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Antioxidant properties of probiotic fermented milk supplemented with chestnut flour (*Castanea sativa* Mill)

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Abstract

The effect of sweet chestnut (*Castanea sativa* Mill) flour in stimulating the growth of probiotic bacteria in fermented skim milk produced with different probiotic strains, namely *Lactobacillus acidophilus*, *L. rhamnosus* and *Bifidobacterium animalis* subsp. *lactis* was evaluated. Microbial counts, pH, total titratable acidity (LA %) and syneresis were measured in fermented skim milk samples. Additionally, the antioxidant capacities of the samples were measured by Trolox equivalent antioxidant capacity (TEAC), free radical scavenging activity (DPPH), and Ferric Reducing-antioxidant Power (FRAP) assays. The viability and growth proportion index (GPI) of *L. rhamnosus* were significantly higher than those of *L. acidophilus and B. lactis* in all samples during storage. Results indicated that all probiotic fermented milks enriched with chestnut flour displayed significant probiotic viability (>7 $\log_{10}$ cfu/g) with high antioxidant capacities. *L. acidophilus*, *L. rhamnosus* and *B. lactis* survived throughout the shelf life of the chestnut-fermented skim milk, and remain at this satisfactory viability level even after 21 days of storage. The antioxidant capacity and phenolic contents were dependent on probiotic strains used.

Practical applications

Nowadays the focus is rather on the effects of foods on maintenance of health, well-being and prevention of certain diseases than simply satisfaction of appetite or nutrition. The consumers' health consciousness due to the scientific knowledge of the interactions between diet and health is a driving factor to develop products with health-related claims such as probiotic foods. This paper investigated the effects of chestnut flour supplementation not only on viability of probiotic bacteria but also the antioxidant capacity and phenolic contents in fermented milk throughout predicted shelf life. The results indicated that chestnut flour could be used as prebiotic for further researches to develop dairy products to deliver probiotics.

KEYWORDS
antioxidant capacity, probiotic, sweet chestnut (*Castanea sativa* Mill) flour

1 | INTRODUCTION

Sweet chestnut (*Castanea sativa* Mill.) is a good source of many bioactive compounds that have been associated with prevention of cancer, cardiovascular disease and neurological function disorders, as well as anti-inflammatory effects (Barreira, Ferreira, Oliveira, & Pereira, 2008). It is a native deciduous seasonal tree of the Mediterranean countries from the genus of long-lived trees in the Fagaceae family. It produces edible nuts which have been used since ancient times. Asia, Southern Europe and Turkey, and North-America are the three main chestnut cultivar growing areas in the world. In Asia, mainly in China, *C. mollissima* is found naturally as well as in cultivation; in Southern Europe and Turkey *C. sativa* is predominant and in North-America *C. dentate* is widespread naturally (Bounous, Botta, & Beccaro, 2000; Comba, Gay, Piccarolo, & Aimonino, 2009). Turkey, having numerous genotypes and cultivars, is one of the leading countries in the world with an annual production of 60,000 tons (Anonymous 2015). Bursa Region is well-known for either fresh or industrially processed forms of chestnuts and these commercial products have a high economic value.

Chestnuts are generally consumed fresh, cooked, steamed, grilled, roasted, boiled or fried, being the most common cooking methods. The fruit can be peeled and eaten raw, or can be used to stuff vegetables,

poultry, and fowls; can be dried and milled into flour to be used in breads, cakes, pastas, soups, and sauces; and can be candied known as "marron glacé." In order to extend the consumption it is necessary to obtain derived products along with fresh and processed chestnuts (Demiate, Oetterer, & Wosiacki, 2001; De Vasconcelos, Bennet, Rosa, & Ferreira-Cardoso, 2010a; De Vasconcelos et al., 2010b).

These derived products such as flour and starch present the advantages like serving as sources of gluten-free contents and essential fatty acids. The nutritional composition of chestnut consists of complex carbohydrates mainly starch, proteins, vitamins and minerals as well as antioxidants, fatty acids (mostly monounsaturated and polyunsaturated) and fiber ingredients, which makes it a good prebiotic source. Chestnuts have high lysine, threonine and a considerable quantity of γ-amino butyric acid (Yildiz, Ozcan, Calisir, Demir, & Er, 2009).

Chestnut flour (CF), obtained by grinding dried chestnuts after the pericarp and the endocarp have been removed, is done mainly for valorization of small chestnuts or chestnuts with double embryos. Aside with valorization CF presents high levels of dietary fiber, vitamin E and B group vitamins and is usually preferred as a basic ingredient for the confectionery paste (Sacchetti, Pinnavaia, Guidolin, & Rosa, 2004).

