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Effect of Greek-style yoghurt manufacturing processes on starter and probiotic bacteria populations during storage

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ABSTRACT

This study compared viable counts of *Streptococcus thermophilus* (starter) and *Lactobacillus helveticus* R0052 (probiotic), as a function of two manufacturing processes of Greek-style (GS) yoghurt: centrifugation of a curd (GS-CF process) or ultrafiltration of milk prior to fermentation (GS-UF process). Fresh GS-UF and GS-CF yoghurts had between 3 and 7 times higher counts of *Lb. helveticus* R0052 and *S. thermophilus* than the regular stirred yoghurt (Control). Strain R0052 was three times more stable in the GS-CF yoghurt than in the Control over the 44 d storage period. The highest lactose content (44 g L^{-1}) was obtained in the Control, while 60% less was obtained in the GS-UF yoghurt; the opposite trend was observed for lactic acid and galactose. Free amino acid levels were 33% higher in the GS products. GS yoghurt can be 10 times better than traditional yoghurt to deliver probiotic bacteria, as a function of the process used.

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1. Introduction

High protein yoghurts are consumed worldwide, with diversity in composition and denominations, depending on the place of origin (Aryana & Olson, 2017). These strained yoghurts are best known as Greek-style yoghurts in North America and are characterised by protein content usually around 9%–10%. Their creamy texture and their natural, nutritive and low-fat attributes have made them very popular in the past few years (Wouters, 2012).

Traditionally, the total solids content of these yoghurts was increased by straining in a cloth bag. Concentration of the curd can now be done more efficiently by centrifugation (Tamime & Robinson, 2007). Other techniques, such as addition of dry milk ingredients or ultrafiltration of milk preceding fermentation, can also be used to increase the protein content (Jørgensen et al., 2019). The method used for Greek-style yoghurt production will define the composition of the final product (Ozer, Stenning, Grandison, & Robinson, 1999a; b).

Traditional yoghurt has health attributes, an example being improved lactose assimilation in individuals with lactose maldigestion (EFSA, 2010). Over the last few decades, probiotic bacteria have increasingly been added to yoghurt (Aryana & Olson, 2017), ice cream (Balthazar et al., 2018) or cheese (Silva et al., 2018) to further enhance health benefits. *Lactobacillus helveticus* is a species that is used in cheese making and which has potential health benefits (Drake, Boylston, Spence, & Swanson, 1996; Taverniti & Guglielmetti, 2012). The particular *Lb. helveticus* R0052 strain used in this study was selected because of its ability to grow in milk (Champagne, Green-Johnson, Raymond, Barrette, & Buckley, 2009) as well as for its safety (Foster, Tompkins, & Dahl, 2011) and potential health effects (Arseneault-Breard et al., 2012; Diop, Guillou, & Durand, 2008; Gilbert et al., 2013; Messaoudi et al., 2011; Ten Bruggencate, Girard, Floris-Vollenbroek, el Bhardwaj, & Tompkins, 2015).

By definition, probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill et al., 2014). Thus, to have a bioactive impact on the host, it is considered that, in most cases, a minimal number of living probiotic cells must be present in the product. No universal number

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of probiotic cells guarantee efficacy (Martinez, Bedani, & Saad, 2015). However, some regulatory agencies require that a billion cells per portion must be present in the food product (Health Canada, 2009; Hill et al., 2014), even at the best-before date (CFIA, 2016). Thus, probiotics must survive during the production and storage of the carrier product. Evaluation of the survival of probiotic bacteria in yoghurt has been done mostly with regular stirred or set yoghurt. Therefore, the effect of protein concentration and production processes of Greek-style yoghurt on viable counts during storage are still unknown. The addition of whey proteins or caseins in several forms is known to promote the growth and viability of some probiotic lactobacilli (Akalin, Gönç; Ünal, & Fenderya, 2007; Antunes, Cazetto, & Bolini, 2005; Qingli et al., 2013). Moreover, the increased buffering capacity of milk containing higher protein content (Li & Corredig, 2014), contributes to maintaining higher pH values in a fermented dairy product (Kailasapathy & Supriadi, 1996). Thus, knowledge about milk proteins suggests that Greek-style yoghurts produced by centrifugation or ultrafiltration could improve probiotic bacteria survival and stability in comparison with regular stirred yoghurt, but no data are available on the effect of manufacturing processes of high-protein yoghurts on stability of probiotics during subsequent storage.

Most yoghurts marketed are of the “stirred” type rather than the “set” type. In the manufacturing process of stirred yoghurts, a “smoothing” step is carried out to break the curd and prevent sensory perception of particles. This step incorporates some air. Some probiotic bacteria are very sensitive to oxygen (Talwalkar & Kailasapathy, 2004), and it is unknown to what extent yoghurt smoothing will affect the viability of probiotics.

The aim of this study was to assess the effect of manufacturing Greek-style yoghurts by ultrafiltration or centrifugation on the viable counts of *Lb. helveticus* R0052 in fresh products and during storage, comparing with regular stirred yoghurt as a Control.

2. Materials and methods

2.1. Microorganism and media

The *Lb. helveticus* R0052 probiotic culture was obtained freeze-dried from Lallemand Health Solutions (Montreal, QC, Canada) and stored at 4 °C. Its population was determined individually by the pour plate technique in BD Difco™ MRS agar (BD Diagnostic Systems, Sparks, MD, USA) with 48 h incubation at 37 °C in an anaerobic chamber. Precisely, dried cultures were rehydrated 1:10 in a 37 °C-preheated broth made of BD Bacto™ Peptone (BD Diagnostic Systems), Tween® 80 (Sigma–Aldrich Canada Co., Oakville, ON, Canada), sodium ascorbate (Sigma–Aldrich Canada Co) and L-cysteine (Sigma–Aldrich Canada Co). A homogenisation was carried out at 27,000× *g* for 30 s with an Omni THQ Homogeniser (Omni International, Kennesaw, GA, USA), followed by incubation at 37 °C for 15 min. Ten-fold dilutions were made in 1% peptone water, the first one being homogenised again. This methodology, and other methods in the following lines, followed recommendations for analysis of probiotics in supplements and foods (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011). This technique was not used for enumeration of *Lb. helveticus* in yoghurt; the methods used for this purpose are fully described in section 2.4 below.

A commercial mix of exopolysaccharide-producing yoghurt cultures was used as a starter. The YO-MIX® T11 yoghurt cultures was obtained freeze-dried from DuPont Nutrition & Health (Mississauga, ON, Canada).

