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

Paloma Delgado-Fernández, Nieves Corzo*, Agustín Olano, Oswaldo Hernández-Hernández, F. Javier Moreno

Department of Bioactivity and Food Analysis

Institute of Food Science Research CIAL, (CSIC-UAM)

CEI (UAM + CSIC), C/ Nicolás Cabrera, 9, E-28049 Madrid (Spain).

*Author to whom correspondence should be addressed:

C/ Nicolás Cabrera 9, E-28049 Madrid (Spain).

Tel: +34 910017954

e-mail: nieves.corzo@csic.es

ABSTRACT

This work investigates the feasibility of manufacturing yogurts with three prebiotics: fructooligosaccharides (FOS), galactooligosaccharides (GOS) or lactulose added at two concentrations (2 and 4%, w/v). Physicochemical and microbial characterization 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 hours) and cold storage (for 28 days). The main findings revealed that GOS and FOS remained unaltered throughout fermentation and cold storage, while the viability of starter culture was similar to that observed in the control yogurt. Yogurts 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 to a significant increase (2.2 $\log_{10}$ cfu/mL) in *L. delbrueckii* ssp. *bulgaricus* and a relatively lower decrease (1.0 $\log_{10}$ cfu/mL) 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 yogurt manufacture. These microorganisms metabolize lactose to produce organic acids and simple carbohydrates like glucose and galactose. Acidification is responsible for texture, and aroma of yogurt (Costa, Balthazar, Franco, Márs ico, Cruz & Conte, 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 yogurt 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 yogurts. Some studies have reported that lactulose and inulin promoted the growth of *Lactobacillus delbrueckii* subs. *bulgaricus* and

58 *Lactobacillus casei* (Akalin et al. 2004; Desai, Powell, & Shah, 2004; Shin, Lee, Pestka,
59 & Ustunol, 2000). However, none of these studies assessed stability of the added prebiotics
60 throughout the manufacture and their subsequent storage. Moreover, the information available is
61 fragmented since, to the best of our knowledge, no other reports on the performance of the main
62 available prebiotics at different doses have been reported. Consequently, there is a lack of
63 knowledge on the potential contribution of prebiotics to lactic acid bacteria behavior in yogurt
64 production. In this context, several studies on the structure-function relationship of prebiotic
65 oligosaccharides and their degradation by bacteria have shown the influence of the type of
66 prebiotic, highlighting the role of its chemical structure on the fermentation pattern (Cardelle-
67 Cobas et al. 2012; Mussatto & Mancilha, 2007). However, more research is needed for a better
68 understanding of the role of prebiotics in yogurts.

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

74

75 ## 2. Materials and methods

76 *2.1 Microbial cultures and prebiotics*

77 Commercial starter culture *(YoFlex® Advance 2.0)* containing a mixture of *Streptococcus*
78 *thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* in a lyophilized form was from Chr.
79 Hansen (Denmark), and stored at -80ºC until use.

80 Lactulose, trade name "Galactofructose®", was kindly provided by Solactis Group (Paris,
81 France) with a composition of 74.5% of lactulose, 7.5% of galactose, 8% of lactose and 10% of

epilactose, tagatose and fructose per 100g of total carbohydrates. Vivinal-GOS® was obtained from Borculo Domo (Hanzeplein, Groningen, The Netherlands) composed of 74% GOS, 16% monosaccharides, and 10% lactose per 100g of total carbohydrates. FOS was provided by Wako Pure Chemical Industries (Osaka, Japan) composed of 97% of FOS, 0.8% of monosaccharides, and 2.2% of sucrose per 100g of total carbohydrates.

2.2 Preparation of yogurt starter culture

Skim powder milk (25 g) (Sveltesse®, Nestlé Spain) was added to one liter of pasteurized milk and the mixture heated at 75°C for 45 min and cooled down to 45ºC before inoculation. Then, working culture (0.2 g/L) was added to the mixture and incubated at 45ºC during 7 hours. Later, it was held 12 hours at 4ºC and pre-stored until its inoculation into milk prior to yogurt-making.

