NFS Journal 29 (2022) 16–25

Contents lists available at ScienceDirect

NFS Journal

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

Original article

Development of probiotic yoghurts with high protein content by ultrafiltration

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ARTICLE INFO

Keywords:
Bifidobacterium animalis
Fermented milk
Ultrafiltered milk
Greek-style yoghurt
High protein content

ABSTRACT

The ultrafiltration technique (UF) was used to achieve skim milk protein concentration and develop probiotic Greek-style yoghurt. Two yoghurts were prepared from ultrafiltered milk (with concentration factors 3 and 1.5, CF1 and CF2 samples, respectively) added with probiotic (*Bifidobacterium animalis* subsp. lactis) and compared to a control yoghurt. Proximal composition and physicochemical analyses were carried out after one day of processing. The microbiological viability of the starter and probiotic cultures, physicochemical composition, and technological properties of yoghurts during 28 days of storage were also evaluated. CF1 yoghurts showed the highest increase in protein content compared to the yoghurt CF2 and control. Consequently, this feature favoured lower post-acidification, better texture, higher water holding capacity, and absence of syneresis. Ultrafiltration was also satisfactory for enhancing the mineral content, especially calcium, phosphorus, and zinc. The probiotic bacteria remained viable throughout the refrigerated storage period, hence the use of ultrafiltered milk proved to be appropriate for producing high protein content probiotic yoghurts.

1. Introduction

Dairy products represent a promising market for the supply of processed foods with health benefits. For thousands of years, different cultures worldwide have consumed these products due to their natural wealth of essential nutrients and energy for maintaining good health [1]. Yoghurt is one of the most popular fermented dairy products, whose consumption has grown worldwide due to the nutraceutical effect and the properties of convenience, the practicality of consumption, and sensory profile, such as pleasant texture, aroma, and flavour [2]. It constitutes an excellent matrix for the application of technological innovations.

The FAO/WHO Commission of the *Codex Alimentarius* defines yoghurt as fermented milk obtained through the action of symbiotic cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* [3]. Its composition varies according to the addition of cream, natural milk derivatives (skim milk powder, whey concentrate, or caseinates), and the application of techniques to concentrate milk or milk proteins [4]. Due to the impact of health and well-being trends, yoghurts are included in the category whose consumption will rise in the coming years [1].

Traditionally, it is necessary to increase the solid contents of milk for yoghurt production; therefore, it is common to fortify the milk with 3 to 4% skim milk powder (SMP). However, the addition of SMP is limited since high levels of SMP can cause sensory defects and elevate the lactose content, resulting in a more acidic product [5,6]. This practice also limits the production of clean label yoghurt made from genuine goat, sheep, donkey, buffalo, and other milk, when the introduction of proteins from another animal species is not desirable. Furthermore, the spray drying technique (used for producing milk powder) is a thermal process that may result in nutritional losses [7]. Dry heating leads to chemical modifications of amino acids, such as oxidation and glycation, reducing their bioavailability and functionality [8]. An alternative for fortifying milk in yoghurt production is ultrafiltration [4,9]. Proteins

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https://doi.org/10.1016/j.nfs.2022.09.003
Received 23 February 2022; Received in revised form 20 September 2022; Accepted 26 September 2022
Available online 2 October 2022

concentrated by ultrafiltration have better nutritional value than products made by the traditional method and avoid the addition of solids from other milk. Another advantage is that ultrafiltered milk can contribute to a reduction in lactose levels when compared to non-processed milk [10,11].

Milk and dairy products, such as powdered milk, yoghurts, cheese, and ice cream, are excellent vehicles for the incorporation of probiotic microorganisms. Traditional yoghurts recipes have been reformulated to include strains of L. *acidophilus* and species of *Bifidobacterium* in addition to conventional yoghurt microorganisms [12].

The International Scientific Association for Probiotics and Prebiotics (ISAAP) defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [13]. Two benefits are often associated with probiotics: (1) supporting a healthy digestive tract and immune system (2) preventing allergic and infectious diseases [13]. The main probiotic species added to fermented products belong to *Lactobacillus* and *Bificibacterium* genera. The consumption of *Bifidobacterium animalis* subsp. *lactis* has been associated with a reduction of upper respiratory tract infections, positive modulation of the intestinal microbiota, and improvement of the immune system [14]. To achieve health-promoting status, probiotics must reach the intestine viable and in adequate quantities (around 6 to 7 log CFU/g of product) [15,16]. Depending on the amount ingested and taking into account the storage effect on the viability of the probiotic, the daily intake of 10^8–10^9 CFU probiotic microorganisms is essential to accomplish probiotic action in the human organism [11,17].

In response to consumers' demand for healthy products and aiming at preserving the integrity of milk proteins, Greek-style probiotic yoghurts were produced with *Bifidobacterium animalis* subsp. *lactis* from ultrafiltered high-protein milk. Two protein concentration factors were pursued, and the results compared to the traditional production process that uses fluid milk fortified with skim milk powder. The evaluation of the products was carried out during the period of cold storage to study the effect of ultrafiltration on the technological, microbiological, physical, physicochemical, and nutritional characteristics of yoghurts.

2. Material and methods

2.1. Material

Pasteurised skim milk (Xandô, Brazil) and Molico® skim milk powder (Nestlé, Brazil) were acquired from a local market (Campinas, São Paulo, Brazil). Conventional yoghurt starter milk culture with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (Lyofast Y450B from Sacco Brazil,) and probiotic culture *Bifidobacterium animalis* subsp. *lactis* - BB12®, (Chr. Hansen, Brazil) were purchased directly from the manufacturer.

2.2. Preparation of starter and probiotic cultures

The starter culture was activated in one litre of sterile whole milk. Aliquots of 20 mL were divided into 50 mL centrifuge tubes and stored at −20 °C for yoghurt production. The total lactic bacteria count in the aliquot was 10^9 CFU/mL. The BB12 probiotic culture (1 g) was added directly to 2 L of milk from each formulation, along with 40 mL of the yoghurt starter culture. According to the manufacturer's specifications, the probiotic culture contained 10^{11} CFU/g.

2.3. Ultrafiltration procedure for milk and yoghurt production

The ultrafiltration experiments were performed on a laboratory-scale system (Pellicon® Cassette Acrylic Holder and Assembly, Merck, Germany), operating by tangential flow filtration, with cassette-type filters and a molecular weight cut off 10 kDa. The pressure used at the system's inlet was between 20 and 30 PSI. The skim liquid milk was concentrated in two concentration factors: the ratio of the initial volume (milk) to the final volume of retentate, labeled CF1 and CF2. For the concentration of CF1 milk, 6 L l of skim milk were used to obtain 2 L of retentate, and for the concentration of CF2 milk, 6 L l of skim milk were used to obtain 4.1 L of retentate. Thus, CF1 = 3 and CF2 = 1.5.

