**Probiotic viability in yoghurts containing oligosaccharides derived
from lactulose (OsLu) during fermentation and cold storage**

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ABSTRACT

We studied the viability of probiotics *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* and microorganisms of a starter culture for set-style yoghurt, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, during manufacture and cold of storage of synbiotic yoghurts containing lactulose and the emerging prebiotic lactulose-derived oligosaccharide (OsLu). The evolution of the carbohydrate fraction, pH and microbial metabolites, such as organic acids, were assessed. Supplementation of yoghurts with lactulose significantly increased counts of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* during fermentation in comparison to control yoghurt, in agreement with lactulose partial consumption. No changes in OsLu content were observed during fermentation or subsequent storage. These results demonstrated that the chemical structure of the oligosaccharide plays a remarkable role in the fermentation of probiotics, suggesting OsLu as an alternative prebiotic to conventional lactulose in dairy products with adequate cell counts of probiotics while preserving the initial levels of the added prebiotic.

1. INTRODUCTION

In recent years, there has been an increase in the development of functional foods associated with the balance of colonic microbiota. Specific food ingredients can promote growth of beneficial microbes in the gut, providing health benefits beyond the nutritional value (Adebola, Corcoran & Morgan, 2014). Diet is considered one of the most important parameters that influences the composition and metabolic activity of the human gut microbiota (Conlon & Bird, 2014). Yoghurt is one of the most popular fermented milk products and an excellent vehicle for the delivery of functional ingredients (Fabersani et al., 2018).

Synbiotics are functional foods comprising mixtures both of probiotics and prebiotics that benefit the host. In yoghurts, prebiotics may act as a substrate for the growth of gut microbiota, that improves gastrointestinal functions and immune system (Macfarlane, Steed & Macfarlane, 2008). Probiotics are microorganisms with potential health-promoting effects on the host if they are sufficiently resistant to the harsh conditions of the gastrointestinal tract (Sadeghi, Pourahmad & Mokhtare, 2017, Tripathi & Giri, 2014). The most common probiotic strains for synbiotic product formulation are those belonging to the genera *Lactobacillus* and *Bifidobacterium* (Fazilah, Ariff, Khayat, Rios-Solis & Halim, 2018).

Prebiotics are substrates selectively utilized by host microorganisms, that confer a health benefit (Gibson et al., 2017). Different types of prebiotics, such as galactooligosaccharides (GOS) (Delgado-Fernández, Corzo, Olano, Hernández-Hernández & Moreno, 2019a, Vénica, Bergamini, Rebecchi & Perotti, 2015), inulin of different chain lengths (Kip, Meyer & Jellema, 2006, Mazloomi, Shekarforoush, Ebrahimnejad & Sajedianfard, 2011), fructooligosaccharides (FOS) (Ahmadi et al,

2014; Akalin, Gönç, Ünal & Fenderya 2007; Delgado-Fernández et al., 2019a), resistant starch and lactulose (Aghajani, Pourahmad & Mahdavi Adeli, 2014, Delgado-Fernández et al., 2019a, Nobakhti, Ehsani, Mousavi & Mortazavian, 2009, Oliveira, Florence, Perego, De Oliveira & Converti, 2011), have been used in the manufacture of yoghurt and other fermented milks.

Lactulose is a well-recognized prebiotic which is resistant to the action of human digestive enzymes, thus, reaching the colon unaltered where it is selectively fermented by bifidobacteria and lactobacilli (Villamiel, Montilla, Olano, Corzo, 2014). However, its main disadvantage is rapid bacterial fermentation at the proximal colon, which may give rise to gas production as a side effect (Schuster-Wolff-Bühning, Fischer & Hinrichs, 2010). By considering that chemical structure may affect fermentation properties of probiotic microorganisms, lactulose-derived oligosaccharides (OsLu) are a new group of compounds with potential new applications. These oligosaccharides were previously synthesized in our laboratory and exhaustively characterized as disaccharides and oligosaccharides with a degree of polymerization of ≥ 3 . Due to their larger size, OsLu might be fermented more slowly than lactulose and, therefore, be able to reach the distal colon, where many chronic gut disorders originate, and modulate gut microbial composition (López-Sanz, Montilla, Moreno & Villamiel, 2015). *In vitro* and *in vivo* (using rats) assays have demonstrated different properties of OsLu, such as a bifidogenic effect, low digestibility degree and prebiotic properties, being resistant to gut digestion (Cardelle-Cobas et al., 2009, 2011, 2012; Hernández-Hernández et al., 2012). Biological activities such as anti-inflammatory (Algieri et al., 2014), a positive effect on iron absorption in rats (Laparra, Diez-Municio, Herrero, Moreno, 2014), as well as absence of any toxicological adverse effects and good tolerance was confirmed in acute and subchronic (28 days) assays using rats (Anadón et al., 2013). More

recently, chemopreventive effects against colorectal cancer have also been demonstrated (Fernández et al., 2018). All these findings revealed that OsLu could be used as a prebiotic ingredient instead of lactulose in a wide range of functional foods, such as dairy products. Indeed, OsLu was successfully used as functional ingredient in milk and processed apple juice, showing excellent thermal stability (López-Sanz et al., 2015). However, in addition to resistance to heating, further investigation on OsLu performance through fermentation by lactic acid bacteria and the subsequent shelf-life period of value-added dairy products, such as synbiotic yoghurts, is warranted.