The populations of starter organisms were determined by the same rehydration and homogenisation steps described for *Lb. helveticus* R0052. BD Difco™ M17 agar (BD Diagnostic Systems, Sparks, MD, USA) was used for the selective enumeration of *Streptococcus thermophilus*. MRS agar, in which glucose was substituted by fructose as carbon source and with pH adjusted to 5.4, was used for the enumeration of *Lactobacillus delbrueckii* subsp. *bulgaricus*. The plates were incubated at 42 °C for 48 h for *S. thermophilus* and at 45 °C for 48 h in anaerobic conditions for *Lb. bulgaricus*. The latter was not enumerated in the yoghurts because it represented less than 0.1% of the total population. Thus, the starter was essentially composed of streptococci. Yoghurt starters typically have higher contents in lactobacilli than in this product. This starter was designed for very low post acidification, which is mostly linked to *Lb. bulgaricus* (Zhang, Zhang, & Han, 2012).

2.2. Milk preparation

A low heat cow skim milk powder (Dairytown Processing Ltd, Studholm, NB, Canada) was rehydrated in a pilot plant facility to prepare two different milk solutions. The first solution was prepared to obtain a 4.0% protein content (Milk 1) and was stored overnight at 4 °C. For the second solution, skim milk powder was rehydrated to obtain a 3.1% protein content. The 3.1% solution was then pasteurised (75 °C for 18 s) and ultrafiltered (50 °C, 30 psi, 4–6 m s^{-1}) to obtain a final solution with 10.6% protein content (Milk 2). A precautionary pasteurisation step was carried out prior to ultrafiltration to ensure microbiological quality of the milk, as the batch process applied during ultrafiltration could have allowed limited bacterial growth. The ultrafiltration system was equipped with 50-PM Romicon Hollow Fiber Cartridges (Koch Membrane Systems, Wilmington, MA, USA) with a 50,000 Da cut-off. Both milk solutions were finally stored at −20 °C in 4 L plastic buckets for use in subsequent experiments. The protein contents of the milk solutions were confirmed with a MilkoScan™ FT-120 analyser (Foss, Hilleroed, Denmarke) and a Vario Max Cube Analyser (Elementar, Langenselbold, Germany).

The buffering capacity of the milk solutions was obtained by measuring the amount of 0.1 M HCl required to reduce the initial pH level of the sample to pH 4.0. Analyses were repeated three times for each milk solution.

2.3. Yoghurt production processes

2.3.1. Basic production steps for regular stirred yoghurt and Greek-style yoghurts

Both milk solutions (9 L of Milk 1 and 3 L of Milk 2) were thoroughly thawed at 4 °C for 16 h. Freezing can affect milk properties (Arena, Salzano, & Scaloni, 2016; Fransson & Lönnerdal, 1983), but this was necessary because the milk matrices were used over 4 weeks to carry out the independent repetitions; it was feared that microbial spoilage would occur during the 4-week storage period. Once thawed, milks were heat treated (90 °C, 10 min) in an autoclave operated in an isotherm program. After heat treatment, the milk solutions were cooled to 40 °C and separated in sanitised plastic buckets into 3 L fractions. The starter (frozen concentrate) and the probiotic strain (*Lb. helveticus* R0052; freeze-dried powder) were directly added to the milk solutions with inoculation rates of 3×10^7 cfu mL^{-1} for the starter (count based on *S. thermophilus*), and 1×10^7 cfu mL^{-1} for the probiotic. The fermentation was performed at 40 °C in a temperature-programmable incubator (Thermo Scientific MaxQ 480R HP) equipped with an in-house Fermentation Acquisition and Control System (FACS) (Agriculture and Agri-Food Canada, Saint-Hyacinthe, QC, Canada) which records pH and temperature values each minute. When the pH value dropped to approximately 4.7, cooling to 18 °C was initiated. At this point, two types of yoghurt were obtained; regular stirred yoghurt (Control) and Greek-style ultrafiltered yoghurt (GS-UF).

A 6 L portion of the Control yoghurt was distributed in 1 L polypropylene bottles to be centrifuged at 8000× *g* for 15 min at 18 °C (Beckman Coulter Avanti™ J-20XPI centrifuge with a JLA 8.100 rotor). These conditions were selected to concentrate the curd from 4.0% to 10.3% protein, resulting in the Greek-style centrifuged yoghurt (GS-CF).

The three products at 18 °C were then smoothed. This step was carried out by extruding the 3 L batches of products in a food processor typical of meat grinders. It consisted of a funnel that enabled the feed of the yoghurt into a cylinder of 5 cm in diameter which was equipped with an endless screw (KitchenAid, Mississauga, ON, Canada). The screw pushed the yoghurt through the cylinder at the end of which a 4-bladed knife rotating a 50 RPM cut the curd, which was then extruded through a 5 cm diameter plate having 64 perforations of 2 mm in diameter. The smoothed yoghurts were separated into 100 g portions and placed into 125 mL polypropylene containers, which were stored at 4 °C for up to 44 d.

2.3.2. Assessment of the effect of smoothing on viable counts of the probiotic culture

Preliminary assays were conducted to evaluate the effect of the smoothing step on the levels of dissolved oxygen that may have affected the viability of *Lb. helveticus* R0052 during storage. Two 3 L batches of regular stirred yoghurt (4% protein) were prepared as described in section 2.3.1. The starter was inoculated into the milk at 3×10^7 cfu mL^{-1} and the probiotic culture was inoculated at 1×10^7 cfu mL^{-1}. Fermentation was performed at 40 °C until pH 4.7 was reached and products were then cooled to 18 °C.

In the control treatment (Not smoothed), the fermented yoghurt was added into the polypropylene cups in 100 g portions using large spoons, without breaking the curd. In the "Smoothed" treatment, the 3 L batch of yoghurt was smoothed using the process described in above (section 2.3.1) and the blended curd was then transferred into the cups. The cups were then stored at 4 °C for 16 d.

At 6 incubation times over the 44 d storage period, a cup was opened and three analyses were carried out: pH, the level of dissolved oxygen (DO_2) and the viable counts of probiotic bacteria. Viable counts were carried out as described in section 2.5 below. The DO_2 level in the yoghurt gel was measured with an YSI Model 85 Handheld Oxygen, Conductivity, Salinity, and Temperature System (Yellow Springs, OH, USA). The pH level was measured with a portable pH meter (Oakton Instruments, Vernon Hills, IL, USA) equipped with a Fisher Scientific™ Accumet™ refillable glass pH electrode (Fisher Scientific, Ottawa, ON, Canada). Viable counts were carried out as described in section 2.5.1 after evaluation of pH and DO_2.