In this pilot study, one batch of three yoghurts formulations was manufactured at a laboratory scale: the control yoghurt was prepared using 2 L of pasteurised skim milk added with 70 g of skim milk powder to obtain an increase in the total solids content (from 9% to ~13%). The two formulations of test yoghurts were produced with CF1 and CF2 ultrafiltered milks, called CF1 and CF2 yoghurts, respectively. Briefly, milk samples were maintained in a thermostatic bath at 85 °C for 30 min and cooled to 43 °C. Then, 40 mL of the starter culture and one gram of the probiotic culture (BB12) were added to 2 L of milk for each treatment. The three yoghurt formulations were incubated at 43 °C, and the pH was measured during the fermentation period until about pH 4.5 to complete the gel formation. The yoghurt samples were stored for 28 days in a BOD chamber at 8 ± 2 °C.

2.4. Physicochemical analysis

Standard pasteurised skim milk, skim milk + powdered skim milk (used for the control yoghurt), retentates CF1 and CF2, and permeates CF1 and CF2 were evaluated for total dry extract, ash content, total protein, fat (lipids), mineral content (Ca and P) and lactose. Control (standard), CF1, and CF2 yoghurts were subjected to chemical analyses after one day of manufacture. The pH was measured during product fermentation and cold storage (8.0 ± 2,0 °C), together with titratable acidity, from the 1st to the 28th day, every 7 days. The analyses were performed, at least, in duplicate, except for lactose quantification (n = 1).

2.4.1. Proximal composition

Proximal composition was determined according to the official methods of AOAC International [18], and the factor of 6.38 was used for nitrogen-to-protein conversion. The sugar dosage was performed by high performance liquid chromatography (HPLC) [19], and total fat quantified as described by Bligh and Dyer [20].

2.4.2. Total and free amino acid content

The HPLC analysis of total and free amino acids was performed in control, CF1 and CF2 yoghurts (after 1, 14, and 28 days of cold storage), using a reverse phase C18 column (LUNA 100 Å / length of 4.6 mm × 250 mm in diameter from Phenomenex, Torrance, CA, USA), RP-HPLC system (Shimadzu Corporation, Kyoto, Japan), according to White et al. [21] and Hagen et al. [22]. Samples were compared with Thermo Scientific's amino acid standard (Rockford, IL, USA), using DL-2-aminobutyric acid (Sigma-Aldrich®, St. Louis, MO, USA) as an internal standard.

Proteolysis during storage was calculated by subtracting the sum of free amino acids/ 100 g of sample on day 1 from the total free amino acids/ 100 g of sample measured on days 14 and 28.

2.4.3. Titratable acidity and pH

Titratable acidity and pH were measured in samples homogenised at room temperature (25 ± 2,0 °C) [23]. Titratable acidity results were expressed as a percentage of lactic acid per 100 g of the product.

2.4.4. Mineral content

The mineral content in the milk samples (skim milk, skim milk + powdered skim milk, retentates (CF1 and CF2) was determined using an inductively coupled plasma optical emission spectrometer (ICP OES), model 5100 VDV Agilent Technologies (Tokyo, Japan), equipped with a 27 MHz solid state radio frequency (RF) source and nebuliser spray using argon as plasma (Air Liquid, Brazil). The quantification followed the method described by Price and Roos [24], expressed in mg /100 g.

2.5. Physical analysis

The analysis of syneresis, water holding capacity, and texture was determined one day after manufacture and every 7 days during the 28 days of storage of the yoghurts (control, CF1, and CF2). They were expressed as mean from three repetitions.

2.5.1. Syneresis and water holding capacity

To assess susceptibility to syneresis, 10 mL aliquots of sample were stored in conical bottom sterile test tubes (13 × 1.7 cm) at 8 ± 2 °C during the experiment period (28 days). The syneresis was measured in centimetres of desorption on the product surface [25].Approximately 5 g of each yoghurt formulation were centrifuged for 15 min at 2.470 ×*g* (CT 6000R, Cientec), at 10 °C. Water Holding Capacity (WHC) was calculated using the equation:

$$WHC\ (\%) = \frac{1 - W1}{W2} \times 100$$

where *W1* is the weight of the whey after centrifugation and *W2* is the weight of the yoghurt [26].

2.5.2. Texture

The texture of the yoghurts was determined using the Texture Analyzer TA-XT2 Plus (Stable Micro Systems, Godalming, England) with a 25 mm cylindrical probe [27]. The test was carried out with 80 g of yoghurt at 10 ± 0.5 °C. The samples were compressed by 20 mm from their original depth during the analysis. The probe speed was 0.5 mm/s during compression and 2 mm/s during pre-test and relaxation. The following parameters were recorded: firmness, consistency, and elasticity.

2.6. Microbiological analysis

Heat treated (85 °C for 30 min) milk samples (control, CF1, and CF2) were submitted to coliform enumeration at 30 °C and 45 °C [28,29], and moulds and yeast counting. The same microbiological analyses were performed for yoghurts samples (control, CF1, and CF2) after manufacture (1 day), plus selective counting of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and the probiotic *Bifidobacterium animalis* subsp. lactis at days 1, 7, 14, and 28 of cold storage. Moulds and yeasts analyses and starter bacteria counts were performed according to Frank and Youssef [30]. The probiotic microorganisms of the genus *Bifidobacterium* spp. were quantified according to the method of the International Dairy Federation [31].

2.7. Statistical analysis

The difference between the formulations and the effect of the storage time on the samples was accessed by analysis of variance (ANOVA), applying the Tukey test at a 5% of probability level. Data analysis was performed with Minitab software, version 16.1.1.

3. Results and discussion

3.1. Microbiological quality of the ingredients and yoghurts

Microbial count in the pasteurised samples was neglectable (< 3 MPN/mL for coliforms and < 10 CFU/mL for Moulds and yeasts), indicating that the heat treatment was efficient. Therefore, the sanitary practices during the yoghurts manufacturing were conducted correctly, assuring food safety. The microbiological counts for all ingredients and final products were in accordance with the limits established by the Brazilian legislation for milk and yoghurt, in force during the period of the production of the yoghurts [32,33]. Undesirable microbes can affect the quality and cause spoilage of dairy products [34]. Although antibacterial effect against *Escherichia coli* was found for Greek-style yoghurt produced by UF, its high nutritional value and enhanced buffering capacity (due to higher protein content) could potentially promote the growth of spoilage yeasts during storage than regular stirred yoghurts [35]. Thus, quality control of food ingredients and good manufacturing practices are essential for increasing products' shelf-life.