Therefore, the objective of this work was to study the viability of probiotics *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis* and microorganisms of the starter culture of set-style yoghurt, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, during manufacture and cold storage of synbiotic yoghurts containing OsLu at two concentrations (2 or 4%). Evolution of pH and carbohydrates, as well as the formation of organic acids (lactic and acetic acids) was also been evaluated. A comparative study with synbiotic yoghurts containing lactulose as prebiotic was also conducted.

2. MATERIALS AND METHODS

2.1 Materials

The trade name of the lyophilized probiotic starter culture was Lyoflora SYAB 1 from Sacco S.r.L. (Cadorago, CO, Italy) and contained *Lactobacillus acidophilus*, *Bifidobacterium animalis* ssp. *lactis*, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. After procurement, storage and maintenance were carried out according to the recommendations of the manufacturer.

Standards of galactose, glucose, fructose, phenyl- β -D-glucopyranoside, lactulose, raffinose and nystose were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lactose was supplied by Thermo Fisher Scientific (Bremen, Germany).

Pasteurized milk and skim milk powder were purchased in a local supermarket. Commercial Galactofructose[®] (Solactis Group, Paris, France) had a carbohydrate composition of 74.5% lactulose, 7.5% galactose, 8% lactose and 10% epilactose, tagatose and fructose. Oligosaccharides derived from lactulose (OsLu) were previously synthesized in our laboratory according to Cardelle-Cobas et al. (2016), and with a carbohydrate composition of 19.2, 12.4, 1.2% of fructose, galactose, glucose, respectively, 24.7% lactulose and 42.2% of the prebiotic (spanning from OsLu disaccharides to tetrasaccharides plus traces of pentasaccharides) per 100 g of total carbohydrates, respectively. Therefore, OsLu comprise a complex mixture of oligosaccharides containing galactose units linked to a fructose residue at the reducing end and whose predominant glycosidic bond was β (1-6).

2.2 Preparation of yoghurt starter culture

Pasteurized milk (1 L) containing 25 g of skim milk powder was heated at 75°C for 10 min and cooled to 43°C before inoculation. Then, starter culture containing probiotics was added at a concentration of 4 g L⁻¹ (corresponding to 1 UC/100L of milk) according to the specifications of the manufacturer and incubated at 45°C for 7 h. The mixture was refrigerated at 4°C until used to prepare the synbiotic yoghurts.

2.3. Manufacture and cold storage of synbiotic yoghurts

Synbiotic yoghurts were prepared using pasteurized milk containing 2.5 % (w/v) skim milk powder which was heated at 75°C for 10 min. The heat treated milk was

transferred to 250 mL flasks and different batches of control (without added prebiotics) and yoghurt mixes supplemented with the corresponding prebiotic (OsLu or lactulose) at 2% and 4% (w/v) were prepared in duplicate. Once the prebiotics were dissolved, the mixture was cooled to 43°C in an ice water bath and 10 g of yoghurt starter culture previously prepared (Section 2.2) was inoculated. After inoculation, each 250 mL flask was divided into 50 mL portions and incubated at 43°C until the pH decreased to 4.6-4.7 and coagulation took place. Samples were then cooled to room temperature and then stored at 4 °C for 28 days. Samples were taken every hour during fermentation process (0-6 h), as well as during the subsequent storage (at 7, 14, 21 and 28 days) for analysis. Each experiment was carried out in duplicate.

2.4 Analytical determinations

All analyses were carried out in triplicate per prepared yoghurt.

2.4.1 Determination of pH of yoghurt samples

The pH was measured in control and synbiotic yoghurts with an electrode pH-meter (Mettler Toledo GmbH, Schwerzenbach, Switzerland) during fermentation (0-6 h) and cold storage at 4° C (7, 14, 21 and 28 days).

2.4.2 Enumeration of starter culture microorganisms

Samples of yoghurts were collected after fermentation and during cold storage at 4 °C and were analyzed within 24 h after collection. Bacterial counts were determined at the end of fermentation, 7, 14, 21 and 28 days of storage.

