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Effect of selected prebiotics on the growth of lactic acid bacteria and physicochemical properties of yoghurts

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A B S T R A C T

The feasibility of manufacturing yoghurts with three prebiotics, i.e., fructooligosaccharides (FOS), galactooligosaccharides (GOS) or lactulose added at two concentrations (2 and 4%, w/v) was investigated. Physicochemical and microbial characterisation was conducted on the effect of prebiotics on acidification, viability of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, lactose and prebiotic consumption and production of organic acids during fermentation (up to 6 h) and cold storage (for 28 d). GOS and FOS remained unaltered throughout fermentation and cold storage, while the viability of starter culture was similar to that observed in the control yoghurt. Yoghurts manufactured with 4% lactulose showed a significant decrease of lactulose content associated with a lower decrease of lactose relative to the control. This effect was associated with a significant increase (2.2 $\log_{10}$ cfu mL^{-1}) in *L. delbrueckii* ssp. *bulgaricus* and a moderate decrease (1.0 $\log_{10}$ cfu mL^{-1}) in *S. thermophilus* at the end of cold storage.

1. Introduction

Over recent years, considerable progress has been made in understanding the function and potential role of the gut microbiota in health and disease. There is evidence that gut microbiota plays an important role not only in gastrointestinal diseases but also in systemic diseases such as obesity, diabetes, rheumatoid arthritis and autoimmune encephalomyelitis (Cénit, Matzaraki, Tigchelaar, & Zhernakov, 2014). Although highly stable over time, the composition and activities of the microbiota may be influenced by a number of factors, diet being an important one (Power, O'Toole, Stanton, Ross, & Fitzgerald, 2014).

Prebiotics are "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health" (Roberfroid, 2007) and their use for the maintenance and restoration of healthy gut microbiota is considered a topic of great interest (Adebola, Corcoran, & Morgan, 2014).

Streptococcus thermophilus and *Lactobacillus delbrueckii* ssp. *bulgaricus* are the microorganisms traditionally used for yoghurt manufacture. These microorganisms metabolise lactose to produce organic acids and simple carbohydrates like glucose and galactose. Acidification is responsible for texture, and aroma of yoghurt (Costa et al., 2014). With respect to prebiotics, different types of carbohydrates, such as inulin of different chain lengths (Kip, Meyer, & Jellema, 2006), fructooligosaccharides (FOS) (Akalin, Fenderya, & Akbulut, 2004; Akalin, Gönç; Ünal, & Fenderya 2007), galactooligosaccharides (GOS) (Van Leusen et al., 2014), resistant starch and lactulose (Nobakhti, Ehsani, Mousavi, & Mortazavian, 2009) have been used in manufacture of yoghurt and other fermented milks.

Currently, there is controversy over the effect of prebiotics on acidification rate, and viability of bacteria during manufacture and cold storage of yoghurts. Some studies have reported that lactulose and inulin promoted the growth of *L. delbrueckii* ssp. *bulgaricus* and *Lactobacillus casei* (Akalin et al., 2004; Desai, Powell, & Shah, 2004; Shin, Lee, Pestka, & Ustunol, 2000). However, none of these studies assessed stability of the added prebiotics throughout the manufacture and their subsequent storage. Moreover, the information available is fragmented since, to the best of our knowledge, no other reports on the performance of the main available prebiotics at

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different doses have been reported. Consequently, there is a lack of knowledge on the potential contribution of prebiotics to lactic acid bacteria behaviour in yoghurt production. In this context, several studies on the structure-function relationship of prebiotic oligosaccharides and their degradation by bacteria have shown the influence of the type of prebiotic, highlighting the role of its chemical structure on the fermentation pattern (Cardelle-Cobas et al., 2012; Mussatto & Mancilha, 2007). However, more research is needed for a better understanding of the role of prebiotics in yoghurts.

The aim of this study was to determine the evolvement of prebiotics and the formation of organic acids during manufacture and cold storage of set-style yoghurts containing different prebiotics (FOS, GOS or lactulose) added at two different concentrations and their correlation with acidity and viability of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* during fermentation and storage periods.

2. Materials and methods

2.1. Microbial cultures and prebiotics

Commercial starter culture (YoFlex® Advance 2.0) containing a mixture of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in a lyophilised form was from Chr. Hansen (Hørsholm, Denmark), and stored at −80 °C until use.

Lactulose, trade name "Galactofructose®", was kindly provided by Solactis Group (Paris, France) with a composition of 74.5% lactulose, 7.5% galactose, 8% lactose and 10% epilactose, tagatose and fructose per 100 g of total carbohydrates. Vivinal-GOS® was obtained from Borculo Domo (Hanzeplein, Groningen, The Netherlands) composed of 74% GOS, 16% monosaccharides, and 10% lactose per 100 g of total carbohydrates. FOS was provided by Wako Pure Chemical Industries (Osaka, Japan) composed of 97% FOS, 0.8% monosaccharides, and 2.2% sucrose per 100 g of total carbohydrates.

2.2. Preparation of yoghurt starter culture

Skim powder milk (25 g) (Sveltesse®, Nestlé Spain) was added to one litre of pasteurised milk and the mixture heated at 75 °C for 45 min and cooled down to 45 °C before inoculation. Then, working culture (0.2 g L^{-1}) was added to the mixture and incubated at 45 °C for 7 h. Later, it was held 12 h at 4 °C and pre-stored until its inoculation into milk prior to yoghurt-making.