Probiotics are living microorganisms which improves the health of the host when ingested in sufficient amounts. The health benefits are stated as improvement of the gut microflora and stabilization of the gut mucosal barrier, prevention of infectious diseases and food allergies, reduction of serum cholesterol level, enhanced anti-carcinogenic activity and immune properties (Leroy & De Vuyst, 2004; Sanders, 2008; Forssten, Lahtine, & Ouwehand, 2011).

Probiotics must be able to exert their benefits on the host through growth and/or activity in the human body. Inclusion of probiotic bacteria in fermented dairy products enhances their value as health promoting foods. However, insufficient viability and survival of these bacteria remain a problem in commercial food products. By selecting better functional probiotic strains and adopting improved methods to enhance survival, including prebiotics in food systems, which are non-digestible dietary components, mainly carbohydrates, an increased delivery of viable bacteria in fermented products can be achieved as well as using the optimal combination of probiotics and prebiotics (synbiotic) (Chow, 2002; Soccol et al., 2010).

Even though the literature focuses mainly on nutrients in fresh chestnut fruits that are important for health; there is limited information on bioactive non-nutrients such as phenolics and potential applications as sources of antioxidants, prebiotics and dietary fiber. Furthermore the prebiotic potential of chestnut flour and its influence on the growth of probiotic microorganisms has not been investigated.

Therefore, the main objectives of this study were (a) to produce a fermented skim milk fortified with chestnut flour and probiotic cultures; (b) to investigate the survival of the probiotic cultures as affected by the chestnut flour over a 21 day cold storage; (c) to determine the effects of chestnut flour addition on the antioxidant and physicochemical properties of probiotic fermented skim milk.

2 | MATERIALS AND METHODS

2.1 | Preparation of probiotic starter culture

Each lyophilized strain was prepared according to Ozcan, Yilmaz-Ersan, Akpinar-Bayizit, Sahin, and Aydinol (2010) using 1 g of lyophilized culture in 100 ml 12% (w/v) reconstituted sterile non-fat milk (autoclaved at 121°C for 15 min). The probiotic cultures of *Lactobacillus acidophilus*, *L. rhamnosus* and *Bifidobacterium animalis* subsp. *lactis* (Danisco, Madison WI, USA) were incubated at 37 ± 1°C for 72 h in anaerobic jars containing Anaerogen Gas Packs (Oxoid). The necessary inoculums were calculated as to give approximately 9.0 $\log_{10}$ colony forming units ($\log_{10}$cfu/ml) in fermented skim milk after inoculation.

2.2 | Fermented skim milk production

Fermented skim milk samples were manufactured at the Food Pilot Plant of Uludag University-Food Engineering Department (Bursa, Turkey). Skim milk powder was reconstituted in distilled water at 10.70% (w/w) to yield reconstituted skim milk of the same overall composition as the raw skim milk and 2% sweet chestnut (*Castanea sativa* Mill) flour (w/w, Kafkas Comp., Bursa Turkey) was added. The chestnut flour was obtained by dry milling of the dehydrated chestnuts–60 USB Smesh sieve. The gross composition is fat 3.80%, protein 4.61%, carbohydrate 69.31% and dietary fiber 9.5%. The milks were then heat-treated at 90°C for 10 min, cooled to 37°C and inoculated with each probiotic bacteria, denoted as LAY (*L.* acidophilus), LRY (*L. rhamnosus*) and BLY (*Bifidobacterium animalis* subsp. *lactis*), were transferred into 500 ml sterile Schott flasks. After inoculation, the incubation was carried out at 37°C until the final pH value reached 4.7. Once the fermentation is completed, the samples were kept at room temperature (22 ± 1°C) for 30 min and stored at 4 ± 1°C. Each fermentation was performed in triplicate.

2.3 | Enumeration of microorganisms

Probiotic strains were enumerated on selective media at the end of the fermentation, and on 7, 14 and 21 days of refrigerated storage. Samples were diluted 10-fold in 10 ml 1-fourth strength sterile Ringer's solution, 1 ml volumes of appropriate dilutions pour plated in quadruplicate on MRS agar (Biolife, Milano, Italy) and incubated anaerobically at 37°C for 3 days. For *Bifidobacterium animalis* subsp. *lactis* MRS-LP (MRS agar with 0.2% (w/v) of lithium chloride and 0.3% (w/v) of sodium propionate) (Tharmaraj & Shah, 2003), for *L. acidophilus* MRS-Bile (MRS agar with 0.15% (w/v) of bile) (Vinderola, Bailo, & Reinheimer, 2000) and for *L. rhamnosus* MRS-Vancomycin (MRS agar with 20 mg/ml of vancomycin) (Björneholm, Eklöw, Saarela, & Mättö, 2002) were used.

