}	1.88^{ab}	0.46^{f}	0.58^{f}	0.22^{c}	1.08^{f}	0.14^{f}	0.26^{de}	0.38^{e}	0.78^{c}	0.75^{b}	0.15^{de}	0.88^{h}	—	0.55^{a}	1.03^{de}	15.46^{g}
	30	2.57^{b}	5.95^{cd}	0.39^{c}	1.66^{b}	1.02^{b}	0.03^{h}	0.41^{b}	1.43^{e}	0.23^{e}	0.31^{bc}	0.69^{c}	1.07^{a}	0.80^{b}	0.14^{de}	0.84^{h}	—	0.23^{e}	1.21^{cd}	18.72^{f}
SMB4	1	2.36^{c}	5.29^{cd}	0.26^{e}	1.24^{c}	0.58^{e}	2.21^{c}	0.18^{d}	1.49^{e}	0.15^{f}	0.29^{bc}	0.38^{e}	0.77^{c}	1.23^{a}	0.16^{cd}	2.59^{cd}	—	0.43^{c}	1.37^{bc}	20.99^{d}
	30	2.98^{a}	9.53^{a}	0.71^{a}	0.37^{f}	1.02^{b}	2.64^{a}	0.49^{a}	3.04^{b}	0.37^{c}	0.49^{a}	0.47^{d}	1.07^{a}	1.19^{a}	0.13^{e}	2.72^{b}	—	0.56^{a}	2.33^{a}	30.11^{a}
SMB5	1	1.55^{f}	4.24^{e}	0.20^{g}	1.89^{ab}	0.47^{f}	0.87^{e}	0.17^{d}	1.14^{f}	0.14^{f}	0.23^{e}	0.25^{g}	0.68^{d}	0.79^{b}	0.14^{de}	1.23^{f}	—	0.49^{b}	1.18^{d}	15.66^{g}
	30	2.24^{c}	6.98^{b}	0.37^{d}	1.33^{c}	1.11^{a}	0.22^{g}	0.41^{b}	1.65^{d}	0.25^{de}	0.32^{b}	0.45^{d}	0.97^{b}	0.72^{bc}	0.21^{ab}	1.07^{g}	—	0.33^{d}	1.49^{b}	20.12^{e}
Digestion																				
SMB1	1	110.66^{b}	129.97^{b}	15.21^{a}	65.93^{b}	59.76^{a}	48.86^{b}	31.99^{ab}	144.97^{c}	28.63^{b}	71.92^{b}	75.27^{a}	68.69^{c}	47.61^{cd}	34.77^{cd}	70.01^{a}	35.52^{bc}	97.16^{b}	52.29^{b}	1,189.21^{bc}
	30	109.05^{a}	132.08^{a}	14.92^{cd}	101.45^{bc}	57.12^{de}	51.15^{a}	24.78^{bc}	157.37^{ab}	32.54^{a}	72.71^{a}	81.51^{a}	69.27^{a}	48.61^{a}	27.65^{c}	65.58^{b}	31.96^{cd}	96.07^{a}	46.27^{a}	1,220.11^{b}