2.3. Yoghurt preparation and cold storage

Yoghurts were prepared using pasteurised milk (500 mL) with added skim milk powder (2.5%, w/v). The mixture was heated at 75 °C for 45 min and then cooled to 44 °C in an ice water bath. Then, 75 g yoghurt starter culture was inoculated. Each batch of control (without added prebiotics) and yoghurts with prebiotics (FOS, GOS, or lactulose) at 2% and 4% (w/v) were divided into five equal portions of 50 mL per duplicate. Each yoghurt was prepared in duplicate.

Each batch was incubated at 43 °C until pH decreased to 4.6–4.7 and protein coagulation took place. During fermentation, samples for each treatment were taken for analysis every hour during fermentation process (0–6 h). After incubation, samples were taken by breaking the soft coagulum in the centre of each yoghurt, to obtain a representative and homogenous sample before yoghurts were cooled to room temperature and stored at 4 °C for 28 d. During cold storage, these samples were analysed at different time intervals (0, 7, 14, 21 and 28 d). No precipitation of prebiotics was observed during refrigerated storage.

Subsequent analyses were carried out in triplicate per prepared yoghurt, with the exception of viable bacterial count that was performed in duplicate.

2.4. Measurement of pH

The pH was measured in each treatment of control and yoghurts with prebiotics with an electrode pH-meter (Metler Toledo, Five Easy Plus) during fermentation (0–6 h) and cold storage (0, 7, 14, 21 and 28 d).

2.5. Viable bacterial count

Samples collected immediately after fermentation and during cold storage were analysed within 24 h after collection. Bacterial counts were carried out at 0 (end of fermentation stage), 7, 14, 21 and 28 d of cold storage (Tabasco, Paarup, Janer, Peláez, & Requena, 2007). *S. thermophilus* was enumerated on plates of M 17 agar (Sigma Aldrich, Steinheim, Germany) with 1% added lactose, incubated at 45 °C for 24 h. For enumeration of *L. delbrueckii* ssp. *bulgaricus*, the medium was prepared according to the instructions of the manufacturer (Sigma Aldrich). Plates were incubated in anaerobic jars at 45 °C for 72 h and lenticular colonies with 1–2 mm diameter were enumerated as *L. delbrueckii* ssp. *bulgaricus*.

2.6. Analysis of carbohydrates

Mono-, di- and oligosaccharides from control and prebiotic supplemented yoghurts, with the exception of lactose and lactulose in yoghurts supplemented with lactulose, were determined by gas chromatography with flame ionisation detection (GC-FID). Before analysis, 1 g of control and prebiotic supplemented yoghurts (2 and 4% w/v) were precipitated with 10 mL of methanol at 99.9% (Merck Millipore, Madrid, Spain), kept for 1 h at room temperature to remove proteins and fats and centrifuged at 10,000×*g* for 5 min. Five hundred microlitres of the resulting supernatant was evaporated with 400 μL of internal standard solution (phenyl-β-glucoside at 0.5 mg mL^{-1}), prior to derivatisation. Then, trimethyl silylated oximes (TMSO) of carbohydrates present in the samples were determined. Sugar oximes were formed by adding 250 μL of hydroxylamine chloride (2.5%) in pyridine to dried samples and heating the mixture at 70 °C for 30 min and then silylated with hexamethyldisilazane (250 μL) and trifluoroacetic acid (25 μL) and kept at 50 °C for 30 min (Brobst & Lott, 1966). Reaction mixtures were centrifuged at 10,000×*g* for 2 min at room temperature. Supernatants were injected or stored at 4 °C prior to analysis.

Analysis of carbohydrates was performed in an Agilent Technologies 7890A gas ghromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with FID. Separation was performed using a fused silica capillary column DB-5HT, bonded, crosslinked phase (5% phenyl-methylpolysiloxane; 15 m × 0.32 mm i.d., 0.10 μm film thickness) (J&W Scientific, Folson, CA, USA), under the following conditions: 150 °C, then increase at 3 °C min^{-1} to 380 °C and held this temperature during 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-glucoside (0.5 mg mL^{-1}). Response factors were calculated after the analysis of standard solutions over the expected concentration range in samples (0.3–5 mg mL^{-1}) of glucose, galactose, fructose, lactose, raffinose and kestose, the two latter as standards of oligosaccharides with a degree of polymerisation ≥ 3 present in yoghurts with FOS and GOS (Montilla, Van de Lagemaat, Olano, & Del Castillo, 2006).

Due to co-elution of lactose and lactulose, following their analysis by GC-FID, the analysis of these two disaccharides in yoghurts supplemented with lactulose was carried out by liquid chromatography with refractive index detector (LC-RID). Before analysis, yoghurt samples (1 g) were mixed with equal volumes (100 μL) of Carrez I (7.2%, w/v, $K_4Fe(CN)_6.3H_2O$) and Carrez II (14%, w/v, $ZnSO_4.7H_20$) solutions in a 10 mL volumetric flask and diluted in acetonitrile/water 70/30 (v/v). The resulting mixture was centrifuged at 10,000×*g* for 10 min and the supernatant was filtered through a 0.45 μm pore membrane (Moreno, Olano, Santa-Maria, & Corzo, 1999). The analysis of lactose and lactulose was performed in an Agilent Technologies 1220 Infinity LC System 1260 equipped with a RID. Analysis of samples was performed in an aminopropyl silane column, Kromasil (100-5-NH_2) (250 × 4.6 mm and 5 μm particle size) with a pore size of 100 Å (Akzo Nobel, Brewster, NY, USA) thermostated at 30 °C. The separation was in isocratic mode and the mobile phase was acetonitrile-water (70/30, v/v) at a flow rate of 0.8 mL min^{-1}. Quantitative analysis was performed by the external standard method, calibration curves in the range 0.2–25 mg mL^{-1}. Determination coefficients obtained from these calibration curves were linear over the range studied ($R^2 > 0.99$).