2.4. Bacterial enumeration

2.4.1. Plating technique

For bacterial enumeration, 11 g samples were taken from the polypropylene cups after a gentle mixing of the yoghurt curd with a sterile spoon. One sample was put in a Fisherbrand™ Lab Blender polyethylene bag (Ottawa, ON, Canada) with 99 mL of 10 g L^{-1} peptone-citrate trisodium buffer. The sample was homogenised (1 min, 230× *g*) in a Stomacher® 400 Circulator (Seward, Worthing, UK). Ten-fold dilutions were done in 1 g L^{-1} peptone water and 1 mL samples of the appropriate dilutions were plated using the pour plate method. The first dilution in the test tube was homogenised at 27,000× *g* for 30 s with an Omni TH_Q homogeniser (Omni International, Kennesaw, GA, USA). The conditions were adapted for selective enumeration of each bacterial strain (Table 1). Preliminary assays were carried out to confirm the selectivity of the methods for each given strain.

2.4.2. Flow cytometry technique

Enumeration of *Lb. helveticus* R0052 was also carried out by flow cytometry (FCM) using the Live/Dead BacLight Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR, USA) as described in Raymond and Champagne (2015). FCM is very useful in the analysis of probiotic-based products because it can ascertain total bacterial populations, viable counts and viable but non-culturable cells (Wilkinson, 2018). In this assay, the dyes were propidium iodide and SYTO 9. SYTO 9 colours all the cells, and enables the determination of total bacterial counts. Propiodium iodide, on the other hand, only enters cells that have significant membrane damages (Wilkinson, 2018), which are considered dead. Thus, dead cells are coloured by the two dyes, while viable cells are only coloured by SYTO 9.

A significant development in FCM is immunology-based analyses (Champagne, Gomes da Cruz, & Daga, 2018; Wilkinson, 2018). In this technique a specific antibody against a given strain is first added to the cell suspension. A second antibody, carrying the fluorescent dye, is then added to target the antibacterial antibody as a function of the animal that was used to create the antibody. This is similar to some ELISA techniques. In this study, a specific goat-produced antibody labelling for the probiotic strain *Lb. helveticus* R0052 was graciously provided by Lallemand Health Solutions. Briefly, the homogenised samples were diluted in phosphate buffered saline (PBS) + Tween 20 at 2 g L^{-1}, to obtain a bacterial concentration between 5×10^5 bacteria mL^{-1} and 5×10^6 bacteria mL^{-1}, and passed through a Full Flow™ Filter 10 μm (Varion, Cary, NC, USA). The sample (200 μL) was first mixed with 1 μL of the species-specific polyclonal antibody (1 mg mL^{-1} solution, Lallemand Health Solutions) and incubated at room temperature for 30 min. Then, 40 μL of the sample was mixed with 160 μL PBS + Tween 20 (2 g L^{-1}), and 1 μL of a second layer antibody (goat anti-rabbit F (ab')2 IgG fragment Alexa Fluor®647 conjugated 1 mg mL^{-1}, Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA), and incubated in obscurity for 30 min at room temperature. The sample was diluted again in PBS (100 μL in 900 μL), stained by adding 1.5 μL of each SYTO 9 and Propidium iodide from Live/Dead BacLight Bacterial Viability Kit (Molecular Probes Inc., Eugene, OR, USA), and incubated in obscurity for 15 min. Each sample was run twice on an Accuri C6 flow cytometer (Becton Dickinson, Mississauga, ON, CA). Samples were run at slow speed for 2 min with the following threshold filter applied: FL4 (675/25) at 3200 and FL1 (530/30) at 1000. For samples without antibody labelling, FL1 at 1000 and FL3 (620/10) at 1000 were used. A control of Live and Dead cells marked with and without the antibody was made to determine the zone of interest and to confirm the positive marking of the bacterial strain R0052. A yoghurt sample without the probiotic was also analysed to confirm the specificity of the antibody (Fig. 1). For analysis on a SSC-H versus FL4-H graph, a first region of interest (gate D, presence of particles marked with the antibody) was applied on a FL3 versuss FL1 graph, on which the region of interest to live and dead cells were created (gate A: live bacteria and gate B: dead bacteria).

2.5. Compositional and physicochemical analyses

The pH level was measured with two systems. The in-house FACS equipped with Red Rod Radiometer analytical pH electrodes (Hach, London, ON, Canada) was used during the fermentations and a portable pH-meter (Oakton Instruments) equipped with a Fisher Scientific™ Accumet™ refillable glass pH electrode (Fisher Scientific) was used on yoghurt after fermentation and during storage.

The sugars, acids and ethanol content of milk and yoghurt were measured by HPLC (Waters 1525 pump and Waters 717-plus auto sampler; Milford, MA, USA). The following conditions were applied:

Table 1
Selective media and incubation conditions used for the growth and enumeration of the probiotic and starter bacterial strains in yoghurt.[a]

Strain	Selective media	Temperature (°C)	Incubation conditions
Lactobacillus helveticus R0052	MRS-CL	45	Anaerobic
Lactobacillus delbrueckii subsp. *bulgaricus* YO-MIX® T11	MRS-F	45	Aerobic
Streptococcus thermophilus YO-MIX® T11	M17-L	42	Aerobic

[a] Incubation time was 48 h in all cases. MRS agar and M17 agar were from BD Difco™, BD Diagnostic Systems, Sparks, MD, USA; MRS-F, MRS agar assembled from individual components with fructose as sugar; pH adjusted to 5.4 (Tabasco, Paarup, Janer, Pelaez, & Requena, 2007).

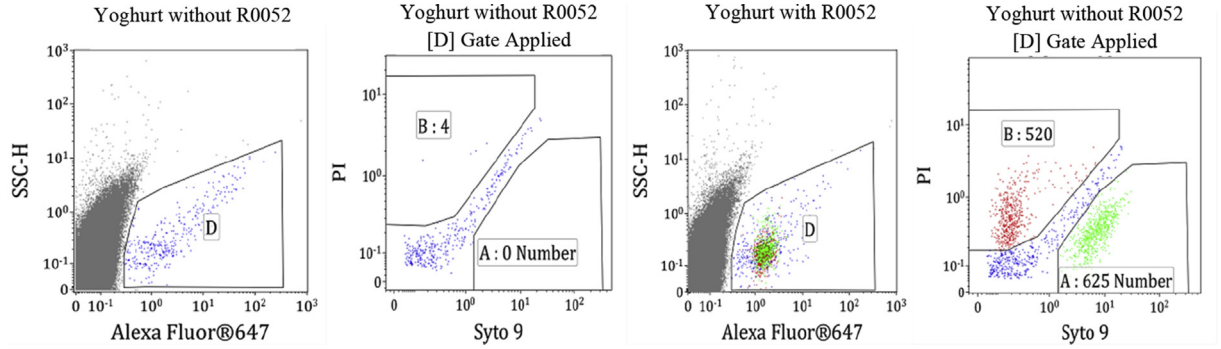

Fig. 1. Antibody specificity and positive labelling of *Lactobacillus helveticus* R0052 in yoghurt. Gate D: antibody labelled R0052, A: Live cells of *Lb. helveticus* R0052, and B: Dead cells of *Lb. helveticus* R0052.