2.3. Yogurt preparation and cold storage

Yogurts were prepared using pasteurized 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 of yogurt starter culture was inoculated. Each batch of control (without added prebiotics) and yogurts with prebiotics (FOS, GOS, or lactulose) at 2% and 4% (w/v) were divided into five equal portions of 50 mL per duplicate. Each yogurt was prepared in duplicate.

Then, each batch was incubated at 43°C until pH decreased down 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 hours). After incubation, samples were taken by breaking the soft coagulum in the center of each yogurt, in order to obtain a representative and homogenous sample before yogurts were cooled down to room temperature and stored at 4°C for 28 days. During cold storage, these samples were analyzed at different time intervals (0, 7, 14, 21 and 28 days). No precipitation of prebiotics was observed during refrigerated storage.

Subsequent analyses were carried out in triplicate per prepared yogurt, 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 yogurts with prebiotics with an electrode pH-meter (Metler Toledo, Five Easy Plus) during fermentation (0-6 hours) and cold storage (0, 7, 14, 21 and 28 days).

2.5. Viable bacterial count

Samples collected immediately after fermentation and during cold storage were analyzed within 24 hours after collection. Bacterial counts were carried out at 0 (end of fermentation stage), 7, 14, 21 and 28 days 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 45ºC for 24 h. For enumeration of *L. delbrueckii* ssp. *bulgaricus*, the medium was prepared according to the instructions of the manufacturer (Sigma Aldrich, Steinheim, Germany). Plates were incubated in anaerobic jars at 45ºC for 72 h and lenticular colonies with 1–2mm diameter were enumerated as *L. delbrueckii* ssp. *bulgaricus.*

2.6. Analysis of carbohydrates

Mono-, di- and oligosaccharides from control and prebiotic supplemented yogurts, with the exception of lactose and lactulose in yogurts supplemented with lactulose, were determined by Gas Chromatography with Flame Ionization Detector (GC-FID). Before analysis, 1g of control and prebiotic supplemented yogurts (2% and 4% w/v) were precipitated with 10 mL of methanol at 99.9% (Merck Millipore, Spain), kept for 1 hour at room temperature in order to remove proteins and fats and centrifuged at 10,000 *g* for 5 min. 500 µL of the resulting supernatant was evaporated

with 400 µL of internal standard solution (phenyl-β-glucoside at 0.5 mg/mL), prior to derivatization. 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 Chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a flame ionization detector (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, California, USA), under the following conditions: 150ºC, then increase at 3ºC/min to 380ºC and held this temperature during 76 min. The temperature of injector and detector were at 280ºC and 385ºC, respectively. Injections were carried out in split mode (1:20) using nitrogen as carrier gas at a flow rate of 1 mL/min. 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 β-phenyl-glucopyranoside (0.5 mg/mL). Response factors were calculated after the analysis of standard solutions over the expected concentration range in samples (0.3-5 mg/mL) of glucose, galactose, fructose, lactose, raffinose and kestose, the two latter as standards of oligosaccharides with a degree of polymerization ≥ 3 present in yogurts 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 yogurts supplemented with lactulose was carried out by Liquid Chromatography with Refractive index Detector (LC-RID). Before analysis, yogurt samples (1g) 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 a LC 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 x 4.6 mm and 5 μm particle size) with a pore size of 100 Å (Akzo Nobel, Brewster, NY, USA) thermostatized at 30ºC. 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. Quantitative analysis was performed by the external standard method, calibration curves in the range 0.2-25 mg/mL. 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 yogurts samples was carried out by LC-UV. Before analysis, 1 mL of yogurt was mixed with 10 mL of 0.0045 M sulfuric acid. The resulting mixture was sonicated for 10 min in a 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 a LC Agilent Technologies 1220 Infinity LC System 1260 equipped with an ultraviolet detector (λ= 210nm). Elution of lactic acid and short-chain fatty acids (SCFAs) was performed in a ion exchange column with sulfonated styrene-divinylbenzene spheres REZEX^{TM} ROA, crosslinked resin (8% hydrogen; 300 x 7.8 mm and 8 μm particle size) (Phenomenex, Torrance, CA, USA) thermostatized at 40ºC. The separation was in isocratic mode and the mobile phase was 0.005 M sulfuric acid at a flow rate of 0.5 mL/min.