2.7. Analysis of organic acids

Determination of organic acids in yoghurts samples was carried out by LC-UV. Before analysis, 1 mL of yoghurt was mixed with 10 mL 0.0045 M sulphuric acid. The resulting mixture was sonicated for 10 min in an ultrasound (frequency of 45 KHz) bath (SONICA® Sweep System Technology, Soltec, Italy). Then, it was centrifuged at 10,000×*g* for 10 min for removal of proteins. The supernatant was filtered through a 0.45 μm pore membrane (da Costa, da Silva Frasao, da Costa Lima, Rodrigues, & Junior, 2016).

The separation of organic acids was achieved in an Agilent Technologies 1220 Infinity LC System 1260 equipped with an ultraviolet detector (λ = 210 nm). Elution of lactic acid and short-chain fatty acids (SCFAs) was performed in a ion exchange column with sulfonated styrene-divinylbenzene spheres REZEX™ ROA, crosslinked resin (8% hydrogen; 300 × 7.8 mm and 8 μm particle size) (Phenomenex, Torrance, CA, USA) thermostated at 40 °C. The separation was in isocratic mode and the mobile phase was 0.005 M sulphuric 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, acetic and propionic acids prepared in concentrations ranging from 0.005 to 2.0 mg mL^{-1}. Determination coefficients obtained from these calibration curves were linear over the range studied ($R^2 > 0.99$).

2.8. Statistical analysis

The experimental data of bacterial counts and carbohydrate, and organic acids concentrations during fermentation and cold storage were presented as mean values ± standard deviations. Mean values of these parameters were submitted using the General Linear Model (GLM) procedure by the Statistica Software 6.0 (SPPS) and, when needed, the different manufactured yoghurts were analysed with factorial-repeated measures ANOVA, considering the influence of the time, the type and the amount of prebiotic, as the main variables, by using the Tukey test at significance level $P < 0.05$.

3. Results and discussion

3.1. Values of pH during yoghurt fermentation and storage

The pH of milk decreased from its initial value 6.7–6.8 to 6.1–6.3 caused by the addition of 15% starter culture, and during fermentation a significant decrease was observed in all prepared yoghurts reaching values ranging from 4.7 to 4.5 at the end of fermentation. The fermentation process lasted for 5 h with the exception of those prepared with lactulose (2 and 4%) whose fermentation period was lengthened up to 6 h (Fig. 1a). Lactose is fermented faster than lactulose when both disaccharides are present in about the same concentration. As the lactose concentration decreases during fermentation, the rate of lactulose fermentation increased, which may be the cause of the slow acidification observed.

The pH of all yoghurts was hardly affected and remained practically stable during cold storage for 28 d (Fig. 1a), reaching pH values of 4.5–4.4 possibly due to the low acidifying activity of the yoghurt cultures. Nevertheless, the major decrease of pH took place in control yoghurt during the last two weeks of the storage period reaching a final content of 4.1, which is in agreement with the highest content of lactic acid found at the end of storage and shown below.

3.2. Effects of prebiotics on lactic acid bacteria count

The bacterial counts throughout the cold storage period are shown in Fig. 1b,c. After the fermentation process at 45 °C (0 days of storage), all manufactured yoghurts contained populations of *Streptococcus* and *Lactobacillus* of 8–9 log_{10} cfu mL^{-1} and 5–6 log_{10} cfu mL^{-1}, respectively. Holcomb, Frank, and Mc Gregor (1991) also found a greater population of *S. thermophilus* than *L. delbrueckii* ssp. *bulgaricus*.

The influence of the type and amount of prebiotic, as well as the time of storage, were evaluated for *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*. In the case of *S. thermophilus*, the time was not a significant variable. However, the other two factors significantly influenced the bacterial cell counts. Lactulose at high dose (4%) significantly ($P < 0.05$) influenced the bacterial counts. This could be explained because at the beginning of storage there were lower counts of *S. thermophilus* (8.0 log_{10} cfu mL^{-1}) comparing with the other yoghurts (Fig. 1b). In the case of *L. delbrueckii* ssp. *bulgaricus* (Fig. 1c), the time, type and amount of prebiotics significantly influenced the bacterial counts. Lactulose, mostly at high concentration (4%), significantly affected the growth of *L. delbrueckii* ssp. *bulgaricus*. This could be explained because there are higher initial bacterial counts (1.2 and 0.7 log_{10} cfu mL^{-1}, at 2 and 4%, respectively), compared with the control yoghurt. In addition, lactulose at 4% significantly increased the survival of bacteria cell counts until the fourteenth day of storage and the total count of this microorganism at the end of storage was 2.2 log_{10} cfu mL^{-1} higher than in the control yoghurt. Therefore, the addition of lactulose promoted the growth of *L. delbrueckii* ssp. *bulgaricus* at different levels depending on the dose, which agrees with previous studies performed with skim milk added lactulose at 5% (Desai et al., 2004) and fermented milk with lactulose at 4% (Oliveira, Florence, Perego, De Oliveira, & Converti, 2011). These authors suggested that *Lactobacillus acidophilus* could metabolise the fructose moiety of lactulose.

