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Galacto-oligosaccharides formation during manufacture of different varieties of yogurt.  
Stability through storage

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1 **GALACTO-OLIGOSACCHARIDES FORMATION DURING MANUFACTURE OF**  
2 **DIFFERENT VARIETIES OF YOGURT. STABILITY THROUGH STORAGE.**

3  
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10  
11 **Abstract**

12 Galacto-oligosaccharides (GOS) have interest in the food industry due to their  
13 recognized functional properties. In this work, we studied the effect of a commercial  $\beta$ -  
14 galactosidase enzyme from *Kluyveromyces lactis* (YNL-2, GODO) and *Lactobacillus*  
15 *acidophilus* La-5, on GOS formation during the manufacture and storage of drinkable and  
16 stirred yogurts. In a preliminary step, GOS synthesis and lactose hydrolysis by  $\beta$ -  
17 galactosidase was evaluated at different initial lactose concentrations and doses of enzyme.  
18 The GOS formation was favored with increasing of lactose concentration and enzyme  
19 doses, while the hydrolysis dominated at lower level of lactose. In turn, the presence of  
20 GOS was already evident at 45 min of fermentation in yogurts with addition of  $\beta$ -  
21 galactosidase. Mean concentrations were 0.36 and 0.62 g/100 g for fresh drinkable and  
22 stirred yogurts, respectively. No changes in the GOS levels were observed through storage,  
23 indicating that they were stable in the products. The probiotic bacteria added were not able  
24 to produce GOS. The diminution of lactose was significant in yogurts with  $\beta$ -galactosidase;

25 contents of residual lactose were around 1.3 g/100 mL. We obtained different varieties of  
26 reduced-lactose yogurts enriched in galacto-oligosaccharides. The presence of probiotic and  
27 prebiotic would increase the functional properties of yogurts.

28 **Keywords:** Galacto-oligosaccharides,  $\beta$ -galactosidase, *L. acidophilus*, inulin, yogurt.

29

## 30 **1. Introduction**

31 Currently, galacto-oligosaccharides have attracted particular interest for research and  
32 applications in the field of food, due to their recognized functional properties. GOS are  
33 non-digestible and non-cariogenic carbohydrates that modulate the colonic microbiota,  
34 promoting the healthy balance (prebiotic effect), among other positive health effects  
35 (Caselato de Sousa, Freitas dos Santos, & Sgarbieri, 2011; Mussatto & Mancilha, 2007).  
36 These compounds are comprised of a variable number of galactose units and, in some  
37 cases, a terminal glucose unit, joined by glycosidic bonds. They are produced from lactose  
38 (or other galactoside) by enzymatic *via* with  $\beta$ -galactosidases. The first step involves the  
39 formation of the galactosyl-enzyme complex and release of the glucose unit. After that, two  
40 reactions can concomitantly occur, hydrolysis and transgalactosylation, depending on the  
41 galactosyl-moiety acceptor present in the reaction medium. When the acceptor is water, the  
42 hydrolysis takes place and lactose is split into glucose and galactose; while, when the  
43 acceptor is galactose (or potentially any sugar), the galactosyl transfer happens and a  
44 complex mixture of GOS is formed (Gosling, Stevens, Barber, Kentish, & Gras, 2010;  
45 Otieno, 2010). The predominance of the GOS synthesis over the hydrolysis, and the yield  
46 and composition of the GOS mixture obtained are significantly affected by the origin of  $\beta$ -  
47 galactosidase enzyme and the operating conditions (lactose concentration, dose of enzyme,  
48 temperature/time and pH) (Boon, Janssen, & van 't Riet, 2000; Gosling et al., 2010).