Quantification of organic acids was carried out by the external standard method using standard solutions of lactic acid, acetic acid and propionic acid prepared in concentrations ranging from 0.005 to 2.0 mg/mL. 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 General Linear Model (GLM) procedure by the Statistica Software 6.0 (SPPS) and, when needed, the different manufactured yogurts were analyzed 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 yogurt 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 yogurts reaching values ranging from 4.7 to 4.5 at the end of fermentation. The fermentation process lasted for 5 hours with the exception of those prepared with lactulose (2 and 4%) whose fermentation period was lengthened up to 6 hours (**Figure 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 increase which may be the cause of the slow acidification observed.

206 The pH of all yogurts was hardly affected and remained practically stable during cold
207 storage for 28 days (**Figure 1a**), reaching pH values of 4.5-4.4 possibly due to the low acidifying
208 activity of the yogurt cultures. Nevertheless, the major decrease of pH took place in control yogurt
209 during the last two weeks of the storage period reaching a final content of 4.1, which is in
210 agreement with the highest content of lactic acid found at the end of storage and shown below.

211

212 *3.2 Effects of prebiotics on lactic acid bacteria count*

213 The bacterial counts throughout the cold storage period are shown in **Figures 1b and 1c**.
214 After the fermentation process at 45ºC (0 days of storage), all manufactured yogurts contained
215 populations of *Streptococcus* and *Lactobacillus* of 8-9 $\log_{10}$ cfu/mL and 5-6 $\log_{10}$ cfu/mL,
216 respectively. Holcomb, Frank & Mc Gregor, (1995) also found more population of *S. thermophilus*
217 compared to *L. delbrueckii* ssp. *bulgaricus.*

218 The influence of the type and amount of prebiotic, as well as, the time of storage, were
219 evaluated for *S. thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*. In the case of *S.*
220 *thermophilus*, the time is not a significant variable. However, the other two factors influence
221 significantly the bacterial cell counts. Lactulose at high dose (4%) significantly influences
222 ($P<0.05$) the bacterial counts. This could be explained because at the beginning of storage there
223 are lower counts of *S.thermophilus* (8.0 $\log_{10}$ cfu/mL) comparing to other yogurts (**Figure 1b**). In
224 the case of *Lactobacillus delbrueckii* ssp. *bulgaricus* (**Figure 1c**), the time, type and amount of
225 prebiotics influence significantly the bacterial counts. Lactulose, mostly at high concentration
226 (4%), affects significantly the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus*. This could be
227 explained because there are higher initial bacterial counts (1.2 and 0.7 $\log_{10}$ cfu/mL, at 2% and
228 4%, respectively), comparing to control yogurt. Besides, lactulose at 4% increases significantly
229 the survival of bacteria cell counts until the fourteenth day of storage and the total count of this
230 microorganism at the end of storage was 2.2 $\log_{10}$ cfu/mL higher than in control yogurt. Therefore,

231 the addition of lactulose promoted the growth of *L. delbrueckii* ssp. *bulgaricus* at different levels
232 depending on the dose, which agrees with previous studies performed with skim milk added
233 lactulose at 5% (Desai et al., 2004) and fermented milk with lactulose at 4% (Oliveira, Florence,
234 Perego, De Oliveira & Converti, 2011). These authors suggested that *L. acidophilus* could
235 metabolize the fructose moiety of lactulose.