In the case of yoghurts with FOS or GOS at different concentrations, the viability of both microorganisms was statistically equivalent to the values observed for the control yoghurt (Fig. 1b,c). These results are consistent with a previous study that showed no effects of FOS on the growth of conventional starter cultures (Akalin, Gönç,

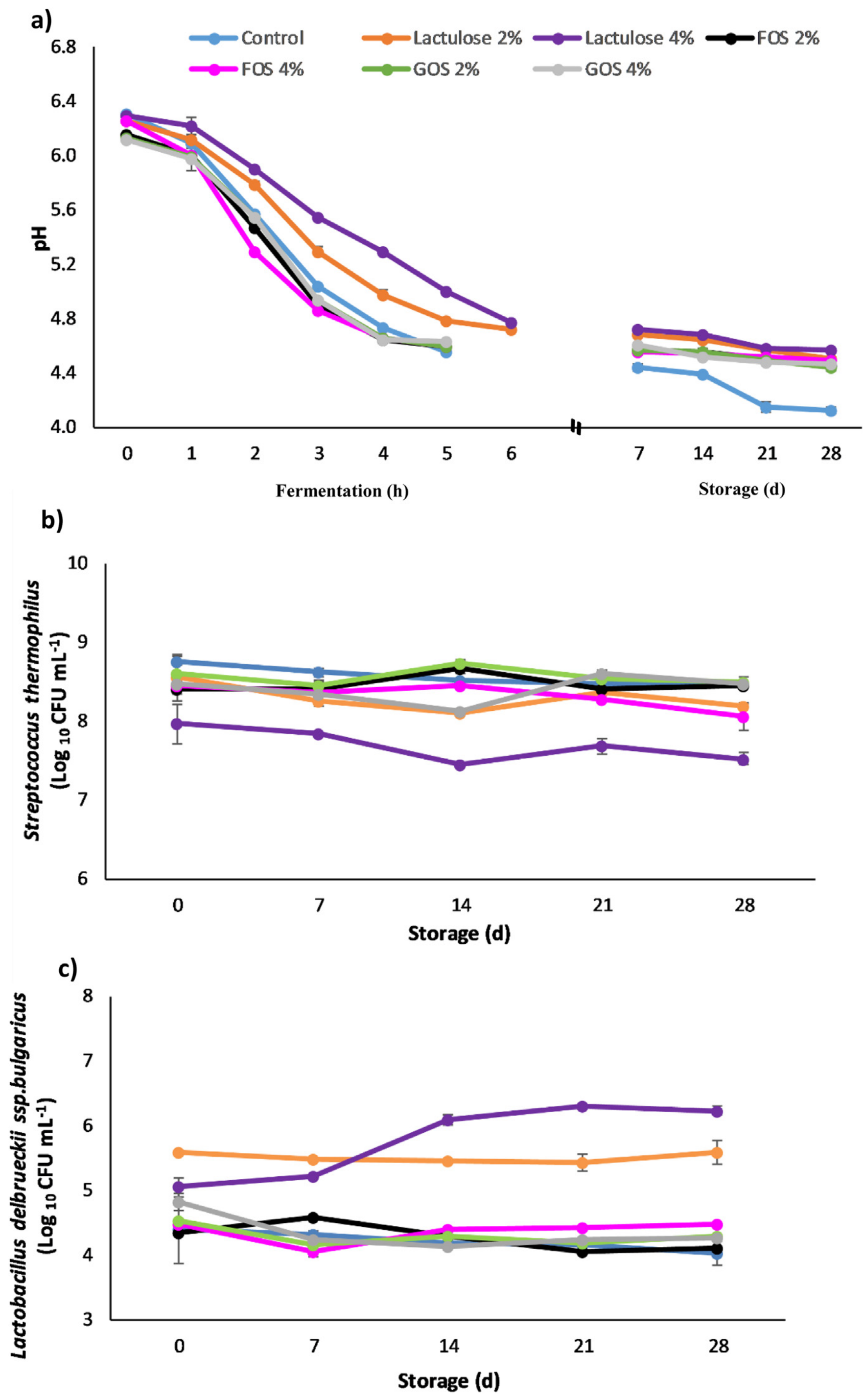

Fig. 1. Evolution of pH (a), viability of *Streptococcus thermophilus* (b) *and Lactobacillus delbrueckii* ssp. *bulgaricus* (c) in control yoghurt and yoghurts with added prebiotics; values are the means ± standard error ($n = 4$).

Ünal, & Fenderya, 2007). In the case of yoghurts with GOS, our results are in agreement with Vénica, Wolf, Bergamini, and Perotti (2016), who did not observe any significant change in lactic acid bacteria counts such as *L. delbrueckii* ssp. *bulgaricus* for 21 d of storage.

3.3. Changes in the carbohydrate fraction during yoghurt fermentation and storage

The content of carbohydrates (i.e., glucose, galactose, lactose and prebiotic oligosaccharides) as well as their changes during fermentation and cold storage was determined both in control and prebiotic supplemented yoghurts.