SMB2	1	118.14^{c}	146.37^{cd}	15.73^{ef}	62.01^{bc}	55.06^{b}	64.63^{c}	38.15^{de}	146.80^{b}	47.78^{b}	88.17^{cd}	94.27^{d}	76.72^{b}	63.79^{bc}	20.05^{a}	69.37^{ab}	39.09^{ab}	114.19^{cd}	63.28^{c}	1,323.58^{a}
	30	107.09^{cd}	129.10^{cd}	15.30^{f}	64.65^{a}	53.83^{cd}	45.51^{b}	35.36^{g}	115.23^{a}	34.02^{b}	73.60^{c}	87.11^{c}	61.69^{b}	37.66^{b}	16.86^{b}	58.25^{c}	37.98^{ab}	95.82^{de}	46.72^{d}	1,115.76^{de}
SMB3	1	110.14^{e}	77.38^{b}	13.87^{b}	55.32^{bc}	38.56^{b}	34.21^{e}	28.70^{a}	92.82^{d}	26.77^{b}	57.49^{d}	81.86^{c}	40.62^{d}	42.33^{de}	27.41^{f}	42.67^{c}	28.14^{d}	98.94^{e}	37.30^{c}	934.55^{f}
	30	106.93^{e}	132.77^{c}	15.99^{c}	67.33^{bc}	58.29^{bc}	45.04^{d}	28.71^{d}	128.49^{c}	32.81^{b}	70.10^{d}	79.69^{cd}	63.00^{e}	40.31^{ef}	13.37^{ef}	64.33^{c}	38.87^{ab}	93.53^{e}	50.87^{c}	1,137.93^{de}
SMB4	1	113.70^{de}	137.30^{cd}	17.86^{def}	71.20^{bc}	63.88^{e}	52.65^{d}	28.72^{cd}	127.26^{d}	33.62^{b}	77.14^{c}	96.81^{b}	67.19^{f}	44.64^{f}	18.67^{cde}	71.48^{d}	39.35^{ab}	102.60^{de}	55.78^{d}	1,232.44^{b}
	30	102.64^{f}	128.56^{d}	15.48^{de}	63.03^{bc}	55.93^{cde}	39.79^{e}	28.73^{c}	110.50^{d}	28.62^{b}	65.86^{e}	81.51^{c}	58.20^{g}	37.68^{f}	18.43^{cd}	58.87^{d}	36.96^{ab}	88.79^{f}	46.19^{d}	1,074.01^{e}
SMB5	1	71.82^{c}	76.33^{e}	11.74^{g}	43.78^{cd}	33.00^{f}	21.06^{f}	28.74^{ef}	80.73^{e}	16.80^{bc}	32.03^{f}	40.21^{c}	34.93^{h}	24.80^{de}	14.06^{b}	41.23^{e}	23.38^{e}	55.85^{c}	27.20^{e}	674.01^{g}
	30	105.85^{g}	136.98^{e}	16.52^{h}	68.26^{d}	60.06^{g}	41.81^{g}	28.75^{fg}	114.84^{f}	32.30^{c}	70.33^{g}	81.85^{e}	64.20^{i}	41.99^{g}	11.29^{def}	65.19^{e}	40.76^{a}	93.55^{g}	52.75^{f}	1,141.05^{cd}