Table 1
Proximal composition (%) of skim milk, retentates and permeates of ultrafiltered milk,and yoghurts prepared with ultrafiltered milks (mean ± SD).

Sample		g/ 100 mL (%)				
		Total Solids	Lactose	Protein	Ash	Fat
Milk	Skim milk	9.24 ± 0.01^{d}	5.12	3.36 ± 0.04^{d}	0.73 ± 0.01^{d}	0.36 ± 0.01^{c}
	Skim milk + powdered skim milk*	12.16 ± 0.03^{b}	6.55	4.48 ± 0.01^{c}	1.04 ± 0.01^{b}	0.33 ± 0.00^{c}
	Retentate CF1	14.79 ± 0.06^{a}	5.30	8.14 ± 0.01^{a}	1.13 ± 0.01^{a}	0.59 ± 0.02^{a}
	Retentate CF2	11.31 ± 0.23^{c}	5.17	5.24 ± 0.05^{b}	0.89 ± 0.01^{c}	0.48 ± 0.02^{b}
Permeates	CF1	4.39 ± 0.01^{b}	–	0.14 ± 0.00^{a}	0.39 ± 0.01^{b}	0.04 ± 0.06^{a}
	CF2	5.40 ± 0.01^{a}	–	0.16 ± 0.03^{a}	0.45 ± 0.01^{a}	0.008 ± 0.01^{a}
Yoghurts	Control	11.35 ± 0.16^{b}	4.57	5.11 ± 0.17^{c}	1.04 ± 0.01^{b}	0.36 ± 0.01^{b}
	CF1	13.92 ± 0.09^{a}	3.19	8.48 ± 0.01^{a}	1.11 ± 0.01^{a}	1.40 ± 0.06^{a}
	CF2	10.79 ± 0.14^{c}	3.41	6.01 ± 0.10^{b}	0.45 ± 0.01^{c}	0.39 ± 0.01^{b}

* Used for the control yoghurt formulation. Control: standard yoghurt prepared with fluid skim milk + powdered skim milk; CF1: yoghurt prepared with ultrafiltered milk at a concentration factor of 3; CF2: yoghurt prepared with ultrafiltered milk at a concentration factor of 1.5. Means followed by the same letters in the same column, for the same type of sample, did not differ statistically ($p < 0.05$). All analyzes were performed in duplicate ($n = 2$) except for lactose ($n = 1$).

3.2. Fermentation process

During the fermentation curve of the three yoghurt formulations (control, CF1, and CF2), the pH lowering was quite similar for all the samples (data not shown). Thus, the differences in the milk composition (discussed in the next section) did not affect the drop in pH and the development of acidity during fermentation. The ideal pH (4.6–4.7) was reached in a time between 3.5 (CF2) and 4 h (CF1 and control) (data not shown). The short fermentation time has been related to the temperature and type of starter culture [4].

In a Greek-style yoghurt produced with a 10.6% protein UF milk, the fermentation period required to reach a pH level of 4.8 was 2.4 times longer than control yoghurt (4% protein) fermentation [35]. This effect was explained by the buffering capacity (BC) of proteins and other milk components concentrated by the UF. In our study, the yoghurts CF1 and CF2 did not present a significant difference in the fermentation time compared to the control yoghurt, probably because protein content was not high enough to delay the acidification process by buffering effect.

The BC of calcium and phosphate could also influence a prolonged fermentation time. According to Kim and coworkers [36], the equilibrium of colloidal calcium phosphate alters BC. When phosphate salts were added to milk, a "high-buffering yoghurt" was obtained, resulting

Table 2
Mineral content (mg/100 g) in skim milk and ultrafiltered milks (mean ± SD; $n = 3$).

Sample	Calcium	Phosphorus	Potassium	Sodium	Zinc	Iron	Magnesium
Skim milk	112.83 ± 0.92^{c}	95.12 ± 0.45^{d}	149.72 ± 0.78^{b}	41.87 ± 0.80^{b}	0.51 ± 0.01^{d}	$< 0.02 \pm 0.00^{c}$	10.02 ± 0.06^{d}
Skim milk + powdered skim milk*	169.04 ± 0.72^{b}	124.68 ± 0.33^{c}	191.12 ± 4.21^{a}	53.37 ± 1.47^{a}	0.65 ± 0.03^{c}	0.23 ± 0.01^{a}	17.91 ± 0.03^{a}
CF1 Milk	247.70 ± 1.90^{a}	174.07 ± 1.25^{a}	160.64 ± 3.03^{a}	44.45 ± 1.33^{b}	1.22 ± 0.00^{a}	0.03 ± 0.00^{b}	15.58 ± 0.13^{b}
CF2 Milk	168.10 ± 1.77^{b}	127.52 ± 1.18^{b}	151.74 ± 2.75^{b}	44.00 ± 0.95^{b}	0.79 ± 0.02^{b}	$< 0.02 \pm 0.00^{c}$	12.51 ± 0.03^{c}

* Used for the control yoghurt formulation. CF1 Milk: ultrafiltered milk at a concentration factor of 3; CF2 Milk: ultrafiltered milk at a concentration factor of 1.5. Values followed by the same letters in the same column did not differ statistically ($p < 0.05$).

in a lower speed of acid production, with an increase of 4 h in the fermentation time to reach pH 4.5 in comparison to control yoghurt. As shown in Table 1 and discussed later, calcium and phosphate contents were significantly higher in CF1. Nevertheless, the modification of chemical composition observed in milk concentrated by UF did not affect the fermentation process.

3.3. Physicochemical composition

The proximal composition of milk, retentates (CF1 and CF2), permeates, and yoghurts are given in Table 1. The different concentration factors (3 and 1.5) resulted in significantly different ($p < 0.05$) composition profiles in the retentates CF1 and CF2. All components in CF1 and CF2 were increased compared to pasteurised skim milk, except for lactose, which remained almost unaltered after the ultrafiltration process. The increase in protein and fat content was expected as the UF membrane separates compounds of lower molecular weight in the permeate, keeping the protein and fat in the retentate. However, the protein concentration in the retentate indicated that the CF reached only 2.4 ×, which is below the theoretical targeted CF of 3.0 ×. Protein concentration in CF1 permeate was 0.14 g/ 100 g and in CF2 was 0,16 ± 0,03 g/ 100 g, which is expected for a low cut-off ultrafiltration membrane (10 kDa). Although, lactose and mineral levels in CF1 were similar to those found by Valencia and coworkers [37], who successfully reached a 2.9 × concentration (a CF of 3.0 was pursued). The differences might be due to distinct ultrafiltration settings. Our results indicate the need for UF process optimisation, aiming for better performance.