3.3.1. Lactose

Lactose, the major carbohydrate in yoghurts, was readily hydrolysed into galactose and glucose by lactic acid bacteria through the Embden–Meyerhof–Parnas pathway. The consumption of lactose was observed from the first hour of fermentation process (Fig. 2a), this decrease being concomitant with the high acidification of all yoghurts found after the first hour of fermentation (Fig. 1a). At the beginning of fermentation, the concentration of lactose was in the narrow range of 5.4–5.6 g 100 g^{-1} yoghurt in all yoghurt studied, and then significantly decreased to reach percentages of consumption that ranged from 20 to 28% at the end of fermentation. At the end of the cold storage, the total decrease in lactose was higher in the control yoghurt (38% of decrease with a final content of lactose of 3.4 g 100 g^{-1} yoghurt) as compared with yoghurts with prebiotics (25–33% of decrease with

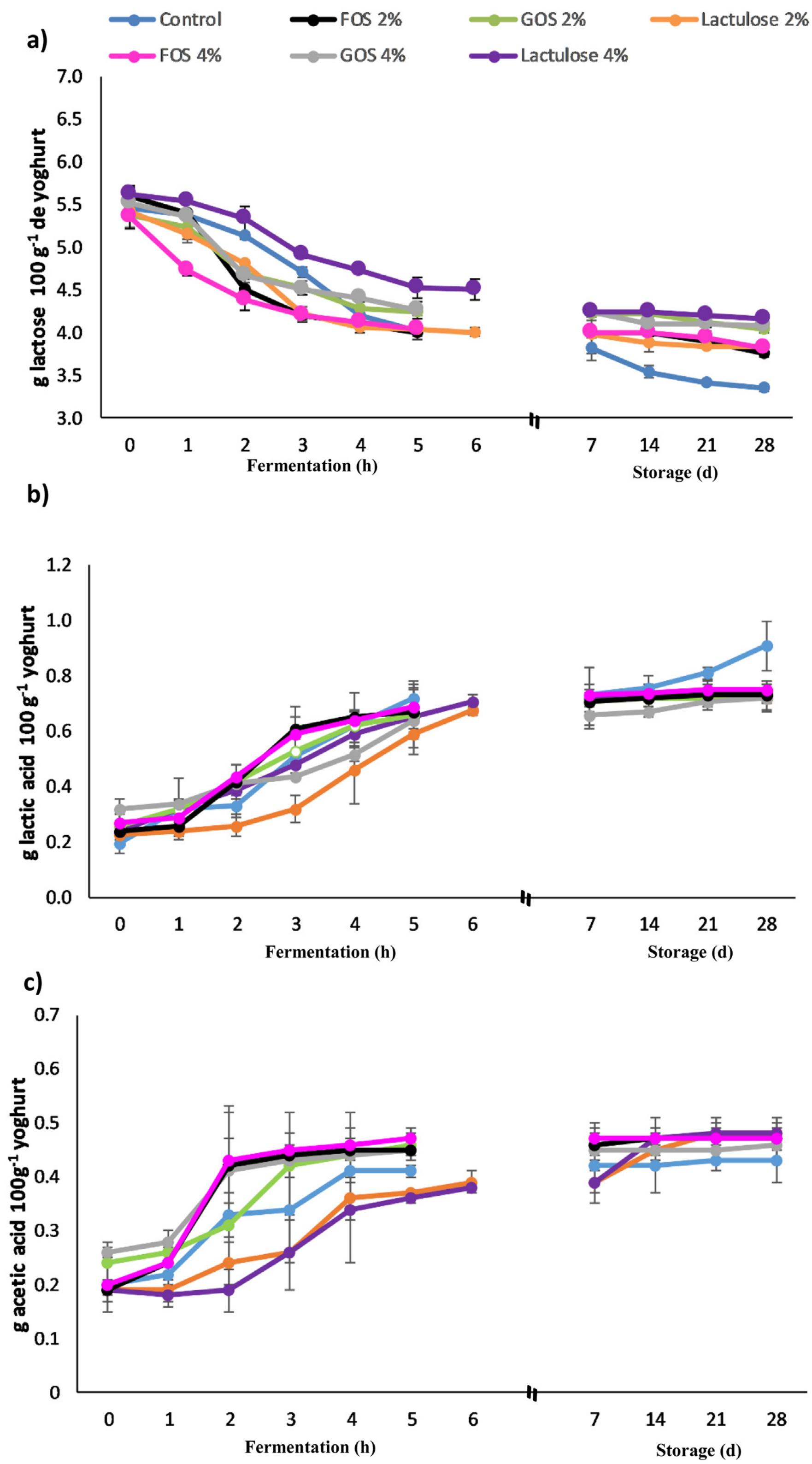

Fig. 2. Change of lactose (a), lactic acid (b) and acetic acid (c) in control yoghurt and yoghurts with added prebiotics during fermentation at 45 °C and cold storage at 4 °C; values are the means ± standard error ($n = 6$).