Phosphate buffer saline (PBS) 1X was used to prepare dilutions for microbiological analysis. *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus* were quantified according to Tabasco, Paarup, Janer, Peláez, & Requena (2007), but with some modifications. *Streptococcus*

thermophilus was incubated at 37°C for 72 h on M-17 agar, containing 1% lactose (M17-lactose) and supplemented with 1.2% bacteriological agar (Scharlab, Barcelona, Spain). For enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus* appropriate dilutions were pour-plated on MRS-fructose fermentation broth (Condalab, Madrid, Spain), which did not contain meat extract and glucose. It was supplemented with 0.2% w/v Tween[®] 80, 1% w/v fructose, 0.05% w/v cysteine, and 1.2% w/v agar. Plates were incubated in anaerobic jars at 45°C for 72 h and lenticular colonies with 1–2mm diameter were enumerated as *Lactobacillus delbrueckii* ssp. *bulgaricus* whereas non-core shapeless colonies were identified as *Lactobacillus acidophilus*. These colonies with diverse morphology were corroborated with single cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus* using MRS-fructose.

In the case of *Bifidobacterium animalis* ssp. *lactis*, the MRS-LP-modified agar was used for their enumeration according to Fachin, Moryia, Gândara & Viotto (2008). It was supplemented with 0.1% w/v Tween[®] 80, 1% w/v lactose, 0.05% w/v cysteine, 0.2% w/v lithium chloride, 0.3% w/v sodium propionate and 1.2% w/v agar (Scharlab). Plates were incubated in anaerobic jars at 45°C for 72 h.

2.4.3 Determination of carbohydrates

Mono-, di- (with the exception of lactose and lactulose) and oligosaccharides from control and prebiotic (OsLu and lactulose) supplemented yoghurts, were determined by GC-FID. Before analysis, 10 mL of methanol at 99.9% (Merck Millipore, Madrid, Spain) were added to 1g of control and prebiotic supplemented yoghurts (2 and 4% w/v) and kept for 1 hour at room temperature to precipitate proteins and fats. Later, the supernatant was centrifuged at 10,000 x g for 5 min and then, prior to derivatization, 500 µL of supernatant was evaporated with 400 µL of a phenyl-β-D-

glucopyranoside (internal standard) solution (0.5 mg mL^{-1}). Carbohydrates were analysed as trimethyl-silylated oximes (TMSO) prepared following the method of Brobst & Lott (1966). Sugar oximes were formed by adding $250 \mu\text{L}$ of hydroxylamine chloride (2.5% w/v) in pyridine to dried samples and heating the mixture at 70°C for 30 min and then silylated with hexamethyldisilazane ($250 \mu\text{L}$) and trifluoroacetic acid ($25 \mu\text{L}$) and kept at 50°C for 30 min. Reaction mixtures were centrifuged at $10,000 \times g$ for 2 min at room temperature. Supernatants were injected or stored at 4°C prior to analysis.

Chromatographic analysis was then carried out following the method of Montilla, Van de Lagemaat, Olano & Del Castillo (2006) using an Agilent Technologies 7890A Gas Chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector (FID) using a fused silica capillary column DB-5HT, bonded, crosslinked phase (5% phenyl-methylpolysiloxane; $30 \text{ m} \times 0.32 \text{ mm i.d.}$, $0.10 \mu\text{m}$ film thickness) (J&W Scientific, Folson, CA, USA). The oven temperature was initially 150°C and increased at a rate of 3°C min^{-1} to 380°C and held at this temperature for 76 min. The temperature of injector and detector were at 280°C and 385°C , respectively. Injections were carried out in split mode (1:20) using nitrogen as carrier gas at a flow rate of 1 mL min^{-1} . Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software.

Quantification of each sugar was performed by internal standard calibration using phenyl- β -D-glucopyranoside (0.5 mg mL^{-1}). Response factors were calculated after analysis of standard solutions of glucose, galactose, fructose, lactose, raffinose and nystose over the expected concentration range in samples ($0.1\text{-}5 \text{ mg mL}^{-1}$).

In synbiotic yoghurts supplemented with lactulose and OsLu, analyses of lactulose and lactose were carried out by LC-RID due to co-elution in GC-FID analysis of these two disaccharides. First, proteins in yoghurt samples were precipitated according to the method of Moreno, Olano, Santa-Maria & Corzo (1999) with some modifications. Yoghurt sample (1 g) was mixed with equal volumes (100 μ L) of Carrez I (7.2% w/v $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$) and Carrez II (14 % w/v $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) diluted to 10 mL with acetonitrile/water 70/30 (v/v). The resulting mixture was centrifuged at 10,000 x g for 10 min and the supernatant was filtered through a 0.45 μ m filter. Chromatographic analysis was carried out following the method proposed by Ho Ko & Cheong (2001) with some modifications using a LC Agilent Technologies 1220 Infinity LC System 1260 equipped with a Refractive Index Detector (RID). Analyses of samples were performed in an aminopropyl silane column, Kromasil (100-5-NH₂) (250 x 4.6 mm and 5 μ m particle size) with a pore size of 100 Å (Akzo Nobel, Brewster, NY, USA). Separation was in isocratic mode at 30°C and the mobile phase was acetonitrile-water (70/30, v/v) at a flow rate of 0.7 mL min⁻¹. Quantitative analysis was performed by the external standard method, using galactose, glucose, fructose, lactose, lactulose, raffinose and nystose in the range 0.2-25 mg mL⁻¹. Linear regression curves for each standard were always greater than 0.99. Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software.