236 In the case of yogurts with FOS or GOS at different concentrations, the viability of both
237 microorganisms was statistically equivalent to the values observed for the control yogurt (**Figures**
238 **1b and 1c**). These results are consistent with a previous study that showed no effects of FOS on
239 the growth of conventional starter cultures (Akalin et al. 2007). In the case of yogurts with GOS,
240 our results are in agreement with Vénica, Wolf, Bergamini & Perotti, (2016) that did not observe
241 any significant change in lactic acid bacteria counts such as *L. delbrueckii* ssp. *bulgaricus* for 21
242 days of storage.

243

244 *3.3 Changes in the carbohydrate fraction during yogurt fermentation and storage*

245 The content of carbohydrates (i.e. glucose, galactose, lactose and prebiotic
246 oligosaccharides) as well as their changes during fermentation and cold storage has been
247 determined both in control and prebiotic supplemented yogurts.

248 *3.3.1. Lactose*

249 Lactose, the major carbohydrate in yogurts, was readily hydrolyzed into galactose and
250 glucose by lactic acid bacteria through the Embden–Meyerhof–Parnas pathway. The consumption
251 of lactose was observed from the first hour of fermentation process (**Figure 2a**), being this
252 decrease concomitant with the high acidification of all yogurts found after the first hour of
253 fermentation (**Figure 1a**). At the beginning of fermentation, the concentration of lactose was in
254 the narrow range of 5.4 to 5.6 g/100g yogurt in all studied yogurts, and then significantly decreased

255 to reach percentages of consumption which ranged from 20 to 28% at the end of fermentation. At
256 the end of the cold storage, the total decrease in lactose was higher in the control yogurt (38% of
257 decrease with a final content of lactose of 3.4 g/100g yogurt) as compared to yogurts with
258 prebiotics (25-33% of decrease with a final content of lactose ranging from 3.8 to 4.2 g/100g
259 yogurt) (**Figure 2a**), which are in agreement with the content of lactic acid at the end of storage
260 (see section 3.4). These findings are agreed with studies of Oliveira et al., (2009) who observed
261 higher levels of lactose in yogurts with oligofructose, corresponding to a less production of lactic
262 acid at the end of storage comparing to the control.

263

264 *3.3.2. Prebiotic oligosaccharides*

265 The quantification of prebiotic carbohydrates (di- tri-, tetra, and pentasaccharides) in
266 yogurts containing GOS, FOS or lactulose, during fermentation and storage processes, is shown
267 in **Figures 3** and **4**. In yogurts with GOS added at 2 (**Figure 3a**) and 4% (**Figure 3b**) the level of
268 disaccharides, trisaccharides and tetrasaccharides remained fairly constant ($P<0.05$) throughout
269 fermentation and cold storage. These results clearly indicated that, when lactose is available, the
270 starter culture used for yogurt production does not metabolize GOS, regardless their degree of
271 polymerization and concentration. These results were in agreement with other studies that did not
272 find changes on GOS level during storage of traditional yogurts with added GOS (Vénica et al.
273 2016).

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

277 Concerning yogurts made with lactulose, a different behavior depending on the initial
278 lactulose concentration was observed (**Figure 4**). Whilst yogurts 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/100g yogurt, 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/100g yogurt) to the fifth hour of the fermentation process (2.4 g/100g yogurt). A much more moderate decrease in lactulose content was observed during the storage period reaching values of 2.2 g/100g yogurt. The decrease of lactulose content in yogurts with 4% was in agreement with the increase of *Lactobacillus delbrueckii* count (**Figure 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 yogurt with 4% of lactulose by lactic acid bacteria as compared to the rest of yogurts (**Figure 2a**). Olano, López-Covarrubias, Ramos & Suárez, (1986) studied the influence of lactulose on the growth of several starters during yogurt manufacture and observed a similar decrease in lactulose content (that is, from 3.6 g/100 mL to 2.7 g/100mL) during the fermentation process.