[a–i]Different superscript letters in the same columns (within storage or digestion rows) indicate a significant difference ($P < 0.05$) among samples × storage time.

[1]Values are expressed as mean ± SD.

[2]SMB1 = yogurt beverage; SMB2 = potential probiotic culture beverage; SMB3 = potential probiotic culture inulin beverage; SMB4 = potential probiotic culture potato starch beverage; SMB5 = potential probiotic culture inulin + potato starch beverage.

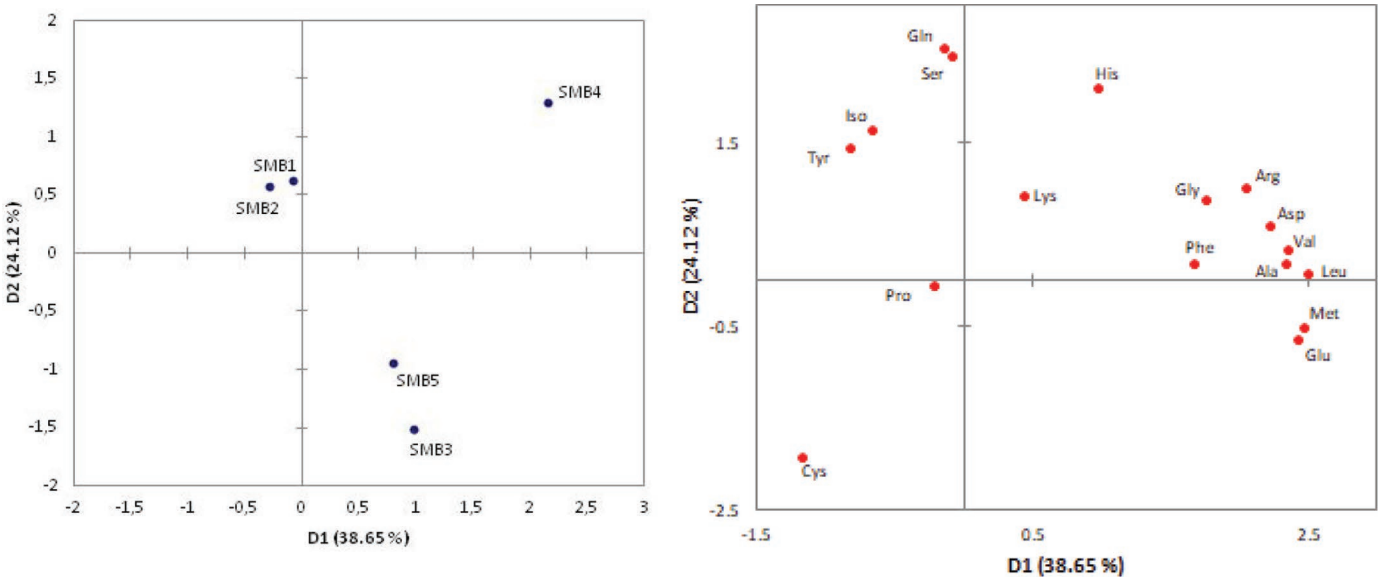

Figure 3. Principal component analysis bidimensional (D1, D2) map of fermented semi-skimmed sheep milk strawberry beverages free AA profile during storage (4 ± 0.5°C). SMB1 = yogurt beverage; SMB2 = potential probiotic culture beverage; SMB3 = potential probiotic culture inulin beverage; SMB4 = potential probiotic culture potato starch beverage; SMB5 = potential probiotic culture inulin + potato starch beverage.

with the DPPH data, the in vitro antihypertensive activity had a similar trend: samples containing Lp B2 had higher ACEI activity, probably related to the metabolic activity of the potentially probiotic strain. Some studies concerning the ACEI activity of probiotic cheeses during ripening and sheep milk ice cream also have shown an increase in antihypertensive properties compared with conventional products (with no addition of probiotics and fibers; Balthazar et al., 2018a; Silva et al., 2018a), as reported in this study.

Milk proteins are the most important sources of functional peptides that have been shown to exert beneficial effects on the cardiovascular system, providing antihypertensive effect mainly by inhibiting the activity of endogenous enzymes such as α-amylase, α-glucosidase, and ACEI. The content of potent bioactive compounds in fermented milks depends on the processing conditions, the bacteria used in the fermentation step, and storage time. Several key factors can affect the formation of ACEI peptides in fermented products, including pre-treatment of milk, cultures, processing conditions, and ripening time (Erkaya and Şengul, 2015). The ACEI in milk may be attributed to high proteolytic activity, supporting our assumption that sheep milk proteins are more susceptible to hydrolysis by proteolytic enzymes produced by Lp B2 and yogurt bacteria.

The inhibition of α-amylase and α-glucosidase by the beverage formulations during storage are illustrated in Figure 4C. In general, beverages containing Lp B2 presented higher inhibition of the digestive enzymes than the yogurt bacteria. Similarly, the formulations containing Lp B2 and inulin (SMB3) or potato starch (SMB4) or both fibers (SMB5) showed higher inhibition of digestive enzymes ($P < 0.05$). Nevertheless, beverage SMB4 presented higher inhibition of α-glucosidase than beverage SMB3 (36.13 and 31.97%; $P < 0.05$). Beverage SMB5 presented the highest inhibition of enzymes: mean values were twice that of those observed in beverages SMB3 and SMB4, and 6 times higher compared with beverage SMB1. The same behavior was observed during storage for all beverages containing Lp B2.

Type 2 diabetes mellitus has risen dramatically, and it is expected to affect 438 million people by 2030, with 70% of the whole cases occurring in poor countries (Weber, 2010). The inhibition of α-amylase activity and α-glucosidase together is seen as an effective strategy for controlling this chronic disease. Probiotics might improve insulin tolerance by reducing inflammatory response in the body, because oxidative stress can lead to insulin resistance and the decrease of glucose uptake by peripheral tissue (Ostadrahimi et al., 2015).