49 GOS are used as functional food ingredients, alone or with fructo-oligosaccharides or  
50 inulin, into infant formulas to mimic the beneficial effects of human milk oligosaccharides  
51 (Bode, 2009). Other processed foods that are important for the inclusion of GOS are  
52 beverages, bakery and dairy products because their functional and technological aspects  
53 (high solubility, clean taste, stability, low glycemic index) (Torres, Gonçalves, Teixeira, &  
54 Rodrigues, 2010). However, GOS can also be formed *in situ* during the manufacture of  
55 fermented dairy foods as a result of the metabolic activity of strains (Gosling et al., 2009).  
56 The formation of oligosaccharides in yogurts prepared by using yogurt cultures combined  
57 with bifidobacteria strains has been reported (Lamoureux, Roy, & Gauthier, 2002). In turn,  
58 Martínez-Villaluenga, Cardelle-Cobas, Corzo, and Olano, (2008a) tested the GOS contents  
59 in commercial products: traditional yogurts, yogurts containing bifidobacteria and ready-to-  
60 drink yogurts with *Lactobacillus casei*. In both studies, it was found a wide variation  
61 among samples analyzed; probiotic yogurts showed higher amount of GOS compared to  
62 traditional ones. The stability of GOS in the dairy matrix is an important aspect to be  
63 considered. Mozaffar, Nakanishi, and Matsuno (1985) detected a disappearance almost  
64 complete of GOS at the latter stage of milk incubation with a commercial  $\beta$ -galactosidase  
65 enzyme. However, Lamoureux et al. (2002), Martínez-Villaluenga Cardelle-Cobas, Corzo,  
66 Olano, and Villamiel (2008b) and Yadav, Jain and Sinha (2007) indicated that no  
67 hydrolysis of GOS occurred through storage. Hence, the results reveal that the amount of  
68 GOS produced depends on the strains and the processing parameters used in the preparation  
69 of different varieties of fermented milks.

70 On the other hand, the direct addition of  $\beta$ -galactosidase enzyme in the production of  
71 reduced-lactose products could lead to simultaneous production of GOS. Delactozed dairy  
72 foods are destined for individuals who are affected by lactose intolerance, because they are

73 deficient of the lactase enzyme in the digestive tract needed to properly absorb the lactose.  
74 The problem of lactose intolerance is well-known and widespread in more than half of the  
75 Latin American population (Ruiz-Matute et al., 2012). Some studies evaluate different  
76 conditions in order to obtain low-lactose milks containing GOS (Chen, Hsu, & Chiang,  
77 2002; Mahoney, 1998; Ruiz-Matute et al., 2012). However, according to our knowledge,  
78 there are scarce data about this topic in fermented milks. The yogurt market in Argentine  
79 has experienced steady growth in recent years and different varieties of products have been  
80 launched; nevertheless, reduced-lactose yogurts with increasing amounts of GOS are yet  
81 absent.

82 The aim of this work was to study the effect of the inclusion of commercial  $\beta$ -  
83 galactosidase from *K. lactis* and the probiotic bacteria *L. acidophilus* La-5 on the GOS  
84 formation during the manufacture and storage of drinkable and stirred yogurts. In a  
85 preliminary step, GOS synthesis and lactose hydrolysis by the  $\beta$ -galactosidase enzyme was  
86 evaluated at different initial lactose concentrations and doses of enzyme.

87

## 88 **2. Materials and methods**

### 89 ***2.1. Enzymatic hydrolysis/transgalactosylation from lactose in buffer***

90 Enzymatic hydrolysis and synthesis of GOS from lactose solution was studied at three  
91 different concentrations of initial lactose and three different doses of enzyme at laboratory  
92 trials. A commercial food grade  $\beta$ -galactosidase enzyme derived from *K. lactis*, YNL-2  
93 GODO (50000 U ONPG/g) produced by Shusei Company Limited (Tokyo, Japan) and  
94 kindly donated by Milkaut S.A. (Santa Fe, Argentine), was employed. These preliminary  
95 experiences were performed to know the ability of this enzyme for GOS production, in  
96 order to apply it for the obtaining of different varieties of yogurts enriched in GOS.