a 20 μL injection on a separation column ICE-Ion 300 (Transgenomic, Omaha, NE, USA), kept at 80 °C; mobile phase of 0.02 N H_2SO_4 injected at 0.4 mL min^{-1}; RI detector (Waters 2414, Milford, MA, USA) at 40 °C coupled with a PDA detector (Waters 2998, Milford, MA, USA) at a wavelength of 208 nm. To do the extraction, 8 g of the sample was mixed with 1 mL of 36% trichloroacetic acid and 30 μL of 5% sodium azide, incubated overnight at 37 °C, and centrifuged at 10,000×*g* for 20 min at 37 °C (Beckman Coulter Avanti™ J-20XPI centrifuge with a JLA 10.5 rotor). Supernatants went through a second 37 °C incubation of 2 h and a second centrifugation with the same parameters. Supernatants were finally filtered on a Millipore filter (Millex-HV, Bedford, MA, USA) of 0.45 μm in a GSLC vial.

Total nitrogen (N_{tot}) was ascertained with a Vario Max Cube Analyser (Elementar, Langenselbold, Germany). Protein content was calculated by the conversion of the total nitrogen content using a nitrogen conversion factor of 6.38 attributed for milk proteins (FAO, 2003). The level of alpha amino-nitrogen (AAN) and degree of hydrolysis (DH) were measured by titration with a formaldehyde solution ACS reagent, 37% (w/w) in H_2O (Sigma–Aldrich® Canada Co) adjusted to pH 9.0 with sodium hydroxide (DILUT-IT® Analytical Concentrate, 0.1 N, J.T.Baker®; VWR International, Montreal, QC, Canada) following the USP 23-NF18 (1995) protocol. Briefly, 5 mL of the sample was diluted in 20 mL of water and adjusted to pH 7.0. Then, 10 mL of formaldehyde was added and the sample was titrated with the sodium hydroxide to a final pH of 9.0. A solution of glycine 20 mg NA 100 mL^{-1} was used as standard to verify the titration. The AAN levels were calculated following the equation of the same protocol. The DH was calculated following the equation of the formol titration protocol of Navarrete del Toro and García-Carreño (2001).

The syneresis index was measured following the method of Harwalkar and Miloslav (1986) with modifications. Briefly, 25 g of sample was centrifuged at 210×*g* for 20 min at 4 °C (Thermo Scientific™ Sorvall™ ST 40 Centrifuge with a Tx-750 rotor). The whey extracted was weighed. The syneresis index was calculated by determining the % of the weight of the sample which was obtained in whey.

2.6. Statistical analyses

The study was separated in two experimental designs. The first one consisted of regular stirred yoghurt produced to determine the impact of the smoothing process on the probiotic strains *Lb. helveticus* R0052 for 16 days of storage (design #1). The other consisted of regular stirred yoghurt (Control) and Greek-style yoghurts (GS-UF and GS-CF) produced to determine the impact of the products on the probiotic strain *Lb. helveticus* R0052, the starter *S. thermophilus* and physicochemical properties for 44 days of storage (design #2).

A split-split-plot design was used to compare the enumeration method and the influence of the production processes and days of storage on *Lb. helveticus* and *S. thermophilus* counts in yoghurt. The three products were considered as the main plot, the two enumeration methods as the sub-plot, and the days of storage as the sub-sub-plot factor. ANOVA and least squares means analyses with a probability threshold of 0.05 were performed on data from the previously described designs to compare the effects of the several factors on the microbial counts, using SAS software (Toronto, ON, Canada).

For the statistical analyses of the smoothing process assays (dissolved oxygen and bacterial counts), one-way ANOVA was carried out with "Product" as parameter, using SigmaPlot software (Systat Software Inc., San Jose CA, USA). For the analyses of the chemical compounds (sugars, acids, amino compounds) and syneresis, two-way ANOVA was carried out with "Time" and "Product" as the parameters, using SigmaPlot software as well.

3. Results and discussion

3.1. Influence of dissolved oxygen and smoothing process on probiotic survival

Since probiotics may be sensitive to oxygen during storage (Talwalkar & Kailasapathy, 2004), it was first verified if the smoothing method introduced oxygen into the yoghurt curd and if this could affect viability. The smoothing step initially resulted in a high level of dissolved oxygen in the yoghurt (Fig. 2). In the

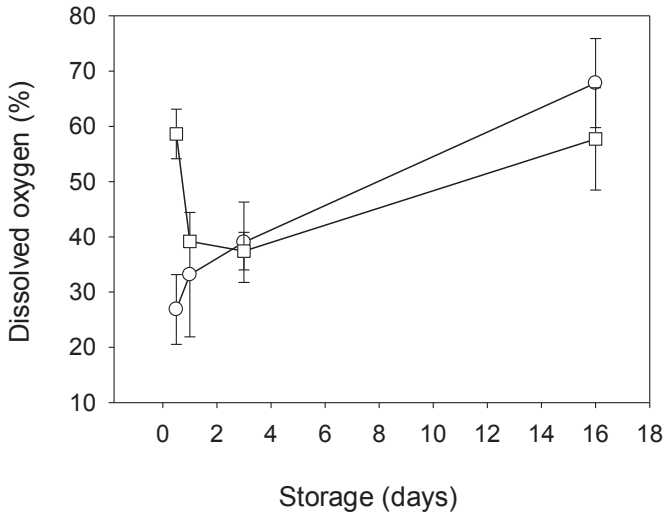

Fig. 2. Influence of the smoothing process of regular stirred yoghurt (□, smoothed; ○, not smoothed) on the level of dissolved oxygen during storage at 4 °C. Error bars represent standard error of the means.

smoothed product, there was a rapid DO_2 decrease during the first day of storage. Other studies obtained similar results, with a drop of the oxygen content after fermentation that was attributed to the metabolic activities of starter bacteria that require dissolved oxygen (Damin, Minowa, Alcântara, & Oliveira, 2008; Dave & Shah, 1997). However, after only one day of storage, the DO_2 levels became identical at approximately 38%. Between 3 and 16 days of storage, the DO_2 levels almost doubled, but there was no significant difference ($p > 0.05$) between treatments (Fig. 2). The oxygen increase during storage was consistent with the results of other studies, which attributed the phenomenon to oxygen permeation through the plastic containers (Damin et al., 2008; Dave & Shah, 1997).

The viable counts of *Lb. helveticus* R0052 were 4×10^7 cfu mL^{-1} at the beginning of storage, and remained at that level until day 16. There was no significant effect of the smoothing process on viable counts of the probiotic culture. Presumably, this is because the effect of smoothing on DO_2 levels only occurred during the first day, which was not a sufficiently long exposure period to generate high viability losses. As a result, the smoothing step was applied to all subsequent manufactures of Control, GS-CF and GS-UF yoghurts.