3.3.3. Monosaccharides

Figure 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 yogurts. With respect to galactose, a moderate increase was observed during fermentation and storage in most of prebiotic supplemented yogurts (**Figure 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 & Woychik, (1976) showed the consumption, preferentially by *L. bulgaricus*, of low concentrations of galactose during fermentation, whereas *S. thermophilus* consumed both glucose and lactose. Moreover, it should be noticed that, in yogurts containing initial levels of lactulose at 4%, galactose underwent the highest

304 increase during fermentation and storage, reaching at the end of storage levels of up to 0.8 g/100g
305 yogurt. These results agreed with the significant decrease of lactulose previously observed in
306 yogurts prepared with 4% of lactulose (**Figure 4**).

307 Glucose content was in the low range of 0.1-0.2 g/100g yogurt in all yogurts except in those
308 containing GOS, which presented initial levels of 0.6 and 1.1 g/100g yogurt depending on the
309 initial dose of GOS (**Figure 5b**). This is due to the fact that the tested GOS contain free glucose.
310 Only in the case of yogurt with 4% of GOS, there was a decrease of glucose up to 0.9 g/100g
311 yogurt observed throughout fermentation and cold storage.

312 Finally, in yogurts with 4% added FOS, fructose was found in trace amounts (up to 0.04
313 g/100g yogurt), remaining stable during fermentation and storage (data not shown). In yogurts
314 with lactulose (4%), fructose was also detected and slightly increased during fermentation up to
315 0.1 g/100g yogurt and then remained constant until the end of storage. This slight increase could
316 be partly associated to the consumption of this prebiotic at 4% by the starter cultures.

317

318 *3.4 Formation of organic acids during yogurt fermentation and storage*

319 The main detected organic acids derived from the metabolism of lactic acid bacteria were
320 lactic (peak 1) and acetic (peak 2) acids (**Figure S1** in Supplementary material). LC-UV
321 chromatographic profiles of organic acids were qualitatively quite similar in all batches of prepared
322 yogurts. Also, propionic acid (peak 3) was detected but it was already present in the milk used to
323 yogurt manufacture at a very low amount (0.1 g/100g yogurt) and no significant changes ($P<0.05$)
324 during fermentation or cold storage processes and type of manufactured yogurt were observed.

325 Concentration of lactic and acetic acids during fermentation and cold storage in all
326 manufactured yogurts is shown in **Figure 2b and 2c**. Lactic acid was the most abundant organic
327 acid found and its increase mostly took place during the fermentation process reaching maximum
328 values about 0.7 g/100g yogurt (**Figure 2b**). This significant increase ($P<0.05$) was concomitant

with the maximum decrease of pH (**Figure 1a**) and the degradation of lactose (**Figure 2a**) by *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in all manufactured yogurts. During the cold storage of yogurts, lactic acid values ranging from 0.7 to 0.9 g/100g yogurt were found. These results are in good agreement with several authors that reported levels of lactic acid of 0.8-0.9 g/100g yogurt at the end of storage (28 days) (Fernandez-García & McGregor, 1994; Vénica, Perotti, & Bergamini, 2014). Concretely, the main, but slight, increase in lactic acid during the cold storage was found in the control yogurt as compared to yogurts with prebiotics (**Figure 2b**), which is in line with the higher decrease of lactose observed at the end of cold storage for the control yogurt (**Figure 2a**).

With respect to acetic acid, an increase was observed during fermentation in all yogurts reaching maximum values of 0.4-0.5 g/100g yogurt, remaining fairly constant during the storage period (**Figure 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 (Venica et al., 2014). Levels of 0.6% of acetic acid in yogurts with inulin and resistant starch added at 0.5, 1 and 1.5% were found, remaining stable until the end of storage (28 days) (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 yogurts. This behavior is in agreement with the resistance of all prebiotics to fermentation by *S. thermophillus* and *L. delbrueckii* ssp. bulgaricus with the exception of the yogurt prepared with lactulose at 4%.