The strong positive correlation between bioactive compounds (Table 3) suggests that the peptides released by the proteolytic action of Lp B2 may have potentially functional benefits. Similarly, fiber addition (inulin or potato starch) increased the proteolytic activity of this microorganism, providing more bioactive peptides in the functional beverages. The mix of fibers enhanced the proteolytic activity of Lp B2.

Distinctions appear when lactic acid bacteria species and strains are compared in relation to proteolytic activity (El-Salam and El- Shibiny, 2013). Moslehishad

et al. (2013) reported that the peptide fraction is correlated with its functionality, implying that the functional effects of the fractions depend on the strain used in the fermentation process. The authors showed that peptide fractions <3 kDa had higher bioactivity than larger molecules (3 to 5 kDa). In addition, the peptide KLPGF showed in vitro inhibition of α-amylase and α-glucosidase. Similarly, other studies in dairy foods inhibited these digestive enzymes (Ostadrahimi et al., 2015; Yousaf et al., 2016).

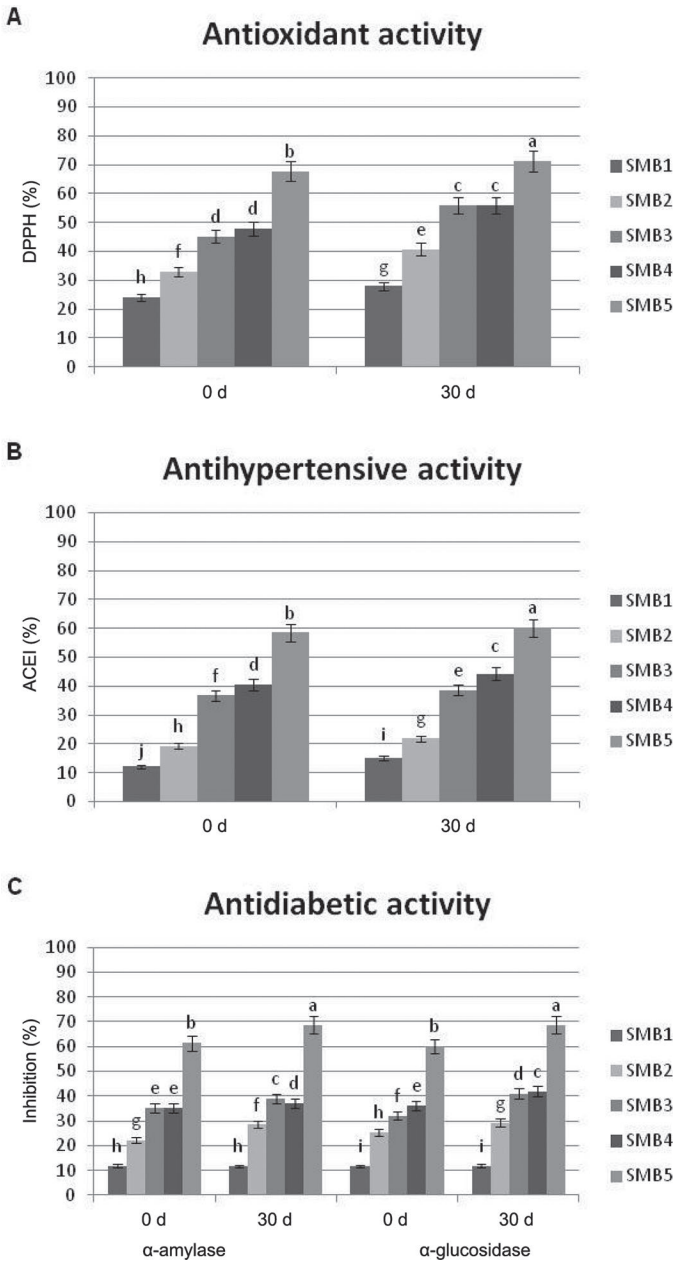

Figure 4. (A) Antioxidant, (B) antihypertensive, and (C) antidiabetic activities of fermented semi-skimmed sheep milk strawberry beverages during storage (4 ± 1°C). SMB1 = yogurt beverage; SMB2 = potential probiotic culture beverage; SMB3 = potential probiotic culture inulin beverage; SMB4 = potential probiotic culture potato starch beverage; SMB5 = potential probiotic culture inulin + potato starch beverage. DPPH = 2,2-diphenyl-1-picrylhydrazyl; ACEI = angiotensin I converting enzyme inhibitor.