Although *Lb. helveticus* was not affected by smoothing, other strains might. In this instance, the addition of enzymes, such as catalase (Hull, Roberts, & Mayes, 1984) or glucose oxidase (Batista et al., 2015), could be considered to eliminate oxygen or its by-product (H_2O_2). The enzymes could be added in the product itself or in plastic materials (Cruz et al., 2013b).

3.2. Chemical attributes of milk and yoghurts

The fermentation time was longer in Milk 2, which had higher protein content resulting from ultrafiltration, and higher buffering capacity than Milk 1 (Table 2). In Milk 2, the acidification rate started to slow down below pH 5. This corresponds to the maximal buffering capacity of caseins, which are found in high concentration in ultrafiltered milk (Domagala, 2012; Moreno-Montoro et al., 2015; Salaun, Mietton, & Gaucheron, 2005).

Yoghurts containing the probiotic strain *Lb. helveticus* R0052 were stored for up to 44 days. The sugar and acid profiles were analysed in the initial milk solutions (day 0) and in the three yoghurts containing *Lb. helveticus* at the beginning (day 3) and at the end (day 38) of storage at 4 °C (Fig. 3). Ethanol and acetic acid levels remained below 0.3 g L^{-1}. Glucose concentration varied between 1.6 and 3.0 g L^{-1}, but these differences were not found to be statistically different, and there was no effect of storage. However, statistical analyses revealed significant differences ($p < 0.05$) between yoghurt types for lactose, galactose, lactic acid and citric acid concentrations (Fig. 3).

Lactose concentration was 12 g L^{-1} higher in Milk 1 than in Milk 2 (Fig. 3) and the GS-UF yoghurt consequently had a lower lactose concentration than did the Control and GS-CF products. For Milk 2 with the ultrafiltration process, skim milk powder was rehydrated at a 3.1% protein level, a concentration that simulates fresh milk, and which results in approximately 47 g L^{-1} lactose. Although lactose concentration in the retentate varies according to the operating conditions of the UF system (Brazuelo et al., 1995), the major portion is lost in the whey permeate. For Milk 1, however, the skim milk powder was rehydrated to obtain a 4% protein level, to simulate enriched fresh milk, which resulted in a lactose level 20% higher than in ultrafiltered Milk 2 (Fig. 3). All these data are in accordance with the average concentrations found in cow milk (Haug, Hostmark, & Harstad, 2007).

In all products, the decrease of lactose during the fermentation corresponded to the quantities of lactic acid and galactose produced. Lactose assimilation and lactic acid production levels in the GS-CF yoghurt were 10% higher than those of the Control at days 3 and 38 (Fig. 3). Nevertheless, data on substrates and metabolites from the GS-CF yoghurt were much closer to those of the Control than were those of the GS-UF product. During storage, there were further significant ($p < 0.05$) changes in metabolite concentrations and the patterns were similar in all yoghurt products. Data for the regular stirred yoghurt (Control) are in accordance with observations of other studies (Adhikari, Gruen, Mustapha, & Fernando, 2002; Vénica, Perotti, & Bergamini, 2014). Ethanol and acetic acid levels were negligible in the three yoghurt types, and did not increase during storage which is consistent with the homofermentative metabolism of the *Lactobacillus* species used.

Along with the lactose uptake, galactose increased during storage (Fig. 3) while glucose remained stable. Unlike glucose, the

Table 2
Characteristics of the milk and the three yoghurts produced for the comparative study of probiotic populations and physicochemical properties.[a]

Product	Protein content (%)	pH level	Buffering capacity (mol HCl 100 mL^{-1})	Average fermentation time (h)
Milk 1	4.0 ± 0.01[a]	6.75 ± 0.01[a]	0.008 ± 0.00[a]	5.5 ± 0.02[a]
Milk 2	10.6 ± 0.02 [bc]	6.86 ± 0.01[a]	0.016 ± 0.00[b]	9.5 ± 0.00[b]
Control yoghurt	4.1 ± 0.03[a]	4.66 ± 0.01[b]		
GS-CF yoghurt	10.3 ± 0.09[b]	4.66 ± 0.01[b]		
GS-UF yoghurt	11.0 ± 0.02[c]	4.73 ± 0.01[c]		

[a] Protein content was calculated by converting the total nitrogen content as described in section 3.4.6. The pH readings of milk were taken at 40 °C at the beginning of fermentation; the pH of yoghurt was taken just prior the smoothing step, when the product was at 18 °C. Average fermentation time represents the time required to reach a pH of 4.7 at 40 °C. The Control (regular stirred) and the Greek-style yoghurt produced by centrifugation (GS-CF) yoghurts were obtained from the fermentation of Milk 1, while the Greek-style yoghurt produced by ultrafiltration GS-UF yoghurt resulted from the fermentation of Milk 2. For a given column, values that are followed by the same letter are not statistically different.

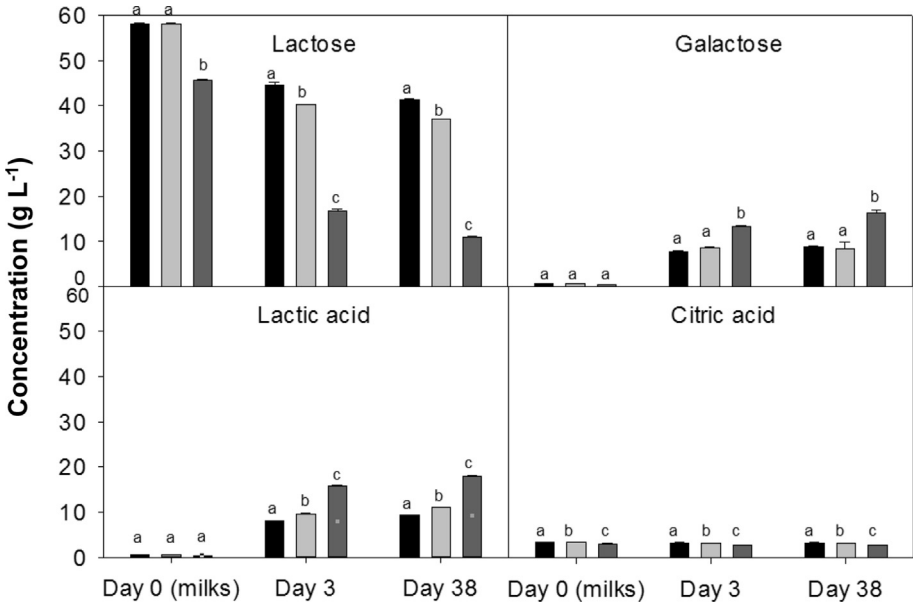

Fig. 3. Sugar and organic acid profiles of the 4% protein milk (Milk 1), 10% protein milk (Milk 2), regular stirred yoghurt (Control, ■) and Greek-style yoghurts made by centrifugation (GS-CF, ■) or ultrafiltration (GS-UF, ■) during storage at 4 °C. All products contained the probiotic strain *Lactobacillus helveticus* R0052. For each day of storage, letters indicate if the concentrations are significantly different ($p < 0.05$) between each yoghurt. Error bars represent standard error of the means (SEM). Black, control yoghurt; light grey, GS-CF; dark grey, GS-UF yoghurt.