2.4.4 Determination of microbial metabolites (organic acids)

Determination of organic acids in yoghurts samples was carried out by HPLC-UV following the method of da Costa, da Silva Frasao, da Costa Lima, Rodrigues & Junior (2016). Before analysis, 1mL of yoghurt was mixed with 10 mL of 0.0045 M sulfuric acid. The resulting mixture was treated with ultrasound and centrifuged at

10,000 x g for 10 min for removal of proteins and the supernatant was filtered through a 0.45 µm filter.

Separation of organic acids was achieved using the LC Agilent Technologies 1220 Infinity LC System 1260 equipped with an ultraviolet detector ($\lambda = 210\text{nm}$) and using an ion exchange column with sulfonated styrene-divinylbenzene spheres REZEXTM ROA, crosslinked resin (8% hydrogen; 300 x 7.8 mm and 8mm particle size) (Phenomenex, Torrance, CA, USA). Separation was in isocratic mode at 40°C and the mobile phase was 0.005 M sulfuric acid at a flow rate of 0.5 mL min⁻¹.

Quantification of organic acids was carried out by the external standard method using standard solutions of lactic acid and acetic acid prepared in concentrations from 0.005-2.0 mg mL⁻¹. Linear regression curves for each standard were always greater than 0.99. Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software.

2.5. Statistical Analysis

Experimental data of bacterial counts and carbohydrate evolution as well as organic acids formed during fermentation and cold storage of synbiotic yoghurts are presented as mean values $\pm$ standard deviations. Mean values of these parameters were submitted to analysis of variance using General Linear Model (GLM) by the Statistica Software 6.0 (SPSS Inc, Chicago, IL, USA). Factorial-repeated measures ANOVA were also used to explore the influence of time, type and amount of prebiotic. They were compared using the Tukey test at significance level $P < 0.05$.

3. RESULTS

3.1. Evolution of pH during fermentation and cold storage of control and synbiotic yoghurts

The decline in pH during fermentation and the subsequent storage of all prepared yoghurts is shown in **Figures 1a-e**. The initial pH of synbiotic and control yoghurts was $\text{pH} \approx 6.6$ and decreased to 4.7-4.6 at the end of fermentation in all yoghurts. The sharpest pH decrease was observed after 2 h of fermentation, with coagulation taking place around 4 h in control and prebiotic supplemented yoghurts, except for yoghurts supplemented with lactulose at 4% which took 6 h to reach pH 4.6 (**Figure 1e**).

During cold storage, pH values decreased slightly reaching values of $\text{pH} \approx 4.4$ due to the low acidifying activity of the lactic acid bacteria at 4 °C and in an acidic environment.

3.2. Changes in the counts of lactic acid bacteria during cold storage

Viable cell numbers of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and probiotic microorganisms (*Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis*) during cold storage (28 days) of manufactured yoghurts are shown in **Figures 2a-d**.

Viable counts of *Streptococcus thermophilus* ranged from 9.2 to 9.4 log CFU mL⁻¹ at the end of fermentation for all studied yoghurts (**Figure 2a**) and minimal changes were observed during the storage period.

Lactobacillus delbrueckii ssp. *bulgaricus* numbers were significantly higher in the two synbiotic yoghurts containing lactulose (achieving values of 7.4 and 7.8 log CFU mL⁻¹ for 2 and 4%, respectively) compared to the control and synbiotic yoghurts supplemented with OsLu, at the end of fermentation and also during the entire storage period ($P < 0.05$) (**Figure 2b**). From the fourteenth day of storage, *Lactobacillus*

delbrueckii ssp. *bulgaricus* count showed a decrease in yoghurts supplemented with lactulose (especially at 4%) reaching values of ≈ 7.0 log CFU mL⁻¹ at the end of storage (28 days).