The cell concentrations were expressed in logarithm of colony forming units per gram of product ($\log_{10}$ cfu/g). Growth proportion index (GPI) of probiotic microorganisms for each growth interval assessed was calculated as following (Shafiee, Taghavi, & Babalar, 2010b):

$$\text{GPI} = \frac{\text{Final cell population (log10 cfu/g)}}{\text{Initial cell population (log10 cfu/g)}}$$

2.4 | Analytical methods

The acidification activity during the fermentation (data not given) and 21 days of cold storage was determined through pH measurements by means of a digital pH meter (Analyzer model 315i/SET, WTW, Germany). The titratable acidity (LA %) of fermented milks was determined according to AOAC methods No. 947.05 (AOAC, 2000). Syneresis was expressed as volume of drained whey (ml/25g sample) (Wu, Hulbert, & Mount, 2001).

2.5 | Total phenolic content and antioxidant activity

Fermented skim milk samples (2 g) were blended with 20 ml extraction solution (methanol/water, 70:30, v/v) and stirred at 20 ± 1°C for 4 hr in the dark with the help of a magnetic stirrer. The suspension was centrifuged at 3,500 × g for 10 min and filtered through sheets of qualitative filter paper (75 g m^2, 0.2 mm thickness). These supernatants were used for determination of total phenolic contents and antioxidant capacity ABTS, DPPH and FRAP (Isik, Sahin, & Demir, 2013).

The total phenolic contents of probiotic samples were determined spectrophotometrically at 725 nm according to Folin-Ciocalteu (FC) colorimetric method developed by Singleton, Orthofer, and Lamuela-Raventos (1999). The supernatants were diluted with ethanol/acetic acid solution (1:20, v/v). After adding 0.25 ml extracts, 2.3 ml distilled water, 0.15 ml Folin-Ciocalteu reagent the solution was vortexed for 15 s. After 5 min 0.30 ml 35% Na_2CO_3 added and content was mixed and left to stand at room temperature in dark for 2 hr. A standard calibration curve was plotted using gallic acid (Merck, Germany). The results were expressed as "milligrams of gallic acid equivalents (GAE) per 100 g of dry weight."

The antioxidant capacity of fermented milks was evaluated using three different approaches. The scavenging rates of 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals were measured by the procedures reported by Re et al. (1999) and Skrede, Bryhn-Larsen, Aaby, Skivik-Jorgensen, and Birkeland (2004) whereas ferric reducing antioxidant power (FRAP) analysis was performed by the method described by Lucas et al. (2006).

The ABTS radical cation was produced by reacting 20 mM ABTS stock solution with 2.45 mM potassium persulfate and kept at room temperature in the dark for 12–16 hr before use. To 1 ml of diluted ABTS solution x mL of sample or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as control and (4-x) ml ethanol were added and incubated at 30°C for 6 min. Scavenging of the ABTS radical was followed spectrophotometrically by monitoring the decrease in absorbance at 734 nm during 6 min against the solvent blank which was run as negative control in each assay. All determinations were carried out in triplicate. Standard curve was prepared using different concentrations of Trolox and to calculate the Trolox Equivalent Antioxidant Capacity (TEAC) the gradient of the plot of the percentage inhibition of absorbance versus sample concentration was divided by the gradient of the plot for Trolox to give TEAC at a specific time. A calibration curve was prepared with different concentrations of Trolox to calculate TEAC (Murcia et al., 2002).

For DPPH radical scavenging capacity of samples, 0.1 ml prepared supernatant or 1.5 ml of sodium phosphate buffer (control; 0.1 M, pH 7.0, containing 1% (w/v) Triton X-100) and 1.5 ml DPPH (100 μM) solution were mixed, shaken vigorously, left in the dark at room temperature for 30 min and the decrease in absorbance at 517 nm was measured. The percentage of DPPH decrease in absorbance of the sample relative to the control was calculated using the equation:

$$\text{AA } (\%) = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \text{ x } 100$$

Inhibition (%) was calculated according to trolox calibration curve as "μmol Trolox equivalent per gram of sample."

For ferric reducing/antioxidant power (FRAP) assay, after appropriate dilutions, 200 μl of samples were mixed with 1.8 ml of the ferric tripyridyl triazine (TPTZ) reagent (prepared by mixing 300 mmole/L acetate buffer, pH 3.6; 8 mmole/L, 2, 4, 6-tripyridyl-5-triazine in 30 mmole/L HCl; 20 mmole/L $FeCl_3$ in the ratio of 10:1:1). The mixture was incubated at 37°C for 10 min, centrifuged and the TPTZ complex formed with the reduced ferrous ions was measured on the supernatant at 593 nm against the solvent blank. Results were calculated from a standard scale of ferrous sulfate (Sigma Aldrich, France) ranging from 30 to 500 μmol/L Fe^{2+}.

2.6 | Statistical analysis

The experiments were carried out in three different batches of fermented milks ($n = 3$) throughout refrigerated storage. All the data obtained were subjected to statistical analysis using analysis of variance (ANOVA, SPSS 14.0), followed by Duncan's test for mean comparison. The criterion for statistical significance was $p < .01$ and $p < .05$.