Mineral Content and Availability

Minerals are micronutrients that have key roles in the human body with diverse functions and potential in metabolism and homeostasis, from building strong bones to transmitting nerve impulses for a healthy, long life (Gharibzahedi and Jafari, 2017).

Table 4 shows the mineral content of the fermented semi-skimmed sheep milk strawberry beverages. The samples differed significantly in their mineral constituents, but the fermented beverages were important sources of calcium (635.47–880.30 mg/100 g), magnesium (313.60–373.87 mg/100 g), phosphorus (701.57–886.43 mg/100 g), zinc (61.17–81.50 mg/100 g), and potassium (449.00–666.40 mg/100 g).

With respect to changes in mineral availability, the fermented semi-skimmed sheep milk strawberry beverages differed significantly in their mineral content during processing. The mean content of calcium in beverages after manufacturing was 763.45 mg/100 g. After in vitro enzymatic hydrolysis, the availability of calcium reached ~15.5% and ~21.7% for beverages SMB1 and SMB5, respectively, and the averagecalcium availability was estimated to be ~18.1%. The mean content of magnesium in all beverages was 337.45 mg/100 g. After in vitro enzymatic hydrolysis, the mean availability of magnesium reached ~19.2 and ~30.9% for beverages SMB5 and SMB2, respectively, and the average magnesium availability was estimated to be ~26.7%. The mean content of phosphorus was 815.70 mg/100 g. After in vitro enzymatic hydrolysis, the mean availability of phosphorus reached ~33.2 and ~42.3% for beverages SMB1 and SMB5, respectively, and the average availability of phosphorus was estimated to be ~39.1%. The mean potassium content was 577.08 mg/100 g immediately after manufacturing (Table 4). After in vitro enzymatic hydrolysis, the overall availability of potas-

Table 3. Pearson correlations between bioactive compounds presented in semi-skimmed (1.6% vol/vol fat) fermented sheep milk strawberry beverage[1,2]

Variable	DPPH	ACEI	α-Amylase	α-Glucosidase
DPPH	1	0.972	0.966	0.975
ACEI	0.972	1	0.964	0.963
α-Amylase	0.966	0.964	1	0.993
α-Glucosidase	0.975	0.963	0.993	1

[1]All r-values are $P < 0.05$.

[2]DPPH = 2,2-diphenyl-1-picrylhydrazyl; ACEI = angiotensin I converting enzyme inhibitor.

Table 4. Mineral content and changes in mineral availability from semi-skimmed (1.6% vol/vol fat) fermented sheep milk strawberry beverage