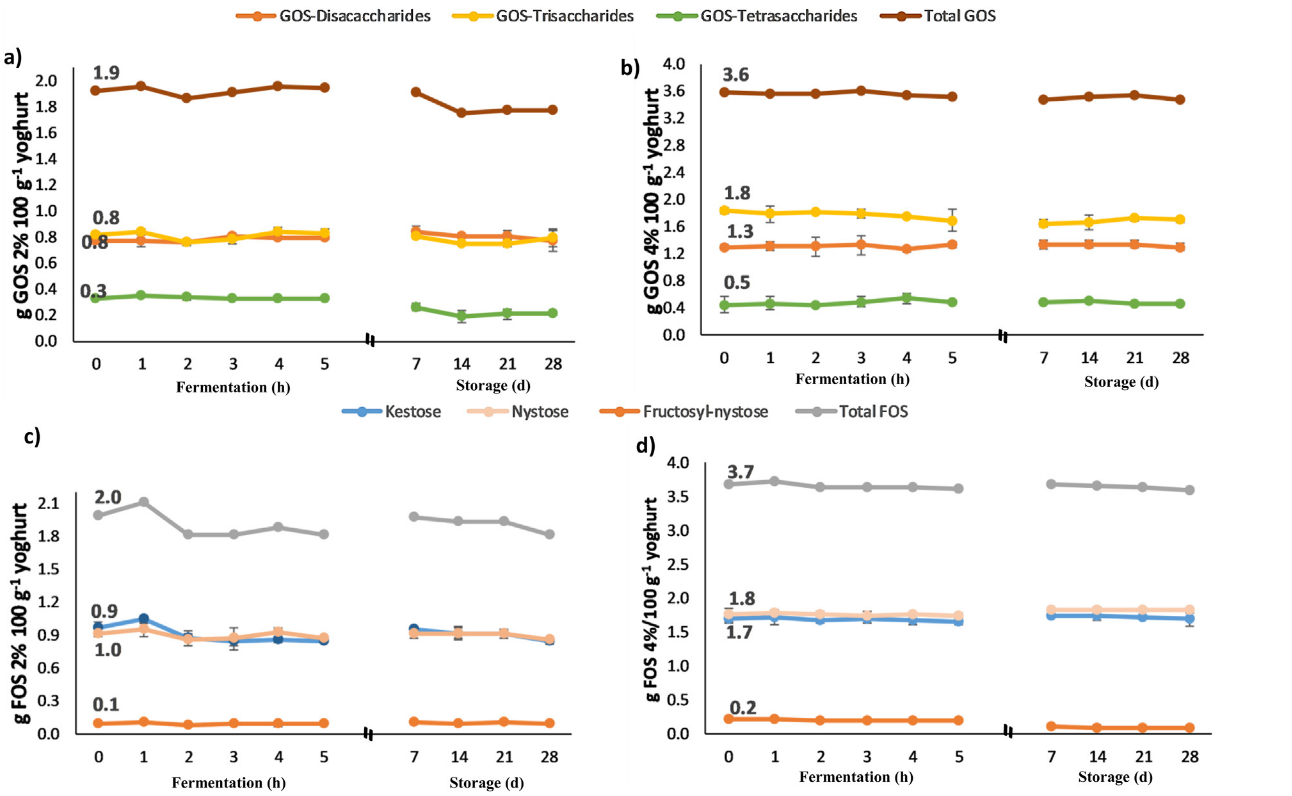

Fig. 3. Change of GOS and FOS content in yoghurts following their addition at (a) and (c) 2% and at (b) and (d) 4%, respectively, during fermentation at 45 °C and cold storage at 4 °C; values are the means ± standard error ($n = 6$).

a final content of lactose ranging from 3.8 to 4.2 g 100 g^{-1} yoghurt) (Fig. 2a), which is in agreement with the content of lactic acid at the end of storage (see section 3.4). These findings agreed with those of Oliveira et al. (2009) who observed higher levels of lactose in yoghurts with oligofructose, corresponding to a less production of lactic acid at the end of storage compared with the control.

3.3.2. *Prebiotic oligosaccharides*

The quantification of prebiotic carbohydrates (di-, tri-, tetra-, and pentasaccharides) in yoghurts containing GOS, FOS or lactulose, during fermentation and storage processes, is shown in Figs. 3 and 4. In yoghurts with GOS added at 2 (Fig. 3a) and 4% (Fig. 3b) the level of di-, tri- and tetrasaccharides remained fairly constant ($P < 0.05$) throughout fermentation and cold storage. These results clearly indicated that, when lactose is available, the starter culture used for yoghurt production does not metabolise GOS, regardless their degree of polymerisation and concentration. These results were in agreement with other studies that did not find changes on GOS level during storage of traditional yoghurts with added GOS (Vénica et al., 2016).

In the case of yoghurts with FOS added at 2 and 4%, kestose, nystose and fructosyl-nystose were quantified and their evolution is shown in Fig. 3c,d. In a similar way to GOS behaviour, the content of FOS remained unaffected during fermentation and cold storage.

Concerning yoghurts made with lactulose, a different behaviour depending on the initial lactulose concentration was observed (Fig. 4). Whilst yoghurts with lactulose added at 2% showed a slight but not significant ($P > 0.05$) decrease of the added prebiotic, that is from 1.7 to 1.5 g lactulose 100 g^{-1} yoghurt, a remarkable and significant decrease in lactulose content was observed ($P < 0.05$), mainly during the fermentation process, when added at 4%. Thus, a 33% of lactulose decrease was observed from the initial (3.6 g 100 g^{-1}

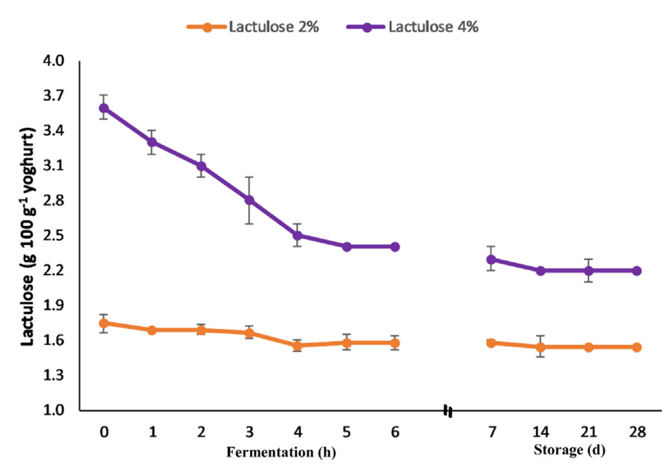

Fig. 4. Change of lactulose content in yoghurts following their addition at 2 and at 4% during fermentation at 45 °C and cold storage at 4 °C, values are the means ± standard error ($n = 6$).