Figure 2c shows the viability of *Lactobacillus acidophilus* during cold storage. At the beginning of storage, it ranged from 7.0 to 7.2 log CFU mL⁻¹ ($n=3$) in control or OsLu added yoghurts, and had no changes during storage. However, counts of *Lactobacillus acidophilus* were greater when lactulose was incorporated, reaching values of 7.5 and 8.0 log CFU mL⁻¹ for 2 and 4%, respectively, at the end of fermentation. There was however a decline in cell counts in synbiotic yoghurts containing lactulose at 4% between the second and third week of storage, decreasing to a final value of ≈ 7 log CFU mL⁻¹.

Finally, viability of *Bifidobacterium animalis* ssp. *lactis* during storage is shown in **Figure 2d**. At the end of fermentation, the counts were ≈ 6.4 log CFU mL⁻¹ in control yoghurt and one with the addition of OsLu. However, higher initial counts were found in yoghurts with added lactulose, having values of 6.7 and 6.8 log CFU mL⁻¹ at 2 and 4%, respectively, in comparison to other yoghurts. This behavior was similar to that previously observed for *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus acidophilus* counts. Furthermore, viability of *Bifidobacterium animalis* ssp. *lactis* remained stable to the end of storage in all yoghurts.

3.3. Evolution of carbohydrates during manufacture and cold storage of synbiotic yoghurts

3.3.1. Monosaccharides

Figures 3a-c shows changes in galactose, lactose and lactulose during fermentation and cold storage of the yoghurts. Negligible levels of glucose were found

during fermentation and storage of control and supplemented yoghurts ($\sim 0.1 \text{ g } 100 \text{ g}^{-1}$). In the case of galactose (**Figure 3a**), the content at the beginning of fermentation was dependent on type of yoghurt, likely due to the presence of galactose in the initial prebiotics used to produce the synbiotic yoghurts. During fermentation, liberation of galactose was detected in all samples. The content of galactose increased during the fermentation process in all yoghurts, reaching values of $0.5\text{-}1.0 \text{ g } 100 \text{ g}^{-1}$, and then it remained constant for the whole storage period.

In addition, initial levels of fructose were found in yoghurts supplemented with OsLu at 2 and 4%, being 0.4 and 0.8%, respectively, at the beginning of fermentation. Lactic acid bacteria metabolized this monosaccharide, with a percentage of degradation of 12-13% after 28 days of storage. Trace amounts of fructose were also found in yoghurts supplemented with lactulose during fermentation due to hydrolysis of lactulose (mostly at 4%) during fermentation (data not shown).

3.3.2. Lactose

Figure 3b shows lactose levels during fermentation and cold storage of control and synbiotic yoghurts. Initial content of lactose was in the narrow range from 5.3 to 5.4 $\text{g } 100 \text{ g}^{-1}$ of yoghurt. Decrease in lactose was significantly higher from the second hour of fermentation, in good agreement with the pH decrease described in **Figure 1**, reaching values of 30-38% of hydrolysis at the end of fermentation. During the storage, lactose decreased slightly in all yoghurts, with final values in the range from 3.0 to 3.6 $\text{g } 100 \text{ g}^{-1}$. However, this trend was less pronounced in yoghurts with lactulose added at 4% (final decrease of 33%), as compared to the other yoghurts (39-43%).

3.3.3. Prebiotics

Changes in lactulose content during fermentation and cold storage are shown in **Figure 3c**. It can be observed, lactic acid bacteria did not significantly hydrolyze lactulose at the lower concentration (only 15% of lactulose was hydrolyzed at the end of storage). However, when lactulose was added at 4% a remarkable and significant decrease ($P < 0.05$) was obtained, mainly from the second hour of the fermentation, decreasing 40.8% and 43.3% at the end of fermentation and storage, respectively.

In contrast to lactulose behavior, initial levels of prebiotic carbohydrates present in OsLu did not exhibit any change ($P < 0.05$) throughout fermentation and cold storage regardless of concentration and degree of polymerization of the prebiotic (**Figure S1**).

3.4 Formation of organic acids during yoghurt fermentation and cold storage

The organic acids detected, because lactic acid bacteria metabolism during fermentation and storage of the yoghurts, were lactic and acetic (**Figures 1a-e**).

Lactic acid was the more abundant organic acid with an initial content from 0.1 to 0.2 g 100 g⁻¹ in all produced yoghurts. This amount increased from the second hour of fermentation, consistent with the decrease of pH previously observed (**Figures 1a-e**), as well as with hydrolysis of lactose (**Figure 3b**). At the end of fermentation, levels of lactic acid in control and synbiotic yoghurts with OsLu added at 2 and 4% were around 0.5 g 100 g⁻¹ of yoghurt (**Figures 1a-c**). Interestingly, production of this organic acid during fermentation was higher in synbiotic yoghurts with lactulose, achieving values of 0.8 g 100 g⁻¹ of synbiotic yoghurt (**Figures 1d-e**). During storage, the acid content increased significantly, attaining final values of 0.7 and 0.8 g 100 g⁻¹ of yoghurt in the case of control and OsLu supplemented yoghurts, whereas in yoghurts with lactulose production of lactic acid was higher, especially when lactulose was added at 4% (1.1 g 100 g⁻¹ of yoghurt).