Beverage[2]	Mineral	Before in vitro hydrolysis (mg/100 g)	After in vitro hydrolysis (mg/100 g)	Amount of mineral released (%)
SMB1	K	449.00^{d} ± 12.0	46.00^{d} ± 0.7	10.25^{ab} ± 0.4
	Mg	313.60^{e} ± 1.4	91.63^{c} ± 1.6	29.22^{b} ± 0.4
	Ca	635.47^{c} ± 1.1	98.63^{e} ± 0.6	15.52^{d} ± 0.1
	P	701.57^{e} ± 1.3	232.80^{e} ± 1.5	33.18^{e} ± 0.2
	Zn	61.17^{e} ± 0.9	4.61^{e} ± 0.1	7.53^{d} ± 0.1
SMB2	K	533.17^{c} ± 19.1	56.40^{c} ± 0.9	10.59^{a} ± 0.5
	Mg	319.33^{d} ± 0.9	98.97^{a} ± 0.6	30.99^{a} ± 0.1
	Ca	723.40^{d} ± 1.1	114.17^{d} ± 0.3	15.78^{c} ± 0.1
	P	812.93^{d} ± 2.1	314.57^{d} ± 1.9	38.70^{d} ± 0.3
	Zn	65.63^{d} ± 1.1	6.44^{d} ± 0.2	9.81^{c} ± 0.3
SMB3	K	612.87^{b} ± 2.3	61.80^{b} ± 0.5	10.08^{ab} ± 0.1
	Mg	324.33^{c} ± 0.9	94.80^{b} ± 0.6	29.23^{b} ± 0.3
	Ca	753.63^{c} ± 1.4	119.23^{c} ± 1.0	15.82^{c} ± 0.2
	P	822.77^{c} ± 1.6	324.67^{c} ± 0.8	39.46^{c} ± 0.1
	Zn	71.27^{c} ± 1.1	7.55^{c} ± 0.2	10.60^{b} ± 0.3
SMB4	K	623.97^{b} ± 1.5	62.60^{b} ± 0.7	10.03^{ab} ± 0.1
	Mg	356.13^{b} ± 1.1	89.03^{d} ± 0.6	25.00^{c} ± 0.1
	Ca	880.30^{a} ± 1.0	188.83^{a} ± 0.7	21.45^{b} ± 0.1
	P	854.80^{b} ± 2.3	355.40^{b} ± 0.8	41.58^{b} ± 0.1
	Zn	76.43^{b} ± 1.0	9.63^{b} ± 0.3	12.60^{a} ± 0.3
SMB5	K	666.40^{a} ± 1.4	65.23^{a} ± 1.0	9.79^{b} ± 0.2
	Mg	373.87^{a} ± 1.6	71.97^{e} ± 0.6	19.25^{d} ± 0.2
	Ca	824.43^{b} ± 1.0	178.93^{b} ± 0.6	21.70^{a} ± 0.1
	P	886.43^{a} ± 1.8	375.24^{a} ± 2.0	42.33^{a} ± 0.1
	Zn	81.50^{a} ± 1.1	10.35^{a} ± 0.4	12.70^{a} ± 0.4

$^{a–e}$Different superscript letters in the same row indicate a significant difference ($P < 0.05$).

[1]Values are expressed as mean ± SD.

[2]SMB1 = yogurt beverage; SMB2 = potential probiotic culture beverage; SMB3 = potential probiotic culture inulin beverage; SMB4 = potential probiotic culture potato starch beverage; SMB5 = potential probiotic culture inulin + potato starch beverage.

sium achieved ~9.8, and beverages SMB5 and SMB2 presented availability rates of ~10.6% and ~10.1%, respectively. The mean content of zinc in all samples was 71.20 mg/100 g. After in vitro enzymatic hydrolysis, the overall availability of zinc was estimated to be 10.65%, but values ranged from ~7.5 to ~12.7% for beverages SMB1 and SMB5, respectively.

Mineral concentrations in fermented semi-skimmed sheep milk strawberry beverages were higher than in fermented goat milk with *L. plantarum* C4 (Bergillos-Meca et al., 2015), possibly because of higher mineral content in sheep milk than goat milk (Balthazar et al., 2017a). Bergillos-Meca et al. (2015) reported digestive system or food manufacture, both suitable for keeping some minerals in soluble form, which may increase dietary casein and improve mineral bioavailability. However, the mechanisms involved in mineral availability are not totally clear, and further studies are needed.

CONCLUSIONS

The present study developed new functional fermented sheep milk strawberry beverages containing *L. plantarum* CECT 8328 and inulin, potato starch, or both. *L. plantarum* CECT 8328 counts remained stable/viable during refrigerated storage up to 30 d and after in vitro simulated digestion; the lactic acid bacteria was also able to improve the antioxidant, antihypertensive, and antidiabetic activities of the beverages, especially when combined with fiber addition. Few attempts have been made to exploit sheep milk in this way and to give scientific support to the use of sheep milk for dairy products other than cheese varieties and yogurt; the results from this study encourage further investigation into both the technological and sensory characteristics of the product and the assessment of its functional properties.

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2017, "Fermented milk beverages from ovine milk," principal investigator A. Santillo.

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