3 | RESULTS AND DISCUSSION

Ensuring a high viability and metabolic activity of probiotic bacteria during the production as well as over the predicted shelf life is important for any probiotic product to be preferred by the consumers. Although there is no world-wide agreement on the minimum viable probiotic cells per gram or milliliter of probiotic product, it is generally accepted that probiotic bacteria must arrive viable and active to different parts of intestine, adhere and colonize. Apart from the viability of probiotics in products until the time of consumption, their survival in food matrices after exposure to gastrointestinal tract (GIT) conditions is the most critical parameter as it determines their health efficiency (Kurmann & Rasic, 1991). So far, only a few studies have been conducted to define the effective dose of probiotic strains, but it is generally accepted that a dose of 10^9–10^{10} cells per day is necessary for optimal functionality (Saarela, Mogensen, Fonden, Matto, & Mattila-Sandholm, 2000). Therefore, the presence of probiotic bacteria at minimum levels of 10^6–10^7 cfu/ml or cfu/g is recommended in functional foods as well

TABLE 1 Viability and growth proportion index (GPI) of probiotic microorganisms in different treatments at the end of fermentation

Probiotic fermented milk	Viable counts during storage (log_{10} cfu/g)				GPI 14				GPI 21		
	0	7	14	21	GPI 0	GPI 7	Day 0	Day 7	Day 0	Day 7	Day 14
LAY	9.59^{aA}	8.58^{bB}	8.60^{bB}	7.30^{cC}	-	0.89	0.90	1.00	0.76	0.86	0.85
LRY	9.30^{bA}	8.85^{aC}	9.18^{aB}	8.72^{aD}	-	0.95	0.99	1.04	0.94	0.99	0.95
BLY	9.60^{aA}	8.70^{abB}	8.46^{cC}	7.70^{bD}	-	0.91	0.88	0.97	0.80	0.89	0.91

Different superscript lowercase letters denote significant differences ($p < .01$) between different probiotic bacteria.
Different superscripts capital letters denote significant differences ($p < .01$) between different times.
LAY = *L. acidophilus* in fermented milks supplemented with chestnut flour; LRY = *L. rhamnosus* in fermented milks supplemented with chestnut flour; BLY = *B. lactis* in fermented milks supplemented with chestnut flour.

as their daily intake (Korbekandi, Mortazavian, & Iravani, 2011). Table 1 shows the viability and growth proportion index (GPI) of probiotic microorganisms in chestnut-fermented skim milk at the end of fermentation and over 21 days of refrigerated storage per 7 day intervals. The viability and GPI of *L. rhamnosus* were significantly higher than those of *L. acidophilus* and *B. lactis* in all chestnut-fermented skim milk samples during storage. There are some reports stating that prebiotic compounds addition into fermented milk products stimulated the intestinal viability of probiotics as well as the viability of probiotics in fermented milks (Heydari et al., 2011; Nobakhti, Ehsani, Mousavi, & Mortazavian, 2008).

Even though the viable counts of *L. acidophilus*, *L. rhamnosus*, and *B. lactis* showed a decrease throughout the whole storage period, it was observed that the populations of all probiotic cultures were higher than recommended satisfactory levels with final counts of 7.30, 8.72, and 7.70 log_{10} cfu/g, respectively, ($p < .01$). The survival of *L. acidophilus* and *B. lactis* decreased about 2 log cycles or more, whereas for *L. rhamnosus* it was higher than 8.00 log_{10} cfu/g and decreased about1 log cycle at 21 days of storage (Table 1). Mortazavian et al. (2008) found out that the survival of *B. lactis* decreased about 2 log cycles or more in fermented skim milk drink supplemented with *L. acidophilus* LA-5 and *B. lactis* BB-12 during 21 days of refrigerated storage. In another research, Christopher, Reddy, and Venkateswarlu (2009) mentioned that viable counts of *B. bifidum* decreased about 2 log cycles from the first day to the 21st day. Comparison of the survival of probiotic strains used in the present study during 21 days of storage with previous related studies revealed that the loss rates of probiotic cells were mostly < 2 log or about 1 log (Table 1).

Many inter-related factors influence the survival of probiotic microorganisms during production and storage, that is, pH, titratable acidity, strains of probiotic bacteria, rate and proportion of inoculation, fermentation type, molecular oxygen, redox potential, hydrogen peroxide, supplementation of milk with nutrients, bacteriocins, microbial competitions, incubation temperature, storage temperature. Researchers have indicated that the tolerance of probiotics both to the product and to the consumer is species- and strain-specific (strain-dependent) (Godward et al., 2000; Talwalker & Kailasapathy, 2004; Tamime, Saarela, Korslund-Sondergaard, Mistry, & Shah, 2005).