Since there were differences among CF1 and CF2 milks, yoghurt prepared with the ultrafiltered milks also showed differences in their composition. Using CF1 for yoghurt manufacture yielded a product with higher amounts ($p < 0.05$) of protein, ash, fat, and total solids in comparison to the use of skim milk + skim powdered milk and the use of a lower concentration factor (CF2). Another successful strategy to reach the desirable high amounts of proteins in Greek-style yoghurts is milk enrichment with bovine colostrum. However, due to the colostrum's unique chemical composition and sensory attributes, preparing yoghurts with bovine colostrum resulted in reduced consumer acceptance [38]. Trained judges perceived sensory changes when analysing Greek-style yoghurt produced with ultrafiltered milk [39]. Yet, it remains unknown if regular consumers would be able to detect these alterations, contributing to a lower acceptance.

As expected, the lactose content and total solids in all yoghurts decreased compared to the respective milk used in their production. During fermentation, the bacteria in the starter culture use lactose as an energy source, metabolising this sugar to lactic acid. Thus, the decrease in lactose and the increase in lactic acid content indicate efficient lactose fermentation and metabolism [40].

3.4. Mineral content

The concentration factor positively influenced the amount of calcium, phosphorus, potassium, zinc, iron, and magnesium (Table 2). These minerals in CF1 milk were found in significantly higher concentrations ($p > 0.05$) than in CF2 and skimmed milk. Apparently, only sodium content is not affected by the ultrafiltration process. For this parameter, CF milks did not differ among themselves or in comparison to skim milk. Copper and manganese measurements for all samples were < 0.02 mg/ 100 g (data not shown).

Regarding the potassium content, CF1 reached similar amounts to those found in the control milk ($p > 0.05$). However, it is worth mentioning that, according to the manufacturer, the powdered skim milk used in the control yoghurt formulation is enriched with calcium, magnesium, and iron. This explains an iron content ten times higher in this sample than the non-supplemented milks.

The highest calcium, phosphorus, and zinc contents were observed in retained milk CF1. The calcium amount in CF2 was similar ($p > 0.05$) to the value in skim milk + powdered skim milk; however, ultrafiltration was able to increase the amount of phosphorus of CF2 in comparison to the non-filtered milk. In milk, two-thirds of the Ca content is bound to the casein micelle as $CaPO_4$ bridges. The remaining portion of the milk Ca is dissolved in the milk serum. During the UF processing, this Ca passes through the membrane into permeate [40]. Even though ultrafiltration usually removes most soluble Ca and P, ultrafiltered milks retained considerable amounts of those minerals, especially CF1. Therefore, milk derivatives prepared with ultrafiltered milk could result in dairy products with desirably higher quantities of essential minerals such as calcium, phosphorus, and zinc, with no changes in sodium content.

Table 3
pH and titratable acidity (g lactic acid/100 g yoghurt) during cold storage of yoghurts prepared with ultrafiltered milks (mean ± SD; n = 2).

Parameter	Yoghurt	Storage (days) 1	7	14	21	28
pH	Control	4.67 ± 0.00^{bA}	4.50 ± 0.06^{aB}	4.44 ± 0.04^{aBC}	4.32 ± 0.01^{aC}	4.19 ± 0.01^{bD}
	CF1	4.72 ± 0.00^{aA}	4.52 ± 0.01^{aB}	4.45 ± 0.04^{aBC}	4.37 ± 0.06^{aC}	4.36 ± 0.03^{aC}
	CF2	4.65 ± 0.00^{cA}	4.47 ± 0.01^{aB}	4.42 ± 0.00^{aC}	4.32 ± 0.0^{aD}	4.23 ± 0.01^{bE}
Titratable Acidity (g lactic acid/100 g yoghurt)	Control	0.77 ± 0.02^{aD}	0.90 ± 0.00^{bC}	1.11 ± 0.00^{bA}	0.93 ± 0.01^{bBC}	0.95 ± 0.01^{bB}
	CF1	0.83 ± 0.21^{aB}	1.17 ± 0.06^{aAB}	1.31 ± 0.04^{aA}	1.48 ± 0.02^{aAB}	1.23 ± 0.01^{aA}
	CF2	0.70 ± 0.05^{aB}	0.89 ± 0.02^{bA}	0.90 ± 0.00^{cA}	0.92 ± 0.01^{bA}	0.89 ± 0.01^{cA}

Control: standard yoghurt prepared with fluid skim milk + powdered skim milk; CF1: yoghurt prepared with ultrafiltered milk at a concentration factor of 3; CF2: yoghurt prepared with ultrafiltered milk at a concentration factor of 1.5. Values followed by the same lowercase letters in the same column or the same uppercase letters in the same row did not differ statistically ($p < 0.05$).

3.5. Titratable acidity and pH during storage

There was a reduction ($p < 0.05$) in pH over the storage time at 8 °C

Table 4
Total amino acids content during cold storage (1, 14 and 28 days) of yoghurts prepared with ultrafiltered milks.

Total Amino Acids	Control (g/ 100 g of sample)			CF1 (% of increase)[1]			CF2 (% of increase)[1]		
	Storage (days)								
	1	14	28	1	14	28	1	14	28
Aspartic Acid	0.43	0.41	0.41	69.77	70.73	78.05	16.28	19.51	19.51
Glutamic Acid	1.09	1.07	1.08	73.39	69.16	74.07	17.43	19.63	15.74
Serine	0.28	0.28	0.28	71.43	64.29	71.43	17.86	14.29	17.86
Glycine	0.10	0.10	0.10	70.00	60.00	60.00	10.00	10.00	20.00
Histidine	0.13	0.14	0.13	92.31	84.62	84.62	23.08	30.41	30.77
Arginine	0.17	0.17	0.17	82.35	70.59	82.35	23.53	23.53	23.53
Threonine	0.23	0.23	0.23	52.17	43.48	47.83	2.17	2.17	4.35
Alanine	0.17	0.17	0.17	64.71	58.82	70.59	17.65	17.65	17.65
Proline	0.47	0.47	0.48	23.40	44.68	77.08	19.15	19.15	18.75
Tyrosine	0.24	0.24	0.24	87.50	79.17	83.33	16.67	16.67	16.67
Valine	0.31	0.31	0.31	77.42	70.97	70.97	19.35	19.35	16.13
Methionine	0.13	0.13	0.13	76.92	69.23	69.23	23.08	15.38	15.38
Cystine	0.07	0.06	0.07	71.43	71.88	71.43	14.29	15.00	14.29
Isoleucine	0.26	0.26	0.26	73.08	65.38	69.23	15.38	15.38	15.38
Leucine	0.47	0.47	0.47	76.60	68.09	74.47	17.02	17.02	17.02
Phenylalanine	0.24	0.24	0.24	70.83	62.50	62.50	8.33	8.33	8.33
Lysine	0.37	0.37	0.37	83.78	75.68	83.78	24.32	24.32	21.62