yoghurt) to the fifth hour of the fermentation process (2.4 g 100 g^{-1} yoghurt). A much more moderate decrease in lactulose content was observed during the storage period reaching values of 2.2 g 100 g^{-1} yoghurt. The decrease of lactulose content in yoghurts with 4% was in agreement with the increase of *Lactobacillus delbrueckii* count (Fig. 1c). This fact could be attributed to stimulation of β-galactosidase activity, which could be responsible for quicker hydrolysis of lactulose to galactose and fructose (Oliveira et al., 2011). Also, the substantial decrease in lactulose could explain the limited consumption of lactose observed during the fermentation process of the yoghurt with 4% of lactulose by lactic acid bacteria as compared with the rest of yoghurts (Fig. 2a). Olano, López-Covarrubias, Ramos, and Suárez (1986) studied the influence of lactulose on the growth of several starters during yoghurt manufacture and observed a similar decrease in lactulose content (that is, from 3.6 g 100 mL^{-1} to 2.7 g 100 mL^{-1}) during the fermentation process.

3.3.3. Monosaccharides

Fig. 5 shows the changes in the levels of the main monosaccharides, galactose and glucose, detected during fermentation and cold storage of control and prebiotic supplemented yoghurts. With respect to galactose, a moderate increase was observed during fermentation and storage in most of prebiotic supplemented yoghurts (Fig. 5a). This fact can be attributed to the combined effect of lactose hydrolysis and the uncompleted consumption of released galactose (Alm, 1982; Goodenough & Kleyn, 1976). In addition, O'leary and Woychik (1976) showed the consumption, preferentially by *L. delbrueckii* ssp. *bulgaricus*, of low concentrations of galactose during fermentation, whereas *S. thermophilus* consumed both glucose and lactose. Moreover, it should be noticed that, in yoghurts containing initial levels of lactulose at 4%, galactose underwent the highest increase during fermentation and storage, reaching at the end of storage levels of up to 0.8 g 100 g^{-1} yoghurt. These results agreed with the significant decrease of lactulose previously observed in yoghurts prepared with 4% of lactulose (Fig. 4).

Glucose content was in the low range of 0.1–0.2 g 100 g^{-1} yoghurt in all yoghurts except in those containing GOS, which presented initial levels of 0.6 and 1.1 g 100 g^{-1} yoghurt depending on the initial dose of GOS (Fig. 5b). This is due to the fact that the GOS tested contain free glucose. Only in the case of yoghurt with 4% of GOS, there was a decrease of glucose up to 0.9 g 100 g^{-1} yoghurt observed throughout fermentation and cold storage.

Finally, in yoghurts with 4% added FOS, fructose was found in trace amounts (up to 0.04 g 100 g^{-1} yoghurt), remaining stable during fermentation and storage (data not shown). In yoghurts with lactulose (4%), fructose was also detected and slightly increased during fermentation up to 0.1 g 100 g^{-1} yoghurt and then remained constant until the end of storage. This slight increase could be partly associated to the consumption of this prebiotic at 4% by the starter cultures.

3.4. Formation of organic acids during yoghurt fermentation and storage

The main detected organic acids derived from the metabolism of lactic acid bacteria were lactic (peak 1) and acetic (peak 2) acids (Supplementary material Fig. S1). LC-UV chromatographic profiles of organic acids were qualitatively quite similar in all batches of