The probiotic cells could be subjected to the enhanced antagonistic effects of starter bacteria when used in a combination for production of yogurt and fermented milk, especially *L. delbrueckii* ssp. *bulgaricus* that results in low pH and relatively high titratable acidity, leading to high loss rates of viable probiotic counts (Samona & Robinson, 2007). Yogurt bacteria can suppress probiotics during yogurt storage via "post-acidification" process which is noticeably intensified in temperatures of more than 5°C (Ferdousi et al., 2013). Mortazavian, Khosrokhvar, Rastegar, and Mortazaei (2010) and Shafiee et al. (2010a) both reported that in a commercial culture containing *Lactobacillus acidophilus* LA-5, *Bifidobacterium animalis* subsp. *lactis* BB-12 and yogurt bacteria, at pH 4.2, probiotic bacteria would shift to their mid-stationary phase and below pH 4.2, starter bacteria enter late stationary or death phase. Therefore, the high viability of probiotic cultures in the present study could be a result of using single-strain culture for production and addition of chestnut flour.

It has been proven that the tolerance of cells to detrimental environmental conditions such as acidic conditions and molecular oxygen is strain specific (Korbekandi et al., 2011). Fermentation process is one of the most limiting factors for the viability of *Bifidobacteria* in milk products. *Bifidobacteria* have shown to be highly sensitive to low pH values, although the acid tolerance varied greatly depending on the species and strains (Martin & Chou, 1992; Sanz, 2007). Similar to Tamime et al. (2005) who reported that *B. animalis* ssp. *lactis* had better survival than other *Bifidobacterial* species in lower pH values, we observed *B. lactis* showed sufficient viable counts at the end of fermentation and throughout storage, indicating the maintenance of the recommended level for potential health benefits to the consumer (Table 1).

Table 1 represents growth proportion index (GPI) in different treatments during 21 days storage. The GPI for all strains at the end of storage ranged between 0.85 and 0.95 and the highest GPI for all strains was in 14th day of storage (Table 1). The change in viable counts of probiotics was significant during storage, however, the counts were still higher than satisfactory therapeutic levels. The decrease in viable counts varied due to the probiotic strain used as a result of different sensitivity to environmental stresses of these bacteria such as low pH and high titratable acidity. Comparing the GPI of probiotic cultures in the current study with previous related studies performed with commercial probiotics, revealed that the strains used were significantly more resistant to low pH and their populations were mostly higher than minimum therapeutic level (Shafiee et al., 2010a; Heydari et al.,

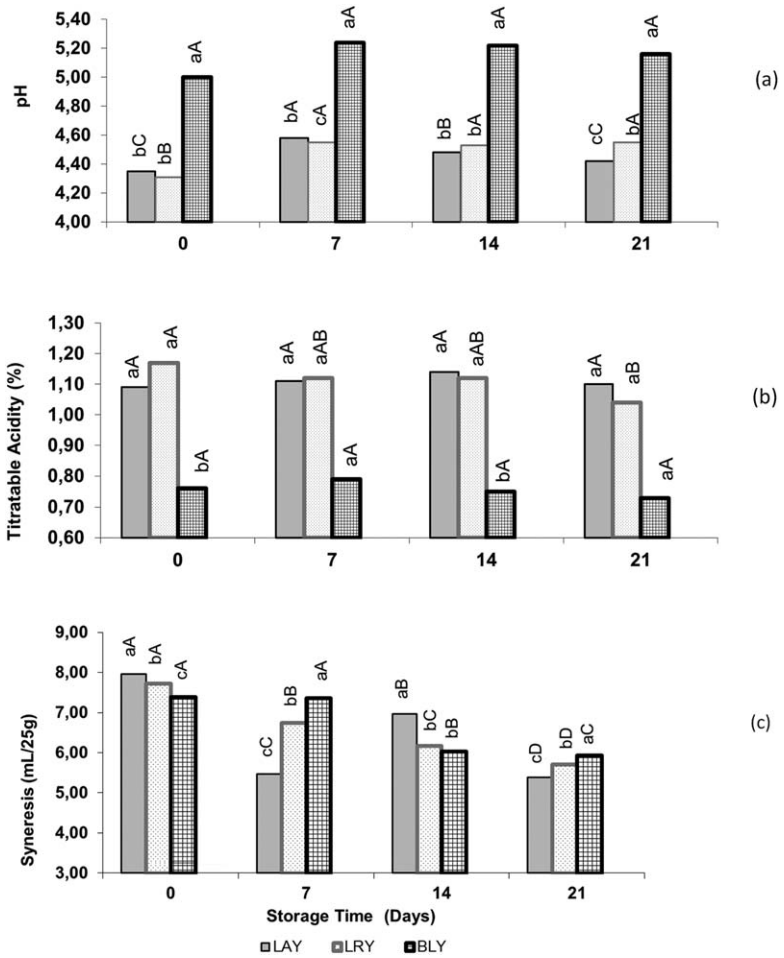

FIGURE 1 Changes in pH (a) and titratable acidity (LA %) (b) and syneresis (c) values during storage. Different superscript lowercase letters denote significant differences ($p < .01$) between different probiotic bacteria. Different superscripts capital letters denote significant differences ($p < .01$) between different times. LAY, *L. acidophilus* in fermented milks supplemented with chestnut flour. LRY, *L. rhamnosus* in fermented milks supplemented with chestnut flour. BLY, *B. lactis* in fermented milks supplemented with chestnut flour