Acetic acid was also produced in all yoghurts during fermentation (**Figures 1a-e**). The initial content in all yoghurts ranged from 0.05 to 0.1 g 100 g⁻¹, then slightly increase from the second hour of fermentation, to values of 0.2 g 100 g⁻¹ at the end of incubation; with the exception of yoghurts with lactulose, where the levels of acetic acid increased to 0.3 and 0.4 g 100 g⁻¹ at 2 and 4% of added lactulose, respectively. All values remained stable during storage.

4. DISCUSSION

Viability of four lactic acid bacteria (including two probiotic strains), as well as changes in carbohydrate fraction, pH and organic acids in set-style control and synbiotic yoghurts supplemented with new oligosaccharides derived from lactulose (OsLu) and lactulose at two concentrations (2 and 4%), were comprehensively assessed. Overall, no microbiological or compositional differences (excluding the added prebiotics) were found between the control and the synbiotic yoghurts with OsLu, which was attributed to the fact that lactose assimilation prevailed over OsLu consumption during the fermentation and subsequent cold storage periods. These results are in line with the recently demonstrated tolerance to OsLu in the manufacture and cold storage of kefir, with no affect on fermentation and composition of the final product (Delgado-Fernandez, Corzo, Lizasoain, Olano & Moreno, 2019b).

In contrast to control and synbiotic yoghurts with OsLu, remarkable differences were observed during the manufacture of yoghurts containing lactulose. Addition of lactulose at 4% extended the time necessary to complete fermentation (**Figure 1e**). Delgado-Fernández et al. (2019a) using a less complex starter culture, i.e. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, also reported a fermentation time of 6 h in yoghurts supplemented with lactulose at 4%. In contrast to

these results, Oliveira et al. (2011) reported shorter fermentation times when lactulose was added at 4% in skim milk inoculated with *Lactobacillus bulgaricus*, *Lactobacillus acidophilus* and *Bifidobacterium lactis* in co-culture with *Streptococcus thermophilus*. This behavior could be explained by the use of different co-cultures, as well as by addition of mixtures of prebiotics also having monosaccharides and lactose instead of pure substrates, which could affect the rate of fermentation. In our work, a longer fermentation time in yoghurts supplemented with lactulose at 4% could be explained by the fact that lactose is fermented faster than lactulose only during the first stages of fermentation when lactose concentration was higher than lactulose levels. Concretely, according to **Figures 3b** and **3c**, lactose was more rapidly hydrolyzed than lactulose during the first two hours of fermentation, whereas between the second and third hour of fermentation both lactulose and lactose were present in similar levels. Thus, as the lactose concentration decreased during fermentation, the rate of lactulose fermentation increased, which could be the cause of the slow acidification observed from the second hour of fermentation (**Figure 1e**) and, therefore, the extension of the fermentation time. Following fermentation, the significant decline of pH observed in all yoghurts during cold storage was in agreement with those observed by Özer, Akin, & Özer (2005) in yoghurts containing *Lactobacillus acidophilus* and *Bifidobacterium bifidum*, where pH values of 4.3-4.4 were reported after 14 days of refrigerated storage.

Concerning the viable counts of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and probiotics (*Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis*), the addition of OsLu neither stimulated nor affected negatively the growth of these lactic acid bacteria, counts were fairly similar to those in the control yoghurt. However, addition of lactulose at 2% and, especially, 4% significantly increased the counts of all probiotics and *Lactobacillus delbrueckii* ssp.

bulgaricus at the end of fermentation (**Figures 2b-d**). Indeed, a concentration-effect relationship was observed in the case of the counts of *Lactobacillus delbrueckii* ssp. *bulgaricus* in which lactulose at 4% promoted growth to a greater extent than lactulose added at 2% (**Figure 2b**). However, lactobacilli cell counts significantly ($P > 0.05$) declined from the fourteenth day of storage in yoghurts supplemented with lactulose (**Figures 2b and 2c**). This trend was also observed by Oliveira et al. (2011) with co-cultures of *Streptococcus thermophilus* and *Lactobacillus acidophilus*, where lactulose at 4% stimulated the growth of these lactic acid bacteria although the cell counts started to decrease at 35 days of storage. Interestingly, the increased counts of *Bifidobacterium animalis* ssp. *lactis* remained stable during storage, which demonstrates the potential bifidogenic effect of synbiotic yoghurts with added lactulose during the whole shelf-life period (**Figure 2d**). Oliveira et al. (2011) also reported higher cell counts of *Bifidobacterium animalis* ssp. *lactis* during storage of fermented skim milk when lactulose was added at 4%. Finally, *Streptococcus thermophilus* counts remained stable ($P > 0.05$) from the end of fermentation and throughout refrigerated storage for all studied yoghurts (**Figure 2a**). In agreement with this, high viability of *Streptococcus thermophilus* during storage of set yoghurts produced by *Lactobacillus rhamnosus* and *Bifidobacterium animalis* subsp. *lactis* in co-fermentation with traditional starters has been previously described (Settachaimongkon et al., 2014). Furthermore, Özer et al. (2005) did not observe any statistically significant difference for the counts of *Streptococcus thermophilus*, in yoghurts with added lactulose (2.5%) during 14 days of storage.