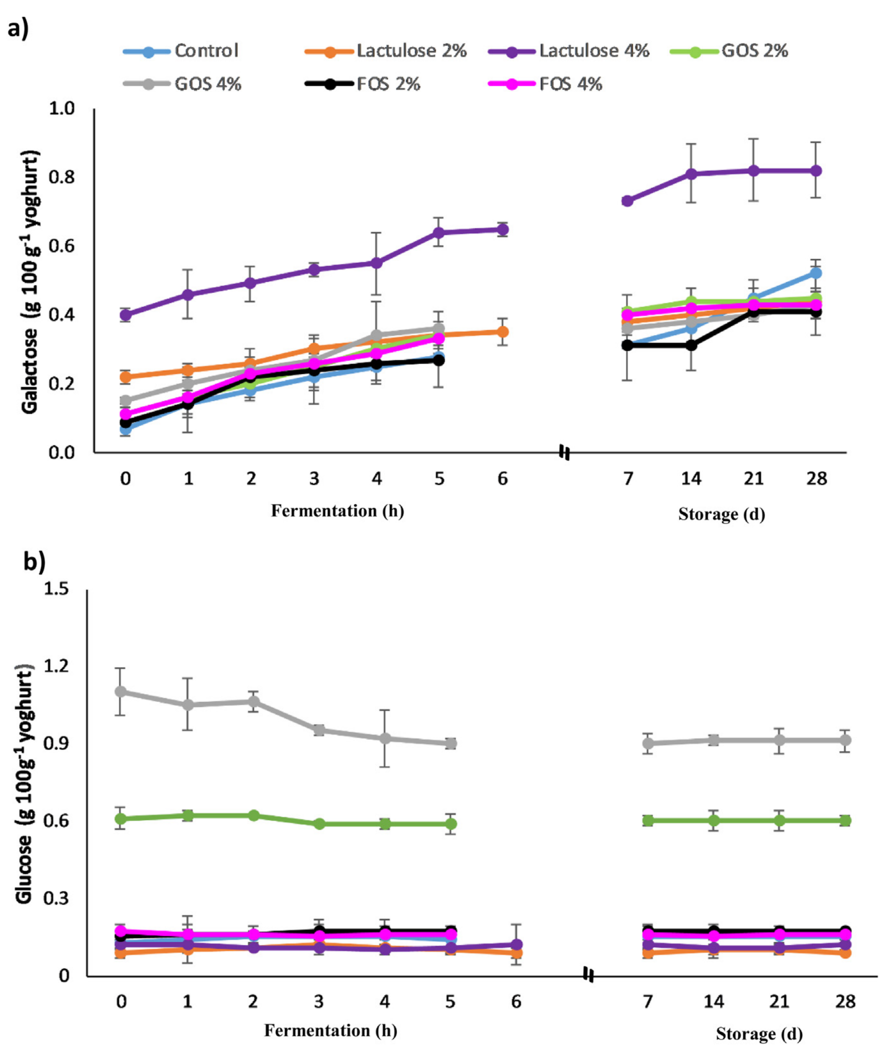

Fig. 5. Change of (a) galactose and (b) glucose content in control yoghurt and yoghurts with added prebiotics during fermentation at 45 °C and cold storage at 4 °C; values are the means ± standard error ($n = 6$).

prepared yoghurts. Also, propionic acid (peak 3) was detected but it was already present in the milk used to yoghurt manufacture at a very low amount (0.1 g 100 g^{-1} yoghurt) and no significant changes ($P < 0.05$) during fermentation or cold storage processes and type of manufactured yoghurt were observed.

The concentration of lactic and acetic acids during fermentation and cold storage in all manufactured yoghurts is shown in Fig. 2b,c. Lactic acid was the most abundant organic acid found and its increase mostly took place during the fermentation process reaching maximum values about 0.7 g 100 g^{-1} yoghurt (Fig. 2b). This significant increase ($P < 0.05$) was concomitant with the maximum decrease of pH (Fig. 1a) and the degradation of lactose (Fig. 2a) by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in all manufactured yoghurts. During the cold storage of yoghurts, lactic acid values ranging from 0.7 to 0.9 g 100 g^{-1} yoghurt were found. These results are in good agreement with several authors that reported levels of lactic acid of 0.8–0.9 g 100 g^{-1} yoghurt at the end of storage (28 d) (Fernandez-Garcia & McGregor, 1994; Vénica, Perotti, & Bergamini, 2014). The main, but slight, increase in lactic acid during the cold storage was found in the control yoghurt as compared with yoghurts with prebiotics (Fig. 2b), which is in line with the higher decrease of lactose observed at the end of cold storage for the control yoghurt (Fig. 2a).

With respect to acetic acid, an increase was observed during fermentation in all yoghurts reaching maximum values of 0.4–0.5 g 100 g^{-1} yoghurt, remaining fairly constant during the storage period (Fig. 2c). Although it is known that *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* are homofermentative microorganisms, they produce small amounts of acetic acid (Adhikari, Grün, Mustapha, & Fernando, 2002). High acetic acid values are associated with the heterofermentative pathway of lactose produced by strains of bifidobacteria (Vénica et al., 2014). Levels of 0.6% of acetic acid in yoghurts with inulin and resistant starch added at 0.5, 1 and 1.5% were found, remaining stable until the end of storage (28 d) (Donkor, Henriksson, Vasiljevic, & Shah, 2007).

Therefore, no effect of any tested prebiotic on the level and type of organic acids was observed during the manufacture and storage of yoghurts. This behaviour is in agreement with the resistance of all prebiotics to fermentation by *S. thermophilus* and *L. delbrueckii* ssp. bulgaricus with the exception of the yoghurt prepared with lactulose at 4%.

4. Conclusions

Overall, the supplementation of yoghurts with GOS or FOS at two different concentrations (2 and 4%) did not have a significant impact on acidification, populations of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*, lactose consumption and production of organic acids during fermentation and cold storage. GOS and FOS were not metabolised by yoghurt lactic acid bacteria under the assayed conditions; lactose was efficiently used as a carbon source. However, supplementation with lactulose at 2 and 4% affected the fermentation rate, increasing the processing time from 4 to 6 h, as well as the viability of the starter cultures. In this sense, lactulose, when added at 4%, significantly increased the population of *L. delbrueckii* ssp. *bulgaricus* (by 2.2 $\log_{10}$ cfu mL^{-1}), which was in accordance with a decrease of around 40% of lactulose observed throughout fermentation and cold storage periods. Lastly, the growth of *S. thermophilus* was unaffected by the presence of any of the tested prebiotic, with the exception of a slight decrease (1.0 $\log_{10}$ cfu mL^{-1}) in yoghurts prepared with added lactulose at 4%. This fact could be attributable to a similar metabolism by *S. thermophillus* and *L. delbrueckii* ssp. *bulgaricus* of the prebiotics assayed with the exception of the yoghurt prepared with lactulose at 4%.

To conclude, this work demonstrates the feasibility to manufacture yoghurts with different doses of GOS, FOS or lactulose. In the case of yoghurts prepared with lactulose, the level of added prebiotic is a critical factor to improve the viability of *L. delbrueckii* ssp. *bulgaricus*, although the stability of lactulose could be affected, especially at the highest lactulose concentration tested.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.09.003.

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