2011; Mortazavian, Ghorbanipour, Mohammadifar, & Mohammadi, 2011).

The pH and titratable acidity values in probiotic chestnut-fermented skim milk did vary depending on the strain used ($p < .01$). The initial pH (day 0) ranged between 4.31 and 5.00 and the final pH ranged from 4.42 to 5.16 in all samples (Figure1a). The initial titratable acidity changed within 0.76–1.17% and final acidity from 0.73 to 1.10% due to the metabolic activity of cultures during refrigerated storage (Figure 1b). The buffering capacity of the product itself, prebiotic and the strain used can result in considerably slow decline in pH through refrigerated storage. Depending on the probiotic strain used the acid production was influenced by chestnut flour addition. Unlike *Lactobacillus* species, *Bifidobacteria* are known to be fastidious organisms that grow poorly in dairy products. The slow acid production and lower decline in pH values of fermented skim milk with *B. lactis* have been attributed to its requirement for specific nutrients in growth media such as free amino acids and peptides (Rybka & Fleet, 1997; Sahadeva et al., 2011).

Syneresis is a common defect in fermented dairy products and is generally defined as the separation of aqueous phase from continuous phase or gel network (Gauche, Tomazi, Barreto, Ogliari, & Bordignon-Luiz, 2009). The initial syneresis values for LAY, LRY, and BLY were 7.96, 7.72, and 7.39, whereas the final syneresis values were 5.38, 5.71, and 5.93, respectively (Figure 1c). The highest syneresis at the end of the storage was determined in *B. lactis*, whereas the lowest value was obtained in *L. acidophilus*. The effect of storage time on syneresis revealed that the value of syneresis decreased throughout refrigerated storage. It could be said that the addition of chestnut flour, having high dietary fiber, improved the gel network of the fermented milks due to casein-particle aggregation leading to gelation, resulting in higher viscosity and lower syneresis levels. This may arise from high water binding capacity of oligosaccharides which are high in chestnut flour. The oligosaccharides may also reduce free releasable water, slightly increase water binding capacity of the molecules (Radi, Niakousari, & Amiri, 2009) and influence the elastic character of the gel, making the yogurt less susceptible to rupture. Lucey, Munro, and Singh (1998) stated that rate of syneresis is directly related to the acidity and therefore is inversely related to pH. In the present research, we observed that syneresis was higher in BLY compared to LAY and LRY at the end of storage due to lower acidity and higher pH levels (Figure 1a–c).

TABLE 2 Total phenolic content and antioxidant capacity of fermented milks supplemented with chestnut flour and different probiotic bacteria

Probiotic fermented milk	Total phenolic content (mg GAE/100 g dry weight)	Total antioxidant capacity (mg Trolox/100 ml)		
		TEAC	DPPH	FRAP
LAY	60.174[b]	12.759[b]	5.874[b]	14.350[a]
LRY	51.403[c]	12.454[c]	6.073[a]	8.665[c]
BLY	62.367[a]	13.312[a]	5.897[b]	13.993[b]

Different superscript lowercase letters denote significant differences ($p < .01$) between different probiotic bacteria.
LAY = *L. acidophilus* in fermented milks supplemented with chestnut flour; LRY = *L. rhamnosus* in fermented milks supplemented with chestnut flour; BLY = *B. lactis* in fermented milks supplemented with chestnut flour.

Although oxidation has not been considered an important problem for fermented milks due to refrigerated storage, lipids may be subjected to oxidative deterioration. Among the factors reported to influence the survival of *L. acidophilus* and *Bifidobacteria* exposure to dissolved oxygen during manufacture and storage is considered most significant in reducing their viability in fermented milk products since most probiotic bacteria are classified as micro aerophilic and strictly anaerobic (Klaver, Kingma, & Weerkamp, 1993). Fermented milks contain high levels of oxygen, which is incorporated during the various homogenization, mixing and agitation steps of manufacture. Additionally, oxygen diffuses through the packaging material during storage (Ainsworth & Gillespie, 2007). Aerobic bacteria can completely reduce oxygen to water, however, in probiotic bacteria the oxygen-scavenging system is either reduced or completely absent. Hence the lack of an electron-transport chain results in the incomplete reduction of oxygen to hydrogen peroxide. Additionally, probiotics do not possess catalase that is essential for the decomposition of hydrogen peroxide. Consequently, exposure to oxygen causes the accumulation of toxic oxygenic metabolites, termed as oxygen toxicity, such as superoxide anion (O_2^-), hydroxyl radical (OH^-), hydrogen peroxide (H_2O_2) in the cell, which lead to cell death and viability loss of probiotics (Brunner, Spillman, & Puhan, 1993; Klaver et al., 1993). *Bifidobacterium* spp. is generally considered more vulnerable than *Lactobacillus* spp. to oxygen toxicity due to their strict anaerobic nature.