Utilization of monosaccharides, lactose and prebiotics by lactic acid bacteria was also investigated during fermentation and storage. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* metabolized lactose as the main carbon

source, being unable to metabolize galactose (Ohlsson et al., 2017; Vaillancourt et al., 2008). Several studies have demonstrated that bifidobacteria (Moineau-Jean, Champagne, Roy, Raymond & LaPointe, 2019) and *Lactobacillus acidophilus* (Leahy, Higgins, Fitzgerald & Van Sinderen, 2005) are saccharolytic microorganisms capable of fermenting glucose, lactose, galactose, apart from other substrates. Indeed, Moineau-Jean et al. (2019) reported that galactose content should be similar to the production of lactic acid as it occurred in our control yoghurt (**Figure 1a**). However, due to the complexity of the co-cultures and use of commercial mixtures of OsLu and lactulose with minor amounts of galactose, a possible small consumption of galactose by probiotic bacteria remains to be ascertained. Apart from that, galactose content increased during the fermentation period in all studied yoghurts (**Figure 3a**), which agrees with findings by Farnworth et al. (2007) in yoghurts containing *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis*. Furthermore, data reported by Delgado-Fernández et al. (2019a) in yoghurts using a starter culture having *Lactobacillus delbrueckii* ssp. *bulgaricus* in co-culture with *Streptococcus thermophilus* and lactulose added at 2% and 4% were also in agreement with the present results. Low quantities of glucose (~0.1 g 100 g⁻¹) were found in control and synbiotic yoghurts during fermentation and storage, which is in line with previous results (Delgado-Fernández et al., 2019a). Thus, lactic acid bacteria quickly metabolized glucose released from lactose during the fermentation and storage stages to produce lactic and acetic acids. In addition, free fructose coming from the mixture of OsLu or released from lactulose at high concentration was also metabolized by *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis*, being a preferably carbon source to produce lactic and acetic acids, as well as CO₂ by different pathways (Gomes & Malcata, 1999).

As expected, efficient hydrolysis of lactose (**Figure 3b**) was observed in control and synbiotic yoghurts. However, lactose was hydrolyzed to a lesser extent in yoghurts supplemented with lactulose, being significantly ($P < 0.05$) different when 4% was added. Lactulose, when incorporated at relatively similar concentration (*i.e.*, 4%) as lactose, promoted growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* and probiotic strains, affecting significantly the hydrolysis of lactose, as compared to control yoghurt. This bifidogenic effect of lactulose in synbiotic yoghurts has been previously corroborated by several authors (Delgado-Fernández et al. 2019a; Lu, Yeung & Yeung, 2018; Olano, López-Covarrubias, Ramos & Suárez, 1986; Rastall & Maitin, 2002). Remarkably, the decrease in lactulose found in our work, especially addition at 4% (**Figure 3c**), was higher than previously reported, which could be due to the complexity of the starter culture used in our study that included two probiotic strains (*i.e.*, *Lactobacillus acidophilus* and *Bifidobacterium animalis* ssp. *lactis*) in co-culture with a traditional yoghurt starter. The substantial decrease in lactulose, 40.8%, at the end of fermentation could explain the limited consumption of lactose observed during fermentation of the yoghurt with 4% lactulose, by lactic acid bacteria as compared with the other yoghurts due to the fact that lactose was not the only major carbohydrate to be metabolized under these particular conditions. In the case of synbiotic yoghurts with lactulose added at 2%, the decrease in lactulose was rather moderate (**Figure 3c**) and, consequently, the lactic acid bacteria preferably utilized lactose which was, indeed, initially present around 2.8-folds higher than lactulose. Whereas, in the case of yoghurts with 4% lactulose, both substrates were available in a much more balanced way. On the contrary, the addition of OsLu at different concentrations did not affect the sugar metabolism of lactic acid bacteria during fermentation and storage, probably due to the

degree of polymerization of these oligosaccharides ($DP \geq 3$) and to the great variety of structurally-related carbohydrates present in OsLu composition.