97 Lactose monohydrate (Sigma-Aldrich, Saint Louise, USA) solutions (100 mL) of 5, 10  
98 and 20 g/100 mL were prepared in 100 mmol/L potassium phosphate buffer (pH 6.8)  
99 (Sigma-Aldrich, Saint Louise, USA) containing 1 mmol/L MgCl<sub>2</sub> (Sigma-Aldrich, Saint  
100 Louise, USA). The enzyme was added at different doses, 0.16, 0.25 and 0.40 g/L  
101 (equivalent to 8000, 12500 and 20000 units, respectively), and the reaction mixtures were  
102 incubated in a water bath at 42 ± 1 °C for 3 h. At different times (40, 60, 100, 140 and 180  
103 min), aliquots (4 mL) were withdrawn and immediately immersed in a boiled water bath for  
104 8 min to deactivate the enzyme. The samples were stored at -18 °C for carbohydrates  
105 analysis. The incubation experiences were carried out in duplicate.

106 The amounts of remaining lactose, and the amount of GOS, glucose and galactose  
107 produced were expressed as percentage by weight of the total carbohydrates content in the  
108 reaction mixtures.

109

## 110 ***2.2 Yogurt manufacture***

111 Two varieties of sweetened yogurts, drinkable and stirred were made at laboratory  
112 scale; stainless steel vats of 5 L of capacity each were employed (Vénica, Perotti, &  
113 Bergamini, 2014).

114 The results obtained in preliminary experiences were taken into account to select the  
115 doses of enzyme for the production of yogurts with high levels of GOS. Therefore, for  
116 drinkable yogurts, whose milk base had approximately 5 g/100 mL of lactose, the lower  
117 dose of enzyme was used, while for the stirred yogurts, with levels of initial lactose around  
118 7 g/100 mL, the intermediate level of enzyme was chosen.

119 A factorial design was used for each variety of yogurt. Two factors were studied, the  
120 addition of  $\beta$ -galactosidase enzyme, and the incorporation of *L. acidophilus* La-5 (Chr  
121 Hansen, Horsholm, Denmark) and inulin (Orafti<sup>®</sup>GR, Mannheim, Germany), at two levels  
122 each, with and without addition. Thus, four different types of yogurt were manufactured:  
123 unhydrolyzed (**C**); unhydrolyzed symbiotic (with probiotic and prebiotic) (**P**); hydrolyzed  
124 (**E**) and hydrolyzed symbiotic (**EP**). These yogurts were performed in triplicate resulting in  
125 a total of 12 experimental units for drinkable and stirred yogurts, respectively.

126 Bulk bovine milk 3 g/100 mL fat content (Milkaut S.A., Santa Fe, Argentine) with  
127 addition of 8 g/100 mL sucrose (Ingenio Ledesma S.A., Tucumán, Argentine) was  
128 tempered until it reached approximately 40 °C. At this moment, 2.25 g/100 mL skim milk  
129 powder (SMP) and 2.00 g/100 mL whey protein concentrate (WPC35) (Milkaut S.A., Santa  
130 Fe, Argentine), were added for stirred yogurts. In symbiotic yogurts, 1.00 g/100 mL inulin  
131 was also aggregated. The ingredients were dissolved by manual agitation for 15 min. Milk  
132 bases were heated at  $90 \pm 2$  °C, stand for 5 min, immediately cooled to  $42 \pm 2$  °C, and  
133 inoculated with freeze-dried direct vat set (DVS) YF-L811 (Chr. Hansen, Buenos Aires,  
134 Argentine) containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.  $\beta$ -  
135 galactosidase enzyme (0.16 and 0.25 g/L, for drinkable and stirred yogurts, respectively)  
136 was added together with the starter culture for hydrolyzed yogurts (**E** and **EP**). The  
137 incubation process was conducted at  $42 \pm 2$  °C until pH  $4.70 \pm 0.10$  was reached. At this  
138 point, freeze-dried DVS culture of *L. acidophilus* La-5 was added in order to give initial  
139 cell count of  $10^7$  CFU/g in symbiotic yogurts (**P** and **EP**). The yogurts were immediately  
140 cooled to 25 °C in an ice water bath, applying intermittent manual agitation, followed by

141 placing in screw cap glass flasks (500 mL). Finally, the yogurts were stored at  $5 \pm 1$  °C for  
142 21 days.

143 Aliquots were removed at different times during fermentation and in freshly made  
144 yogurts to measure pH, concentration of GOS and lactose. In addition, throughout the entire  
145 refrigerated storage period, pH, titratable acidity, and concentrations of lactose, GOS and  
146 lactic acid were determined. Overall composition (total solids, protein and fat) and  
147 microbiological counts were also evaluated.