Prebiotics, non-viable food components that confers a health benefit on the host associated with modulation of the microbiota, can be used to improve the viability of probiotic bacteria in milk products as being nutritive substrates, and can exhibit antioxidant activity probably due to the presence of their phenolic compounds. Total antioxidant capacity is mainly attributed to phenolic compounds; however, some vitamins, minerals and other compounds contribute to it.

Chestnut flour has gained notable interest on account of its composition. Besides being gluten free, chestnut flour with high-quality proteins, essential amino acids (4–7%), a relatively high amount of sugar (20–32%), starch (50–60%), dietary fiber (4–10%), a low amount of fat (2–4%), some minerals (i.e., potassium, phosphorous and magnesium) and vitamins E and B (Yildiz et al., 2009; Demirkesen, Mert, Sumnu, & Sahin, 2010) provides not only health and nutritional benefits but also some functional properties. Its fiber content is responsible for the emulsifying, stabilizing, texturizing and thickening properties, while the sugar content improves the color and flavor properties of the gluten-free products. Thus it could serve as an alternative prebiotic with its fiber content and due to the phenolic compounds; it can act as an oxygen scavenger when incorporated in probiotic food formulas by maintaining low oxidation-reduction potential necessary for the viability of probiotic bacteria.

Neri, Dimitri, and Sacchetti (2010) reported that the total phenolic contents of raw chestnuts were 112.06 mg GAE/g DM for three Italian sweet chestnut ecotypes, and De Vasconcelos, Bennett, Rosa, and De Ferreira-Cardoso (2007) found phenolic content as 15.80 mg GAE/g DM for chestnut samples that were grown North East Portugal. In their study Otles and Selek (2012) stated that the total phenolic contents of chestnuts procured from 16 provinces in Turkey varied between 5 and 32.82 mg GAE/g DM, however, there were no significant differences in terms of total antioxidant capacity. The levels of phenolics in chestnuts might be influenced by environmental factors, cultivar type, location, soil composition, and maturity level (Wakeling, Mason, D'arcy, & Caffin, 2001; Barros, Nunes, Gonçalves, Bennett, & Silva, 2011).

Table 2 shows the total phenolic contents of probiotic fermented milk samples with chestnut flour. It was observed that the strain used was significantly effective on phenolic contents ($p < .01$). BLY had the highest phenolic content whereas the lowest was in LRY. The difference in phenolic contents might be attributed to the enzymes present in probiotic strain used such as β-glucosidase, *p*-coumaric acid decarboxylase, decarboxylase which may help in degrading certain phenolic compounds.

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\cdot -}$), hydroxyl (·OH), peroxyl (ROO·), and alkoxyl radicals (RO·), hydrogen peroxide (H_2O_2), and singlet oxygen ($O_2^1\Delta g$) may attack biological macromolecules, giving rise to protein, lipid, and DNA damage, cell aging, oxidative stress-originated diseases (e.g., cardiovascular and neurodegenerative diseases), and cancer. Antioxidants, either exogenous or endogenous, are vital substances which possess the ability to scavenge or quench ROS and reactive nitrogen species (RNS) in order to protect the body from the potent injuries caused by these radicals. Measuring the antioxidant capacity of foods by several detection methods is carried out for the meaningful comparison of the antioxidant potential. Table 2 shows the results of the antioxidant capacity of the samples. For the determination of antioxidant properties we have chosen three methods, which allowed us to estimate, both the ability to reduce prooxidant metal ions (FRAP assay), and radical scavenging activity (TEAC and DPPH assays). The DPPH and FRAP methods are widely

used to investigate the shelf life of food products, for example, as a sensitive tool for monitoring of the oxidation changes in dairy products during storage (Jimenez, Murcia, Parras, & Martinez-Tome, 2008; Zulueta et al., 2009; Ferdousi et al., 2013). The $ABTS^{\cdot-}$ radical solution was generated from ABAP and $ABTS^{2-}$ at 60°C and the absorbance was measured at 734 nm. Subsequently samples were added and the decrease in absorption was measured. A TEAC value can be assigned to all compounds capable of scavenging the $ABTS^{\cdot-}$ by comparing their scavenging capacity with that of Trolox (vitamin E analogue, water-soluble). Quantitative evaluation of the antioxidant capacity using TEAC can be used to provide a ranking order of antioxidants.