[1] Compared to the control sample. Control: standard yoghurt prepared with fluid skim milk + powdered skim milk; CF1: yoghurt prepared with ultrafiltered milk at a concentration factor of 3; CF2: yoghurt prepared with ultrafiltered milk at a concentration factor of 1.5.

in all formulations (Table 3). This reduction in milk pH affects the dissociation of casein micelle resulting in the formation of a three-dimensional protein network [40]. The initial pH differed ($p < 0.05$) between the formulations after 1 day of storage. On the 7th day of storage, all three yoghurts presented similar pH values ($p > 0.05$), displaying the same behaviour until the 21st day. After 28 days of storage, CF1 yoghurt had the highest pH among the samples, whereas control and CF2 yoghurts did not differ. During cold storage, decreases in the pH of the yoghurts are frequently observed. The increased acidity results from post-acidification of the products are related to the continuity of fermentation by lactic acid bacteria during the storage period, with the production of lactic acid [41,42], especially by *L. bulgaricus* [43].

The post-acidification phenomenon or post-fermentation acidification is undesired, shortening the shelf-life of fermented dairy products and provoking technological defects [44]. The pH drop on treatments from the 1st to the 28th day of storage was 0.48 units for the control sample, 0.42 units for CF2 yoghurt, and 0.36 units for CF1 yoghurt. Thus, the use of ultrafiltered milk for yoghurt production resulted in weaker post-acidification. Considering the increasing consumers' preference for mild fermented products [44], ultrafiltered milk should be explored to mitigate post-acidification in Greek-style yoghurt.

As expected, an increase in the titratable acidity over time was observed in all formulations ($p < 0.05$) (Table 3). After 1 day of storage, the titratable acidity was similar ($p > 0.05$) for all yoghurts. Control and CF2 yoghurts did not differ in terms of titratable acidity at the 7th and 21st days of storage. At the time points 14 and 28 days, the titratable acidity of the formulations significantly differed, with higher levels of lactic acid/100 g yoghurt found for CF1. Despite the variation, the acidity values of all samples along cold storage are in accordance with the Brazilian regulatory standards for yoghurts, which is 0.6–1.5 g of lactic acid per 100 g of product [33]. Probiotic yoghurts developed by Sah and coworkers [45] reached, on the 1st day of storage, higher titratable acidity (1,02 g lactic acid/ 100 g yoghurt) than the samples of the present study; however, a similar increase in acidification was

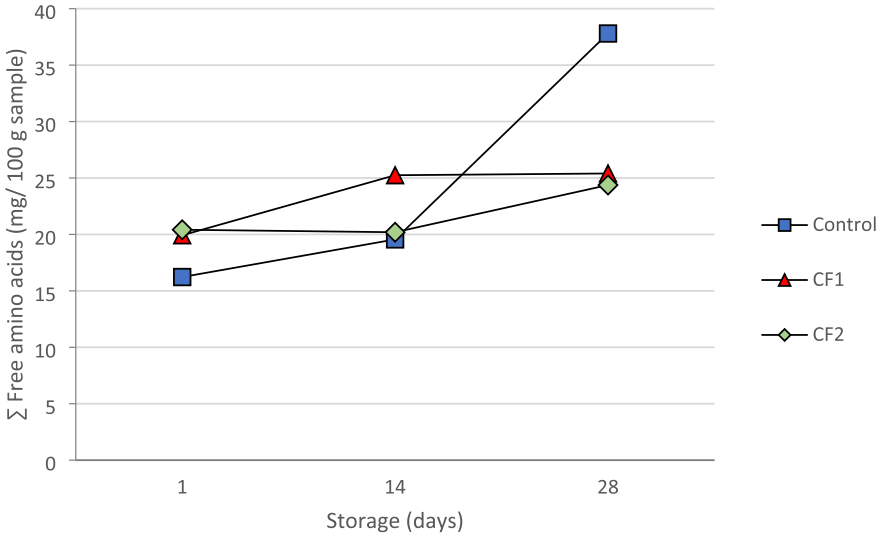

Fig. 1. Sum of free amino acids (mg/ 100 g sample) during cold storage (1, 14 e 28 days) of yoghurts prepared with ultrafiltered milks. Control: Control yoghurt, prepared with fluid skim milk + powdered skim milk; CF1: yoghurt prepared with ultrafiltered milk at a concentration factor of 3; CF2: yoghurt prepared with ultrafiltered milk at a concentration factor of 1.5.