galactose moiety of lactose cannot be used by all bacteria as a carbon source. Utilisation of lactose by most strains of *S. thermophilus* comes with a release and accumulation of galactose in the media (Beal, Louvet, & Corrieu, 1989; Oliveira, Torres, Perego, Oliveira, & Converti, 2012). Lactose consumption and lactic acid production profiles during fermentation and storage of the Control samples are also in accordance with the results of other studies (Adhikari et al., 2002; Beal et al., 1989; Oliveira et al., 2012). The probiotic *Lb. helveticus*, however, can metabolise galactose. Theoretically, the galactose level should be identical to that of lactic acid but it was 10% lower (Fig. 3). Thus, a small fraction of the galactose released could have been fermented by *Lb. helveticus*, or it could have been incorporated in an exopolysaccharide produced by the starter culture. This remains to be ascertained.

There were marked differences ($p < 0.05$) of total nitrogen N_{tot} values between milk solutions prior to fermentation (Table 3).

Table 3
Total nitrogen (N_{tot}) content, α-amino nitrogen (AAN) content and degree of hydrolysis (DH) in the regular stirred yoghurt (Control) and Greek-style yoghurts made by centrifugation (GS-CF) or ultrafiltration (GS-UF) during storage at 4 °C.[a]

Product	N_{tot} (g L^{-1})	AAN (g L^{-1})	DH (%)
Milk 1	6.20 ± 0.02^{A}	0.49 ± 0.00^{A}	2.24 ± 0.01^{A}
Control yoghurt			
Day 3	6.40 ± 0.04^{a}	0.74 ± 0.01^{a}	3.33 ± 0.08^{a}
Day 38	6.40 ± 0.04^{a}	0.78 ± 0.02^{a}	3.53 ± 0.08^{a}
GS-CF yoghurt			
Day 3	16.20 ± 0.15^{b}	1.28 ± 0.11^{b}	2.27 ± 0.17^{b}
Day 38	16.20 ± 0.15^{b}	1.36 ± 0.03^{b}	2.40 ± 0.03^{b}
Milk 2	16.70 ± 0.03^{B}	1.17 ± 0.01^{B}	2.01 ± 0.02^{A}
GS-UF yoghurt			
Day 3	17.30 ± 0.04^{c}	1.45 ± 0.02^{c}	2.40 ± 0.03^{b}
Day 38	17.30 ± 0.04^{c}	1.50 ± 0.02^{c}	2.48 ± 0.04^{b}

[a] All products contained the probiotic strain *Lactobacillus helveticus* R0052. For a given column values with the same superscript letters are not significantly different ($p > 0.05$); capital superscript letters compare values between the two milk solutions, lowercase superscript letters compare values between the three yoghurt types for a given storage period. Statistical results for the effect of storage time for each product are not presented.

Significant increases in N_{tot} ($p < 0.05$) were noted between day 0 and day 3 for the Greek-style yoghurts. The increase in N_{tot} content between UF milk and GS-UF yoghurt suggests a slight loss of moisture during the fermentation and/or during the beginning of the storage. The AAN levels differed between milk solutions prior to fermentation (Table 3). The results were influenced by the processing method and fermentation. Fresh products (day 3) had significantly higher ($p < 0.05$) AAN values than the original milks. Indeed, the highest increase in AAN levels between original milk and the fresh fermented products (day 3) was noted in the GS-CF product. The AAN increases during storage (day 3 to day 38) were not statistically significant ($p > 0.05$) for any of the products. The higher AAN levels observed in the Greek-style products were approximately 33% higher than in the Control (Table 3), which indicates higher proteolysis in the high-protein yoghurts than in regular yoghurt. However, this seems linked to the higher total protein content, since the DH levels in the Greek-style yoghurts are not higher than in the Control products. The AAN concentrations obtained in the regular milk and the Control yoghurt are consistent with the results of another study that used *Lb. helveticus* R0052 (Champagne et al., 2009).

3.3. *Survival of* Streptococcus thermophilus *during storage*

Preliminary tests (data not shown) indicated that the concentration of *Lb. bulgaricus* in the starter mix was at least 6 Logs lower than that of *S. thermophilus*. Thus, for the starter culture, only *S. thermophilus* counts were assessed during storage of the products (Fig. 4).

Between the inoculation step and the first sampling at day 1, *S. thermophilus* counts showed an increase between 1.7 and 2.0 log cfu g^{-1} in the three yoghurt types (Fig. 4). The increase of *S. thermophilus* biomass during fermentation is in accordance with other studies (Mani-López, Palou, & López-Malo, 2014; Ozer & Robinson, 1999). Slightly higher ($p < 0.05$) growth was noted in the GS-UF yoghurt than in the Control yoghurt. The higher counts of *S. thermophilus* in the 10.6% protein Milk 2 could be associated with the

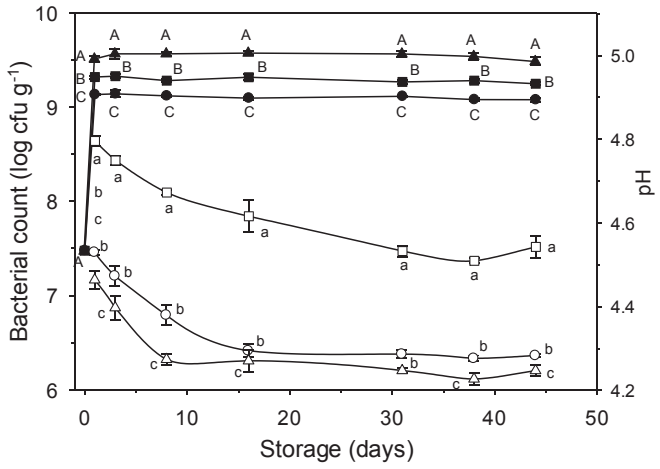

Fig. 4. pH (open symbols) and populations (closed symbols) of *Streptococcus thermophilus* from the YO-MIX® T11 yoghurt cultures in regular stirred yoghurt (Control: ○, ●) and Greek-style yoghurts made by centrifugation (GS-CF: △, ▲) or ultrafiltration (GS-UF: □, ■) during storage at 4 °C. Values at day 0 are the inoculated counts in milk, while values at day 1 represent those in the fresh yoghurts. For a given day of storage, capital letters indicate if the cfu values are significantly different ($p < 0.05$), and lowercase letters indicate if the pH values are significantly different ($p < 0.05$). Error bars represent standard error of the means (SEM).

longer fermentation, presumably due to the higher buffering capacity of Milk 2 (Table 2). Furthermore, the higher growth in Milk 2 could be linked to the content in stimulatory factors of the UF-milk, as casein fractions can improve *S. thermophilus* growth (Qingli et al., 2011; 2013), and as higher AAN levels were present in Milk 2 (Table 3).