148

#### 149 ***2.4. Carbohydrates and lactic acid analysis by HPLC***

150 HPLC equipment for the analysis of carbohydrates and lactic acid consisted of a  
151 quaternary pump, an on-line degasser, UV-visible detector (Series 200), a refractive index  
152 detector and a column oven (Series Flexar) (Perkin Elmer, Norwalk, USA). Data were  
153 collected and processed on a computer with the software Chromera® (Perkin Elmer,  
154 Norwalk, USA).

155 The analysis of GOS, lactose, glucose and galactose in the incubation experiences of  
156 lactose solution with the  $\beta$ -galactosidase enzyme were made on an Aminex HPX-87N  
157 column (300 x 7.8 mm) equipped with a cation  $\text{Na}^+$  microguard cartridge (Bio-Rad  
158 Laboratories, Norwalk, USA). Chromatographic separation was performed using HPLC  
159 water as mobile phase at a flow rate of 0.3 mL/min, maintaining the column at 85 °C.  
160 Aliquots of reaction mixtures were appropriately diluted with distilled water, filtered  
161 through 0.45  $\mu\text{m}$  membranes (Millex, Millipore, São Paulo, Brazil) and injected into the  
162 chromatograph, using a loop of 20  $\mu\text{L}$ .



163 On other hand, the analysis of GOS, lactose and lactic acid during the manufacture (in  
164 milk base, 45 and 150 min of incubation), in fresh yogurts and during storage (7 and 21  
165 days), were made on an Aminex HPX-87H column (300 x 7.8 mm) equipped with a cation  
166 H<sup>+</sup> microguard cartridge (Bio-Rad Laboratories, Hercules, USA), which allow the  
167 simultaneous quantification of sugars and organic acids using UV and IR detectors  
168 connected in series. Chromatographic separation and sample preparation was performed  
169 according to Vénica et al. (2014). Quantification was performed by external calibration  
170 using suitable standards (Sigma-Aldrich, Saint Louise, USA). Regarding the quantification  
171 of GOS, the trisaccharide raffinose was used as standard (Lamoureux et al., 2002; Martínez  
172 Villaluenga et al., 2008b).

173

#### 174 ***2.5. Physicochemical determinations and microbiological counts***

175 The measurement of pH during fermentation (in milk base, 45 and 150 min), in freshly  
176 made yogurts and during storage (7, 14 and 21 days) was done with a digital pH meter  
177 (Orion 3 star benchtop, Thermo Fisher Scientific Inc., Beverly, USA). Titratable acidity  
178 (TA) (1, 7, 14 and 21 days) was determined by titration with 0.1 N NaOH (IDF, 2012). The  
179 results were expressed as Dornic degree (1 °D = 100 mg lactic acid/L). Protein (IDF, 2001),  
180 total solid (IDF, 2005), and fat contents (Bradley et al. 1992) of yogurts with 7 days of  
181 storage were analyzed.

182 Total lactic acid bacteria and moulds and yeasts in freshly made yogurt and at 21 days  
183 were analyzed according to Vénica et al. (2014). The counts of *L. acidophilus* were  
184 determined on MRS agar by Vinderola and Reinheimer (1999).

185

#### 186 ***2.6. Statistical analyses***

187 Data obtained from yogurts were processed by two-way ANOVA in order to detect  
188 differences in pH, TA, lactose, GOS and lactic acid at each sampling time. One-way  
189 ANOVA was also used to detect the effect of storage period on GOS concentration.  
190 Statistical analyses were carried out using SPSS 10.0 software (SPSS Inc., Chicago, USA).

191

### 192 **3. Results and discussion**

#### 193 ***3.1. Enzymatic hydrolysis/transgalactosylation from lactose in buffer solution***

194 Lactose hydrolysis and transgalactosylation reactions by the commercial  $\beta$ -  
195 galactosidase enzyme YNL-2 in the incubation experiences were followed by HPLC-IR  
196 analyses of carbohydrate profiles.