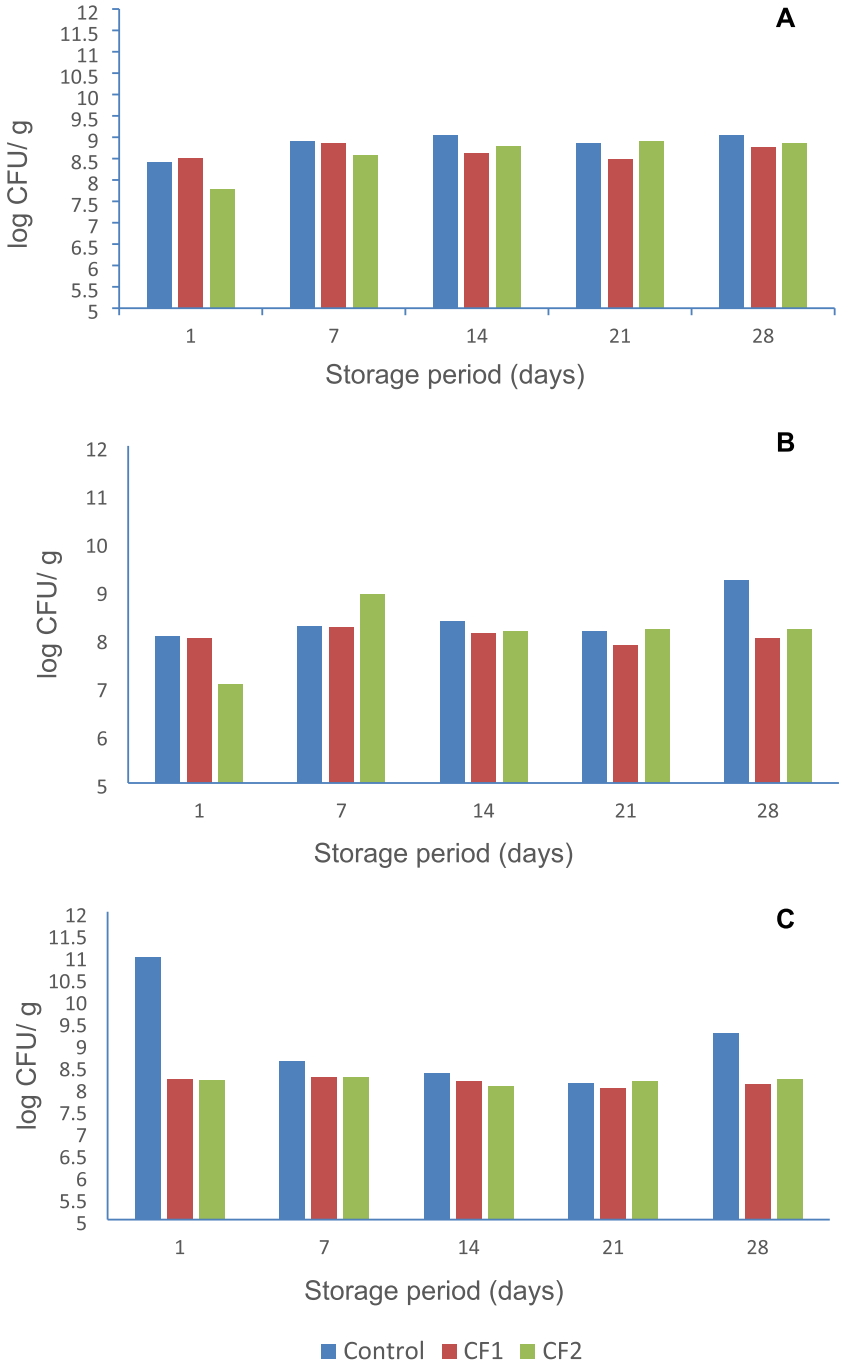

Fig. 2. Viability of microorganisms (A) *S. thermophilus*, (B) *L. bulgaricus,* and (C) *B. animalis* during cold storage (1, 7, 14, 21, and 28 days) of yoghurts prepared with ultrafiltered milks. Control: yoghurt prepared with fluid skim milk + powdered skim milk; CF1: yoghurt prepared with ultrafiltered milk at a concentration factor of 3; CF2: yoghurt prepared with ultrafiltered milk at a concentration factor of 1.5.

observed during 28 days of storage.

3.6. Total and free amino acid content during storage

Milk proteins have a high biological value, and their concentration using the ultrafiltration process promoted an increase in the content of essential amino acids in yoghurts (Table 4). The products, consequently, presented an elevation of branched-chain amino acids that are highly sought after by consumers and practitioners of sports activities. Valine, leucine, and isoleucine were concentrated at approximately 70% and 17% for CF1 and CF2, respectively. In general, the increase of total amino acids in the CF1 sample ranged from 23.40 to 92.31%, while in CF2, the increase was from 2.17 to 30.77% (compared to the control yoghurt). Other amino acids that stood out were histidine, tyrosine, methionine, and cystine, which increased about 68 to 92% on the 28th day of storage of CF1. Unlike thermal processes, modern membrane separation technologies (e.g., ultrafiltration and microfiltration) are gentle and enable protein concentration without provoking oxidative damage to milk proteins. Extensive glycation of the amino acids in milk powder and the formation of irreversible protein aggregates by intermolecular covalent crosslinks are consequences of spray-drying and storage [46]. Aalaei and coworkers [47] analysed lysine availability (used as a marker for glycoxidative damage) in spray-dried stored skim milk powder. They reported a loss of 7.45% of available lysine after

spray drying and registered a decrease up to 42.6% during storage, depending on the temperature and humidity. In our study, lysine content increased 76–84% and 22–24% in CF1 and CF2 samples, respectively. These results confirm that ultrafiltration was able to enhance the nutritional quality of Greek-style yoghurts compared to the traditional formulation prepared with skim milk powder.

The free amino acids production during storage was quantified to better describe the effect of fermentation on proteins. Among the samples, the most intense proteolytic activity on the first day of storage was observed for CF2 (Fig. 1). The control sample showed the highest proteolytic activity of the bacteria during the storage period, achieving an increase of 21.58 mg of free amino acids per 100 g of sample at the end of the experiment, compared with day 1. As presented and discussed in the next section, the counts of L. *bulgaricus* and *B. animalis* in control yoghurt after 28 days of storage are probably related to the higher proteolysis rate.

In the CF1 sample, proteolysis was slightly higher in the first 14 days, remaining practically unaltered at the end of the storage period (total increase of 5.46 mg of free amino acids/ 100 g of sample). No proteolysis was detected in CF2 yoghurt on the 14th day since the amount of free amino acids was similar to the value found on day 1. CF2 released 3.96 mg of amino acids/ 100 g of sample on the 28th day of storage compared to the first measurement. The predominant free amino acids for both control and CF1 yoghurts were, in decreasing order, glutamic acid, proline, alanine, lysine, isoleucine, and histidine (data not shown). The most abundant free amino acid in CF2 yoghurts was alanine, followed by glutamic acid, proline, lysine, histidine, and isoleucine (data not shown).

The proteolytic activity observed in fermented products results from the release of extracellular bacterial proteinases [48]. The proteolysis may generate bioactive peptides during storage, adding extra health benefits to the probiotic yoghurts. Sah et al. [45] reported a positive correlation between the degree of proteolysis and antimutagenic activity, and antioxidant activity in probiotic yoghurts during storage.

Since CF1 yoghurt achieved an impressively high protein content and microbial viability remains high during storage (as discussed later), it is likely that several peptides with potential biological activities are produced. Nyanzi and collaborators [49] gathered consistent scientific literature confirming the release of bioactive peptides by probiotic strains in yoghurt and fermented milk products. Inhibition of angiotensin-converting enzyme (ACE) [50], antimicrobial activity [51], antioxidant activity [50,52], and reduction of cancer cell proliferation [52] were highlighted.