The highest ($p < 0.05$) cfu viable counts were obtained in the GS-CF products, and were in correlation with the concentration factor of the curd. Subsequently, the *S. thermophilus* populations remained stable over all the 44 d of storage, maintaining the significant differences between the three yoghurts cfu values. The slightly higher counts obtained in the GS-UF yoghurt, in comparison with the Control, are in accordance with the results obtained by Ozer and Robinson (1999) who had higher and stable counts of *S. thermophilus* and *Lb. bulgaricus* in a milk that had been concentrated by ultrafiltration. The high lactic acid level probably contributed to stopping *S. thermophilus* growth in the 10.6% protein Milk 2 (Ozer & Robinson, 1999). Therefore, higher counts were obtained in the GS-CF yoghurt. After production, the *S. thermophilus* populations remained stable in all three yoghurt types during all the 44 d of storage. The viable counts in the Control products are consistent with other studies (Damin et al., 2008; Gueimonde et al., 2004; Mani-López et al., 2014; Ozer & Robinson, 1999).

Ozer and Robinson (1999) also found higher acidity levels in products fermented from concentrated milk than in products that were concentrated after fermentation, reflecting a higher acid production. Moreover, the presence of casein fractions, which could have been concentrated during ultrafiltration, has been found to increase the activity of enzymes related to sugar metabolism of *S. thermophilus* and *Lb. bulgaricus*, as well as improve lactic acid production by *S. thermophilus* cells (Qingli et al., 2013, 2014).

After production, the pH level of the GS-UF yoghurt was higher ($p < 0.05$) than in the other yoghurt types by 0.2 pH units (Fig. 4). The pH values of the GS-UF yoghurt remained higher than those of the two other products during all the storage period, and tended to stabilise around 4.5. The pH patterns of the Control yoghurt and GS-CF yoghurts were similar but the GS-CF products were slightly more acid than the control ($p < 0.05$), with an exception for day 31.

3.4. *Survival of* Lactobacillus helveticus *R0052 during storage*

Two enumeration techniques were compared in order to follow *Lb. helveticus* R0052 survival during storage at 4 °C. With the plating technique (cfu), there was a significant increase ($p < 0.05$) in counts during manufacture of yoghurts (Fig. 5). Between the inoculation step and the first sampling after 24 h of storage, the probiotic counts increased by 0.3, 0.9 and 1.2 log cfu g^{-1} in the Control, GS-CF and GS-UF yoghurt, respectively. Growth in Milk 1 was much lower than in Milk 2, which could partially be linked to the shorter incubation time in Milk 1 as well as its lower buffering capacity (Table 2) (Beshkova, Simova, Frengova, Simov, & Adilov, 1998; Shihata & Shah, 2000). At the end of the production, the viable counts (cfu values) of *Lb. helveticus* in the fresh products of GS-CF were 4 times higher than in the Control, which were linked to their concentration during centrifugation, where the bacteria tend to remain in the curd (Nsabimana, Jiang, & Kossah, 2005).

The *Lb. helveticus* population (cfu values) of the GS-UF yoghurt remained relatively stable over the first 16 d of storage, but then suffered a 2.2 log cfu g^{-1} decline until day 44 (Fig. 5). The viable counts in the Control yoghurt had a similar profile, with a decline phase that occurred after day 31. The population in the GS-CF yoghurt remained relatively stable over the storage period, with a slight drop of 0.6 log cfu g^{-1} between days 16 and 44. At the end of the storage, the cfu values of *Lb. helveticus* in the GS-CF yoghurt were 15 times higher than in the Control and 18 times higher than in the GS-UF.

With the use of various fluorescent dyes and labelled antibodies (Chiron, Tompkins, & Burguière, 2018), flow cytometry (FC) allows the estimation of viable (FC_V) or dead (FC_D) cell counts for certain species. The FC_V counts remained stable in all three yoghurt types over the 44 days of storage (Fig. 5). There was no significant difference ($p > 0.05$) between the FC-based viable counts of Greek-style yoghurts. Both increased slightly more than 1 log FC_V g^{-1} between the inoculation step and the first sampling at day 1. The viable counts in the Control yoghurt increased by 0.8 log FC_V g^{-1} during the production phase, and remained lower ($p < 0.05$) than

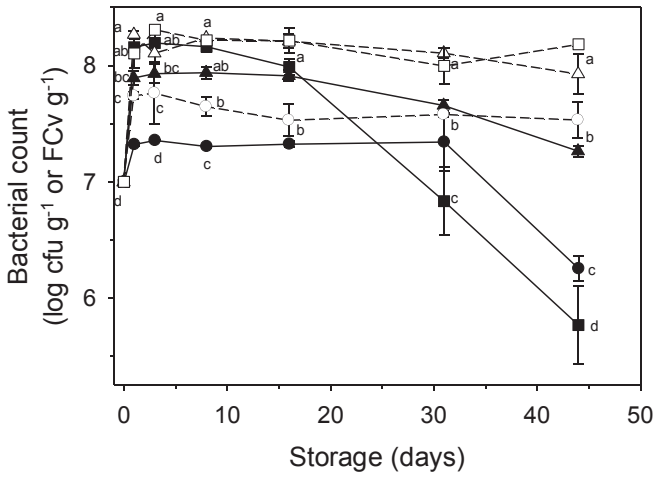

Fig. 5. Comparison of two methods for the enumeration of viable *Lactobacillus helveticus* R0052 in regular stirred yoghurt (Control; ●, ○) and Greek-style yoghurts made by centrifugation (GS-CF: ▲, △) or ultrafiltration (GS-UF: ■, □) during storage at 4 °C. Values at day 0 constitute the inoculated counts in milk matrices, while values at day 1 represent those in the fresh yoghurts. The solid lines and closed symbols represent the counts obtained by plating with selective media (cfu) and the dash lines and open symbols represent those obtained by flow cytometry for viable cells (FC_V). For each day of storage, different letters indicate significantly different counts ($p < 0.05$) in the three yoghurts and between the enumeration methods. Error bars represent standard error of the means (SEM). Solid lines indicate cfu g^{-1} while dashed lines are viable cell counts by flow cytometry (FCv).

the Greek-style yoghurts during storage. The levels of FC_D (data not shown) were significantly ($p < 0.05$) affected by the process. The Control yoghurt contained on average 27% dead cells while the GS-CF and GS-UF contained respectively 19% and 11% of FC_D. As for FC_V counts, FC_D counts remained stable during storage with no significant ($p > 0.05$) effect of time.