4. Conclusions

353 Overall, the supplementation of yogurts with GOS or FOS at two different concentrations
354 (2 and 4%) did not have a significant impact on acidification, populations of *S. thermophilus* and
355 *L. delbrueckii* ssp. *bulgaricus*, lactose consumption and production of organic acids during
356 fermentation and cold storage. GOS and FOS were not metabolized by yogurt lactic acid bacteria
357 under the assayed conditions, being lactose, instead, efficiently used as a carbon source. However,
358 supplementation with lactulose at 2 and 4% affected the fermentation rate increasing the
359 processing time from 4 to 6 h, as well as the viability of the starter cultures. In this sense, lactulose,
360 when was added at 4%, significantly increased the population of *L. delbrueckii* ssp. *bulgaricus*
361 (by 2.2 $\log_{10}$ cfu/mL), which was in accordance with a decrease of around 40% of lactulose
362 observed throughout fermentation and cold storage periods. Lastly, the growth of *S. thermophilus*
363 was unaffected by the presence of any of the tested prebiotic, with the exception of a slight decrease
364 (1.0 $\log_{10}$ cfu/mL) in yogurts prepared with added lactulose at 4%. This fact could be attributable
365 to a similar metabolization of assayed prebiotic by *S. thermophillus* and *L. delbrueckii* ssp.
366 *bulgaricus* with the exception of the yogurt prepared with lactulose at 4%.
367 To conclude, this work demonstrates the feasibility to manufacture yogurts with different
368 doses of GOS, FOS or lactulose. In the case of yogurts prepared with lactulose, the level of added
369 prebiotic is a critical factor to improve the viability of *L. delbrueckii* ssp. *bulgaricus,* although the
370 stability of lactulose could be affected, especially at the highest lactulose concentration tested.
371

372 ## Acknowledgments

373 This work was supported by Spanish Ministry of Economy, Industry and Competitiveness
374 (Projects AGL2017-84614-C2-1-R and AGL2014-53445-R) and the Spanish Danone Institute.
375 The authors gratefully acknowledge Pascal Ronfard from Groupe Solactis and Bert Klarenbeek

376 from Friesland Campina Domo for providing lactulose and GOS, respectively, for the production
377 of yogurts added prebiotics.

References

Adebola, O., Corcoran, O., & Morgan, W.A. (2014). Synbiotics: the impact of potential prebiotics inulin, lactulose and lactobionic acid on the survival and growth of lactobacilli probiotics. *Journal of Functional Foods*, *10*, 75–84.

Adhikari, K., Grün, I. U., Mustapha, A., & Fernando, L. N. (2002). Changes in the profile of organic acids in plain set and stirred yogurts during manufacture and refrigerated storage. *Journal of Food Quality*, *25*, 435-451.

Akalin, A. S., Gönç, S., Ünal, G., & Fenderya, S. (2007). Effects of Fructooligosaccharide and Whey Protein Concentrate on the Viability of Starter Culture in Reduced□Fat Probiotic Yogurt during Storage. *Journal of Food Science*, *72*, 222-227.

Alm, L. (1982). Effect of fermentation on lactose, glucose, and galactose content in milk and suitability of fermented milk products for lactose intolerant individuals. *Journal of Dairy Science*, *65*, 346-352.

Brobst, K., & Lott J, C., 1966. Determination of some components in corn syrup by gas-liquid chromatography of the trimethylsilyl derivatives. *Cereal Chemistry*, *43*, 35-43.

Cardelle-Cobas, A., Olano, A., Corzo, N., Villamiel, M., Collins, M., Kolida, S., & Rastall, R. A. (2012). In vitro fermentation of lactulose-derived oligosaccharides by mixed fecal microbiota. *Journal of Agricultural and Food Chemistry*, *60*, 2024-2032.

Cénit, M. C., Matzaraki, V., Tigchelaar, E. F., & Zhernakova, A. (2014). Rapidly expanding knowledge on the role of the gut microbiome in health and disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1842*, 1981-1992.