3.7. Microbial viability of starter cultures and probiotics during storage

According to Meybodi and coworkers [53], several factors can influence the survival levels of probiotic bacteria in yoghurt, including pH, titratable acidity, oxygen, processing conditions (e. g., heat treatment and fermentation), starter culture, type of probiotic and different ingredients used in yoghurt formulation. In our study, the counts of starter bacteria *S. thermophilus* and L. *bulgaricus* (Fig. 2A and B, respectively) and the probiotic *B. animalis* (Fig. 2C) remained constant in all samples (around 10^8 CFU/ g of yoghurt). These results are in accordance with the Brazilian regulatory standard for yoghurts, which recommends a minimum count of total lactic acid bacteria of 10^7 CFU/ g and, if present, bifidobacteria count must be at least 10^6 CFU/ g [33]. In South Africa, similar counts are required for yoghurt and drinking yoghurt – they must contain at least 10^7 CFU/ g of yoghurt culture in a final product – although the word 'probiotic' is not allowed on the product label [54]. There is no consensus regarding the minimum count of total lactic acid bacteria for probiotic yoghurts, and the list of countries with specific regulatory standards is short. However, according to Nyanzi et al. [49], for therapeutic claims, the generally accepted minimum limit is 10^6 CFU/ mL for probiotic strains in yoghurt for 28 to 30 days of cold storage.

Table 5
Physical analysis (syneresis and water holding capacity) during cold storage of yoghurts prepared with ultrafiltered milks (mean ± SD).

Parameter	Yoghurt	Storage (days) 1	7	14	21	28
Syneresis (cm)	Control	0.25 ± 0.21^{aA}	0.33 ± 0.06^{aA}	0.30 ± 17^{aA}	0.40 ± 0.10^{aA}	0.33 ± 0.06^{aA}
	CF1	0.0^{aA}	0.0^{bA}	0.0^{bA}	0.23 ± 0.21^{abA}	0.0^{bA}
	CF2	0.0^{aA}	0.0^{bA}	0.0^{bA}	0.0^{bA}	0.0^{bA}
Water holding capacity (%)	Control	72.1 ± 0.20^{bA}	48.75 ± 4.40^{bB}	41.54 ± 0.03^{bB}	42.67 ± 0.73^{bB}	41.38 ± 0.5^{cB}
	CF1	87.12 ± 0.10^{aA}	80.87 ± 0.73^{aA}	79.98 ± 5.89^{aA}	77.49 ± 5.28^{aA}	81.95 ± 0.17^{aA}
	CF2	70.54 ± 0.06^{cA}	54.84 ± 0.60^{bB}	50.53 ± 1.41^{bB}	47.91 ± 3.78^{bB}	50.63 ± 0.38^{bB}

Control: standard yoghurt prepared with fluid skim milk + powdered skim milk; CF1: yoghurt prepared with ultrafiltered milk at a concentration factor of 3; CF2: yoghurt prepared with ultrafiltered milk at a concentration factor of 1.5. Values followed by the same lowercase letters in the same column, and for the same parameter, or the same uppercase letters in the same row did not differ statistically ($p > 0.05$) (n = 3 for syneresis; n = 2 for water holding capacity).

The presence of probiotic lactic acid bacteria did not suppress *S. thermophilus* and L. *bulgaricus* viability. Mani-López et al. [55] found similar results only for the viability of *S. thermophilus,* while L. *bulgaricus* decreased about 30 to 50% in probiotic yoghurts. The authors explain that the starter culture population reduction can occur when probiotic bacteria of the same genus are used as the starter culture (e.g., *L. bulgaricus* and *Lactobacillus reuteri*). It is probably caused by the production of bacteriocins, which inhibit microbial growth. Comparable results were reported for probiotic yoghurts supplemented with pineapple peel [45]. They used L. *acidophilus, L. casei, and* L. *paracasei* as probiotic bacteria and observed a significant viability reduction of *S. thermophilus* and *L. bulgaricus* during the 28 days storage period.

During the 28 days of refrigerated storage, the yoghurts maintained probiotic counts $>10^7$ CFU/ mL, achieving the minimal counts (10^6 CFU/ mL) to be considered probiotic products [56]. According to Kumar and Kumar [16], probiotics must reach the intestine in sufficient numbers, between 6 and 7 log CFU/ g of product, to confer health benefits. Therefore, probiotics counts in the yoghurts prepared in the present study remained in appropriate numbers during the storage time even though the titratable acidity increased significantly ($p < 0.05$), which could impair probiotic viability due to acid injury [45].

3.8. Physical analysis (syneresis, water holding capacity, and texture profile) during storage

Syneresis is a technological defect in yoghurts, occurring when a spontaneous release of water from the gel matrix is accompanied by a reduction in its volume [57]. It is considered the main disadvantage regarding the sensory attractiveness of yoghurts [58]. Syneresis occurrence is highly influenced by the water holding capacity (WHC) [59], the physical parameter that determines how many molecules in a matrix can retain water [60]. In our findings (Table 5), control yoghurt showed the highest syneresis value ($p < 0.05$) during storage, and a significant decrease ($p < 0.05$) of water holding capacity (WHC) was observed from the 7th day of storage onwards for control and CF2 yoghurts. WHC in CF1 was constant in all measurements ($p > 0.05$). According to Arab and coworkers, increasing protein levels in yoghurt gels enhances firmness and decreases syneresis. The strategy of raising the water holding capacity can be achieved by adding skimmed milk powder (SMP), whey protein-based, and casein-based ingredients [59]. Hence, it is reasonable to speculate that the high protein content associated with a lower post-