Some differences were therefore obtained in the "viable" probiotic counts between the two enumeration methods (Fig. 5). With the Control yoghurt, the FC_V counts were significantly higher than cfu values ($p < 0.05$), with exceptions for days 16 and 31. For the GS-UF yoghurt, there was no significant difference ($p > 0.05$) between the counts obtained by the two methods at the beginning of the storage until day 16. Subsequently, the cfu readings gave lower ($p < 0.05$) counts that the FC_V method. Within the GS-CF yoghurt, the counts followed two similar trends over the storage period, resulting in non-significant differences ($p > 0.05$) between the two methods for all the storage period except for days 31 and 44. Flow cytometry showed good FC_V values in all three yoghurt types until the end of the storage period. With FC, dead cell counts (FC_D) are based on the loss of cytoplasmic membrane integrity. However, the cfu and FC_V results suggest that the apparent mortality linked to the loss of cultivability (cfu) was not due to the loss of membrane integrity. Conditions in the GS-UF and Control yoghurts prevented cell growth on the selective media without affecting their apparent viability in FC assays. Neither pH nor lactic acid concentration was the sole factor to affect stability during storage, since the highest stability of *Lb. helveticus* was not observed in the product having the highest pH or the lowest lactic acid concentration. Further data are therefore needed to explain the higher stability of the probiotic strain in GS-CF yoghurt. In the regular stirred yoghurt, less lactic acid was produced, the proteolytic activity was very low and there was no concentration process applied. Thus, the cessation of growth in that yoghurt was more likely due to a lack of nutrients able to fulfill *Lb. helveticus* auxotrophies, such as peptide fractions and free amino acids (Christensen & Steele, 2003; Qingli et al., 2011; Shihata & Shah, 2000).

3.5. Syneresis in yoghurts

Syneresis was measured at the beginning and at the end of the storage (Table 4). It was superior ($p < 0.05$) in the Control yoghurt than in the Greek-style yoghurts. There were no significant changes ($p > 0.05$) in syneresis during storage for any product, and there was no interaction between storage time and product. Results obtained from the Control yoghurt were lower than those obtained by other studies that also used the centrifugation analytical method with regular stirred yoghurt (Amatayakul, Sherkat, & Shah, 2006; González-Martínez et al., 2002; Mani-López et al., 2014). However, highly variable values were obtained between the different yoghurts of those same studies, ranging between 23% and 80% depending on the total solids content of the yoghurt and the bacterial cultures used. Moreover, higher syneresis values were obtained in the Control yoghurt than in the Greek-style products, which is in line with most data on high-protein yoghurts (Jørgensen et al., 2019). The increase of total milk solids or proteins and the presence of exopolysaccharides (EPS) are known to reduce syneresis (Amatayakul et al., 2006; Wacher-Rodarte & Farres, 1993). Consequently, the higher protein content of the Greek-style yoghurts and their higher populations of starter bacteria, which were slime-producing cultures, have probably highly contributed to the syneresis reduction in those products. Furthermore, the stability of the syneresis values during storage is in accordance with the results obtained by Mani-López et al. (2014). The novelty of this study is that the manufacturing process does not affect syneresis of yoghurts having approximately 10% protein.

Table 4
Syneresis index of the regular stirred yoghurt (Control) and Greek-style yoghurts made by centrifugation (GS-CF) or ultrafiltration (GS-UF) during storage at 4 °C.[a]

Storage time	Syneresis index (%)		
	Control	GS-CF	GS-UF
Day 3	17.1[a]	2.0[b]	3.9[b]
Day 38	17.2[a]	0.8[b]	1.8[b]

[a] All products contained the probiotic strain *Lactobacillus helveticus* R0052. Syneresis index was calculated as the percentage of the weight of whey obtained after centrifugation divided by the weight of yoghurt sample used for centrifugation; values in a column followed by different superscript letters are statistically different ($p < 0.05$).

3.6. Some commercial implications of the results

This manuscript showed how manufacturing processes have important impacts on the chemical attributes and microbial populations of yoghurts. Three of these attributes could influence consumer preferences: levels of probiotics, level of lactose and lactic acid concentration (Aryana & Olson., 2017).

Consumers have a favourable view of probiotics in yoghurt (Cruz et al., 2013a; Plessas, Bosnea, Alexopoulos, & Bezirtzoglou, 2012) and higher levels in the Greek-style products could improve consumer perception.

Lactose-free products are increasingly brought to market, due to the problems of lactose maldigestion found in many sub-populations. As mentioned previously, classical yoghurt itself helps in reducing lactose maldigestion symptoms (EFSA, 2010). Both GS-UF and GS-CF had higher levels of streptococci than the Control product, which could presumably result in higher levels of β-galactosidase. Furthermore, GS-UF has 3 times less lactose than the Control yoghurt (Fig. 3). These two features could result in lower incidence of lactose maldigestion and attract a sub-population of consumers that do not generally consume dairy products having high levels of lactose.

With respect to consumers, another critical aspect that needs to be addressed is the effect of the processing methods on sensory properties (Cruz et al., 2013a; Esmerino et al., 2017). Lactic acid concentration, in particular, could affect acidity perception by consumers. Sensory attributes are so important that various methodologies are available to specifically analyse them in yoghurt (Desai, Shepard, & Drake, 2013; Pinto et al., 2018). Accordingly, assays are currently underway to quantify over 30 volatile/aromatic compounds of yoghurt that are produced by the cultures, and examine for correlations between chemical attributes (levels of volatiles, lactic acid and sugar) and the sensory attributes ("acid perception", "typical yoghurt aroma" and "off-flavours").

4. Conclusions

In conclusion, the processes used to increase the protein concentration of Greek-style yoghurt influence many attributes of the products, including the growth and the viability of starter and probiotic bacteria. Viable counts of *Lb. helveticus* R0052 can be increased during Greek-style yoghurt production. Moreover, concentration of the yoghurt by centrifugation seems to provide a more suitable matrix to enhance cell activity and growth of probiotic bacteria similar to *Lb. helveticus* during storage. Further research would be necessary to understand the better stability of *Lb. helveticus* in Greek-style yoghurt produce by centrifugation, and to see if the data obtained with *Lb. helveticus* R0052 can be extrapolated to other lactobacilli strains currently used as probiotics. Further research will also be needed to ascertain if the improved stability during storage in some Greek-style products can be extended to a better survival through digestion.

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