399 Costa, M., Balthazar, C., Franco, R., Márcico, E., Cruz, A., & Conte, C. (2014). Changes on
400 expected taste perception of probiotic and conventional yogurts made from goat milk after
401 rapidly repeated exposure. *Journal of Dairy Science*, *97*, 2610-2618.

402 da Costa, M. P., da Silva Frasao, B., da Costa Lima, B. R. C., Rodrigues, B. L., & Junior, C. A. C.
403 (2016). Simultaneous analysis of carbohydrates and organic acids by HPLC-DAD-RI for
404 monitoring goat's milk yogurts fermentation. *Talanta*, *152*, 162-170.

405 Desai, A. R., Powell, I. B., & Shah, N. P. (2004). Survival and activity of probiotic lactobacilli in
406 skim milk containing prebiotics. *Journal of Food Science*, *69*.57-60.

407 Donkor, O. N., Henriksson, A., Vasiljevic, T., & Shah, N. P. (2007). Proteolytic activity of dairy
408 lactic acid bacteria and probiotics as determinant of growth and in vitro angiotensin-
409 converting enzyme inhibitory activity in fermented milk. *Le Lait*, *87*, 21-38.

410 Food and Agriculture Organization of the United Nations and World Health Organization
411 (FAO/WHO). (2001). *Health and nutritional properties of probiotics in food including*
412 *powder milk with live lactic acid bacteria*. FAO/WHO Expert Consultation. Córdoba,
413 Argentina.

414 Fernandez-Garcia, E., & McGregor, J. U. (1994). Determination of Organic Acids During the
415 Fermentation and Cold Storage of Yogurt1. *Journal of Dairy Science*, *77*, 2934-2939.

416 Goodenough, E., & Kleyn, D. (1976). Influence of Viable Yogurt Microflora on Digestion of
417 Lactose by the Rat1. *Journal of Dairy Science*, *59*, 601-606.

418 Holcomb, J. E., Frank, J. F., & Mc Gregor, J. U. (1991). Viability of Lactobacillus acidophilus and
419 Bifidobacterium bifidum in softserve frozen yogurt. *Cultured Dairy Products Journal, 26,* 4–
420 5.

421 Kip, P., Meyer, D., & Jellema, R. H. (2006). Inulins improve sensoric and textural properties of
422 low-fat yoghurts. *International Dairy Journal*, *16*, 1098-1103.

423 Montilla, A., Van de Lagemaat, J., Olano, A., & Del Castillo, M. (2006). Determination of
424 oligosaccharides by conventional high-resolution gas chromatography. *Chromatographia*, *63*,
425 453-458.

426 Moreno, F., Olano, A., Santa-Maria, G., & Corzo, N. (1999). Determination of maltodextrins in
427 enteral formulations by three different chromatographic methods. *Chromatographia*, *50*, 705-
428 710.

429 Mussatto, S., & Mancilha, I.M. (2007). Non-digestible oligosaccharides: a review. *Carbohydrate*
430 *Polymers*, *68*, 587-597.

431 Nobakhti, A. R., Ehsani, M. R., Mousavi, S.M., & Mortazavian, A. M. (2009). Influence of
432 lactulose and Hi-maize addition on viability of probiotic microorganisms in freshly made
433 synbiotic fermented milk drink. *Milchwissenschaft*, *64*, 191-193.

434 Olano, A., López-Covarrubias, S. J., Ramos, M., & Suárez, J. A. (1986). The use of lactulose in
435 the manufacture of low-lactose yogurt. *Biotechnology letters*, *8*, 451-452.

436 O'leary, V. S., & Woychik, J. H. (1976). Utilization of lactose, glucose, and galactose by a mixed
437 culture of Streptococcus thermophilus and Lactobacillus bulgaricus in milk treated with
438 lactase enzyme. *Applied and Environmental Microbiology*, *32*, 89-94.