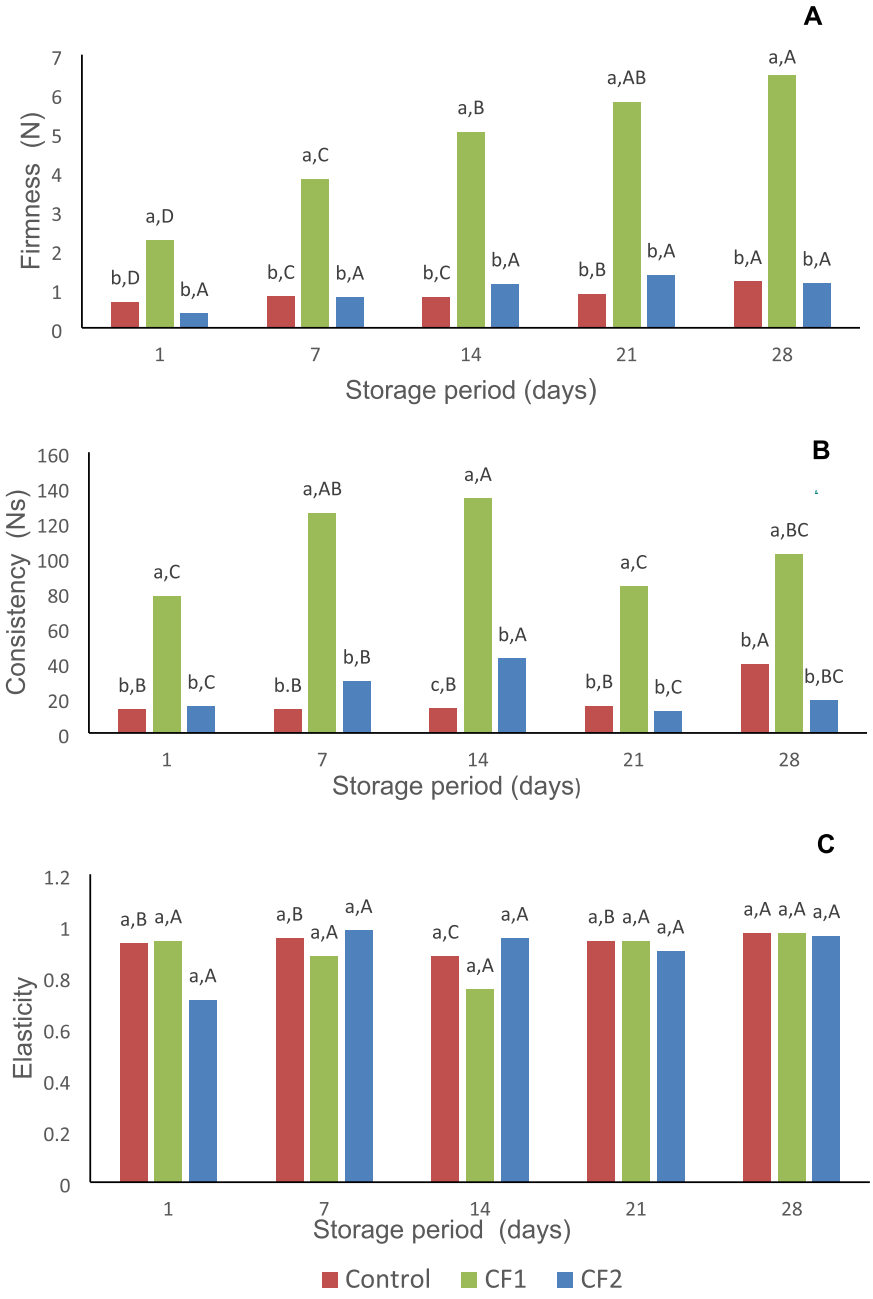

Fig. 3. Textural properties of (A) Firmness, (B) Consistency, and (C) Elasticity during cold storage (1, 7, 14, 21, and 28 days) of yoghurts prepared with ultrafiltered milks. Control: yoghurt prepared with fluid skim milk + powdered skim milk; CF1: yoghurt prepared with ultrafiltered milk at a concentration factor of 3; CF2: yoghurt prepared with ultrafiltered milk at a concentration factor of 1.5. Different lowercase letters represent differences between treatments ($p > 0.05$) and uppercase letters differences over the storage time for each parameter.

acidification in our samples could explain the absence of syneresis observed in CF1 and CF2 yoghurts, once syneresis is a common defect caused by continuous acidification [44]. The increase of protein content by adding albumin, whey protein concentrate, and sodium caseinate (used as agents for improving water holding capacity) also significantly decreased syneresis in a yoghurt-based product [61]. Moreover, the stability to syneresis herein described is in accordance with a previously reported Greek-style yoghurt produced with high content protein milk obtained by UF [62].

Textural parameters of firmness, consistency, and elasticity of the yoghurts are presented in Fig. 3A, B, and C, respectively. Firmness in control and CF2 yoghurts, which did not differ from each other ($p > 0.05$) during storage, was significantly lower ($p < 0.05$) in comparison to CF1 yoghurt. The latter presented a linear increase in firmness along store time. Regarding consistency, again, control and CF2 yoghurts showed the same behaviour ($p > 0.05$), except on the 14th day of storage, where a significant difference was observed. Yet, none reached the same consistency as CF1 yoghurt, even though the latter varied considerably along with storage. Firmness and consistency in CF1 yoghurt might be related to the product's high total solids content (and consequent high protein content)). Similar findings were reported when protein supplementation was performed to fortify protein content in probiotic yoghurts [63]. According to the authors, protein fortification and heat treatment are the most critical parameters determining yoghurt's textural properties. In our samples, the mild heat treatment applied to concentrated milk before fermentation might have promoted the denaturation of the whey proteins and their interaction with the caseins. This interaction causes an increase in the hardness of the gel by improving the water retention capacity and consequently reducing the syneresis [64]. Our findings from the texture analysis confirm that the increase in protein content is related to the rise in gel firmness, as previously discussed. There was no significant difference in the elasticity

values between the samples.

4. Conclusions

The ultrafiltration technique proved to be suitable for preparing probiotic Greek-style yoghurt with *B. animalis* strain since ultrafiltered milk yielded high protein levels, increased the content of essential amino acids, and provided an adequate amount of lactose, which may have had a positive impact on the viability of the probiotic and starter bacteria. The higher concentration factor of CF1 milk was considered responsible for favouring the obtention of yoghurt with desirable nutritional and technological attributes, such as higher content of calcium, phosphorus, and zinc, less intense post-acidification, better texture, greater water retention capacity, and absence of syneresis when compared to the Greek-style yoghurt supplemented with skim milk powder. The release of free amino acids during storage indicates the occurrence of proteolysis, which can be related to the release of bioactive peptides. Further studies focusing on peptide production during the fermentation of yoghurts with ultrafiltered milk should be addressed, aiming to describe possible health-promoting functions in addition to the benefits provided by the probiotic strains, which remained viable during the 28 days of refrigerated storage. Moreover, process optimisation and validation must be performed to confirm the results obtained on a pilot scale. As future perspectives for the study, we intend to evaluate the nonvolatile and volatile flavour compounds produced during the fermentation and storage of yoghurts prepared with ultrafiltered milk. Descriptive analysis of sensory attributes, consumers' acceptance, and purchase intention are other aspects to be explored.

Author statement

The research presented does not involve any animal or human study.

Funding

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) (grant code 001).

CRediT authorship contribution statement

Thaís Marini: Investigation, Methodology. **Darlila Aparecida Gallina:** Conceptualization, Project administration. **Elizabeth Harumi Nabeshima:** Formal analysis. **Alexandre Nunes Ponezi:** Formal analysis. **Katya Anaya:** Visualization, Writing – review & editing. **Adriane Elisabete Costa Antunes:** Validation. **Maria Teresa Bertoldo Pacheco:** Conceptualization, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

Acknowledgments

The graphical abstract was created with http://Biorender.com.

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