According to the data for the ABTS radical cation scavenging potential of fermented milks all samples showed a high scavenging potential against ABTS radical cation. The scavenging potential was significantly different depending on the strain used and ranged from 12.454 to 13.312 mg Trolox/100 ml ($p < .01$). Among the probiotic strains used the maximum activity was determined in BLY followed by LAY and LRY. Jimenez et al. (2008) analyzed the antioxidant activity of several dairy products, that is, yogurt enriched with green tea and lemon, fermented milk, yogurt with strawberry pulp, "low-calorie" yogurt with inulin and milk enriched with vitamin E and their ingredients. They observed that the products, even with lowest TEAC values, could be considered as very good $ABTS^{\cdot-}$ scavengers. In decreasing order, yogurt enriched with green tea and lemon, yogurt with strawberry pulp and low-calorie yogurt with inulin produced the best TEAC results from other dairy products, such as fermented milk and milk enriched with vitamin E. It is known that TEAC is partially dependent on the number of free phenolic hydroxyls, and is also affected by the type of linkage structures in the food matrix.

DPPH, a relatively stable free organic radical, is commonly used to evaluate antioxidant capacity of a particular compound due to utilization from its feature to be scavenged by electron-donating substances such as antioxidants. It has widespread use because of the ease and convenience of the reaction (Lee, Kim, Lee, & Lee, 2003).The DPPH value of LRY (6.083 mg Trolox/100 ml) represents the highest value compared with LAY and BLY ($p < .01$).

Considering the results of TEAC and DPPH assays for fermented milk samples the antioxidant capacity may be attributed to the fortification with chestnut flour, due to its content particularly the phenolics, and the products formed through the metabolic activity of probiotic bacteria that could probably serve as antioxidants.

The FRAP assay measures the absorption at 593 nm of intense blue ferrous (Fe^{2+}) form of ferric-tripyridyltriazine (Fe^{3+}–TPTZ) complex that is formed at low pH in the presence of antioxidant substances in the sample, and is suitable for measuring the antioxidant activity of substances characterized with half-reaction redox potential below 0.7 V (Benzie & Strain, 1996). In the present study, we compared the obtained value from FRAP assay with that measured for the Trolox, a standard antioxidant substance. Among milk components, urate, ascorbate, α-tocopherol and bilirubin were reported to have ferric reducing ability, whereas the activity of other potential antioxidant substances like serum proteins, glutathione and lipoic acid could not be detected during the FRAP assay. It suggests that this assay measures practically only non-protein antioxidant capacity (Chen, Lindmark-Mansson, Gorton, & Akesson, 2003).

Results given in Table 2 indicated that the maximum ferric reducing power activity was in the LAY (14.350 mg Trolox/100 ml) followed by that of BLY (13.993 mg Trolox/100 ml) and was lowest in LRY (8.665 mg Trolox/100 ml).

The Folin-Ciocalteu method tends to overestimate the total phenolic content as it is a non-specific assay in which many other components may interact with each other (Ainsworth & Gillespie, 2007; Wang et al., 2003). According to the results obtained a positive relationship was found between the total phenolic contents and the antioxidant properties. We could conclude that fermented probiotic milks had antioxidant properties which depend mainly on the method of antioxidant determination, ingredients (i.e., chestnut flour) and the type of probiotic bacteria.

4 | CONCLUSION

Most of the beneficial effects of chestnuts are attributed to its non-digestible ingredients (NDIs) with low molecular weight carbohydrates that are intermediate in nature between simple sugars and water extractable materials. Lactobacilli and Bifidobacteria prefer NDIs and utilize them by fermentation, resulting in short chain fatty acids, therefore serving as an energy source. The greatest viability of probiotic bacteria was obtained in *L. rhamnosus*, while the lowest viability was observed in *L. acidophilus*. It is generally accepted that in order to appreciate the therapeutic effects, the probiotic foods should have a minimum concentration of >6 $\log_{10}$ cfu viable cells per ml or g of product at the point of consumption. The probiotic-containing chestnut-fermented milks developed as part of the present study contained levels up to 7 $\log_{10}$ cfu/g, thus satisfying these criteria for a probiotic food product. The antioxidative potential of probiotic fermented milks, evaluated by TEAC, DPPH and FRAP assays, suggested that chestnut flour may serve as a new antioxidant source for functional dairy food applications. In this research, it was observed that chestnut flour could stimulate the viability of probiotic bacteria since it has substrates such as oligosaccharides, however, to be designated as a prebiotic, that complements the metabolic activities of beneficiary bacteria in the gut, the studies should focus on basic and mechanistic studies using in vitro, in vivo, ex vivo, and in silico models.

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