439 Oliveira, R.P., Florence A.C.R., Silva, R.C., Perego, P., Converti, A., Gioielli L.A., & Oliveira
440 M.N. (2009). Effect of different prebiotics on the fermentation kinetics, probiotic survival and
441 fatty acids profiles in nonfat symbiotic fermented milk. *International Journal of Food*
442 *Microbiology*, *128*, 467-472.

443 Oliveira, R.P., Florence, A. C. R., Perego, P., De Oliveira, M. N., & Converti, A. (2011). Use of
444 lactulose as prebiotic and its influence on the growth, acidification profile and viable counts
445 of different probiotics in fermented skim milk. *International Journal of Food*
446 *Microbiology*, *145*, 22-27.

447 Power, S. E., O'Toole, P. W., Stanton, C., Ross, R. P., & Fitzgerald, G. F. (2014). Intestinal
448 microbiota, diet and health. *British Journal of Nutrition*, *111*, 387-402.

449 Roberfroid, M. B. (2007). Inulin-type fructans: Functional food ingredients. *The Journal of*
450 *Nutrition*, *137*, 2493-2502.

451 Shin, H. S., Lee, J. H., Pestka, J. J., & Ustunol, Z. (2000). Viability of bifidobacteria in commercial
452 dairy products during refrigerated storage. *Journal of Food Protection*, *63*, 327-331.

453 Tabasco, R., Paarup, T., Janer, C., Peláez, C., & Requena, T. (2007). Selective enumeration and
454 identification of mixed cultures of Streptococcus thermophilus, Lactobacillus delbrueckii
455 subsp. bulgaricus, L. acidophilus, L. paracasei subsp. paracasei and Bifidobacterium lactis in
456 fermented milk. *International Dairy Journal*, *17*, 1107-1114.

457 Van Leusen, E., Torringa, E., Groenink, P., Kortleve, P., Geene, R., Schoterman, M., &
458 Klarenbeek, B. (2014). Industrial applications of galactooligosaccharides. In F. J. Moreno &
459 M. L Sanz (Ed.), *Food oligosaccharides: Production, analysis and bioactivity* (pp. 35-
460 54).Chichester, UK: Wiley Blackwell.

461 Vénica, C.I., Perotti, M.C., & Bergamini, C.V. (2014). Organic acids profiles in lactose-
462 hydrolyzed yogurt with different matrix composition. *Dairy Science & Technology*, *94*, 561-
463 580.

464 Vénica, C. I., Wolf, I. V., Bergamini, C. V., & Perotti, M. C. (2016). Influence of lactose hydrolysis
465 on galacto□oligosaccharides, lactose, volatile profile and physicochemical parameters of
466 different yogurt varieties. *Journal of the Science of Food and Agriculture*, *96*, 4929-4939.

467 **FIGURE CAPTIONS**

468 **Figure 1.** Evolution of pH **(a),** viability of *Streptococcus thermophilus* **(b)** *and Lactobacillus*
469 *delbrueckii ssp. bulgaricus* **(c)** in control and yogurts added prebiotics. Values are the means ±
470 standard error (*n*=4).

471 **Figure 2.** Change of lactose **(a),** lactic acid **(b)** and acetic acid **(c)** in control and yogurts added
472 prebiotics during fermentation at 45ºC and cold storage at 4ºC. Values are the means ± standard error
473 (*n*=6).

474 **Figure 3**. Change of GOS and FOS content in yogurts following their addition at **(a)** and **(c)** 2% and
475 at **(b)** and **(d)** 4%**,** respectively, during fermentation at 45ºC and cold storage at 4ºC. Values are
476 the means ± standard error (*n*=6).

477 **Figure 4.** Change of lactulose content in yogurts following their addition at 2% and at 4% during
478 fermentation at 45ºC and cold storage at 4ºC. Values are the means ± standard error (*n*=6).

479 **Figure 5.** Change of **(a)** galactose and **(b)** glucose content in control and yogurts added prebiotics
480 during fermentation at 45ºC and cold storage at 4ºC. Values are the means ± standard error (*n*=6).