Concerning the production of organic acids fermentation, lactic acid was found in quantities at the beginning of fermentation in all yoghurts higher than those reported by Shah & Ravula (2000), which could be explained by the different specific conditions and type of commercial starter cultures. Lactic acid increased progressively during fermentation and storage, in parallel with the decline of pH (**Figure 1**) and hydrolysis of lactose (**Figure 3b**) previously observed in control and yoghurts supplemented with OsLu. Nevertheless, higher values of lactic acid were observed in yoghurts supplemented with lactulose (mainly at 4%), which did not fully match the values of pH previously studied (**Figures 1 d-e**). In this case, the addition of lactulose could be an adjuvant factor in the proteolysis of the complex co-cultures used for fermentation, which could affect the buffering capacity of milk. Donkor, Henriksson, Vasiljevic & Shah (2006) reported severe proteolysis by *Bifidobacterium animalis* ssp. *lactis*, *Lactobacillus acidophilus* and *Lactobacillus paracasei* when present in co-culture with yoghurt bacteria, releasing greater amounts of amino acids. We have demonstrated that the consumption of lactulose by probiotics enhanced the growth of the two *Lactobacillus* species and *Bifidobacterium animalis* ssp. *lactis* (**Figures 2b-d**), which could increase the proteolysis-derived compounds in the media. In agreement with our observation, Ramchandran & Shah (2010) reported that addition of oligofructoses to reconstituted skim milk improved the proteolytic capabilities of *Lactobacillus acidophilus* and *Bifidobacterium longum* and both strains produced more lactic acid and acetic acid in the presence of oligofructoses than in the corresponding control. Thus, high proteolysis-derived compounds released from *Lactobacillus delbrueckii* ssp. *bulgaricus* and probiotics could explain the differences in the lactic acid production

between yoghurts with lactulose and the rest of yoghurts without impacting the final pH value.

Acetic acid was produced by *Streptococcus thermophilus* and *Lactobacillus bulgaricus* during fermentation in all yoghurts because of their homofermentative metabolism (Delgado-Fernández et al. 2019a; Donkor, Henriksson, Vasiljevic & Shah, 2007). However, the presence of probiotic bacteria in co-culture with traditional starter cultures is generally associated with a higher production of acetic acid via heterofermentative pathway (Vénica et al., 2015). The higher production of acetic acid in yoghurts supplemented with lactulose as compared to the rest of yoghurts could be produced by *Lactobacillus acidophilus* from degradation of citrate and/or by bifidobacteria using the bifidus pathway (Østlie, Helland & Narvhus, 2003). Finally, the formation of acetic acid was not boosted by addition of OsLu thus avoiding the potential promotion of an unpleasant and undesirable “vinegary” taste which would diminish the organoleptic properties and quality of yoghurts (Chen et al., 2017).

4. Conclusions

Findings of this work showed the feasibility to produce synbiotic yoghurts supplemented with oligosaccharides derived from lactulose (OsLu) at 2 and 4% as the viability of lactic acid bacteria (including probiotic strains) and structural integrity of OsLu were not jeopardized during fermentation and the subsequent 28 day-refrigerated storage. Hence, this new prebiotic could be an excellent functional ingredient to be incorporated into dairy products, as it could reach intact, the descendent colon and stimulate beneficial intestinal bifidobacteria and lactobacilli, being an alternative to lactulose. To conclude, yoghurts and related fermented dairy products may serve as excellent food matrices for OsLu based on their microbiological and compositional

properties, although further studies are needed to evaluate the influence of OsLu on the rheological properties of synbiotic yoghurts, as well as, on their sensorial quality.

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FIGURE CAPTIONS

Figure 1. Determination of pH (black square) and organic acids, lactic (black circle) and acetic acid (black triangle) in: control yoghurts (**a**) and synbiotic yoghurts with oligosaccharides derived from lactulose (OsLu) at 2% (**b**) and 4% (**c**), and lactulose at 2 (**d**) and 4% (**e**) during fermentation and cold storage. Error bars represented standard deviations ($n=6$).

Figure 2. Survival of *Streptococcus thermophilus* (**a**), *Lactobacillus delbrueckii* ssp. *bulgaricus* (**b**), *Lactobacillus acidophilus* (**c**), *Bifidobacterium animalis* ssp. *lactis* (**d**) in control and synbiotic yoghurts with oligosaccharides derived from lactulose (OsLu) and lactulose at 2 and 4% during cold storage. Error bars represented standard deviations ($n=6$).

Figure 3. Evolution of galactose (**a**), lactose (**b**) and lactulose (**c**) during fermentation at 45°C and cold storage of control and synbiotic yoghurts supplemented with OsLu or lactulose at 2 and 4%. Error bars represented standard deviations ($n=6$).

Figure 1

Figure 2

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Figure 3