197 **Fig. 1** shows, by way of example, the HPLC-IR chromatogram of the reaction mixture  
198 containing an initial lactose concentration of 5 g/100 mL and with 0.25 g/L of enzyme,  
199 incubated for 180 min at 42 °C. As expected, glucose and galactose were the main  
200 components due to the hydrolytic activity of the  $\beta$ -galactosidase enzyme. Likewise, it was  
201 possible to detect a first peak with retention time of 14.9 min, which eluted before the  
202 disaccharide fraction (lactose, in this case), corresponding to GOS as a result of the  
203 transgalactosylation activity of enzyme.

204 GOS production (expressed as mean percentage of total sugars) during the time course  
205 of reaction (3 h) in the presence of different doses of  $\beta$ -galactosidase (0.16-0.40 g/L) and  
206 different initial lactose concentrations (5-20 g/100 mL) is shown in **Fig. 2 (A, B and C)**. It  
207 was found that the GOS formation increased with increasing initial lactose concentration  
208 from 5 to 20 g/100 mL, for each dose of enzyme. In particular, for lactose concentrations of  
209 5, 10 and 20 g/100 mL, the maximum GOS contents were 4.2 (reached at 100 min), 6.0  
210 (180 min) and 6.6 g/100 mL (180 min), respectively, for the lower level of enzyme assayed

211 (0.16 g/L); 5.4 (60 min), 8.7 (140 min) and 11.7 g/100 mL (180 min), for the intermediate  
212 enzyme level (0.25 g/L); and 4.9 (40 min), 9.0 (60 min) and 13.1 g/100 mL (140 min), for  
213 the higher enzyme level (0.40 g/L), respectively.. On the other hand, increases of the doses  
214 of enzyme led to maximum amounts of GOS in a shorter reaction time, for each level of  
215 initial lactose tested, as can be seen by the values of reaction times indicated in brackets. In  
216 some cases, a slight degradation of GOS after the maximum reached was observed. In  
217 particular, the decrease of GOS content was more pronounced with the higher doses of  
218 enzyme and the lower concentration of initial lactose in the reaction medium. This behavior  
219 could be attributed to the fact that these compounds are intermediate in the enzymatic  
220 reaction and could be hydrolyzed by the  $\beta$ -galactosidase enzyme when the remaining  
221 lactose contents are low (Čurda, Rudolfová, Štětina, & Dryák, 2006; Rodriguez-Colinas,  
222 Poveda, Jimenez-Barbero, Ballesteros, & Plou, 2011, Splechtna et al., 2006).

223 **Fig. 3 (A, B and C)** illustrates the changes in the percentages of remaining lactose, and  
224 glucose and galactose formed during the incubation period. As expected, the residual  
225 lactose and the glucose and galactose diminished and increased, respectively, as reaction  
226 time elapsed; this effect was more evident with increasing enzyme levels. The diminution  
227 observed in the residual lactose values was more pronounced at lower initial lactose  
228 concentration, which was associated with higher values of glucose and galactose. On the  
229 other hand, the levels of galactose were lower than those of glucose in all cases, above all  
230 in the experiences with higher initial lactose concentration, which is related with the  
231 synthesis of GOS. Mean values of glucose/galactose ratio for all the doses of enzymes  
232 tested were 1.01, 1.15 and 1.32 for 5, 10 and 20 g/100 mL of initial lactose, respectively.  
233 The GOS yields were calculated by dividing the amount of GOS formed by the amount of

234 lactose consumed and multiplying by 100; mean values of the maximum GOS yields were  
235 approximately 8, 15 and 26 %, for 5, 10 and 20 g/100 mL of lactose (data not shown).

236 These results highlight that the reactions of hydrolysis and transgalactosylation occur  
237 simultaneously and the products obtained (glucose, galactose and GOS) are mainly  
238 dependent on the starting lactose concentration in the reaction medium. In addition, we  
239 confirmed that hydrolysis is favored over transgalactosylation at low lactose concentration,  
240 since the amount of hydroxyl groups of carbohydrates is lower as compared to those of  
241 water, while GOS formation dominates at high lactose concentration, since galactosyl  
242 groups have a higher probability of attaching to lactose. Thereby, as the initial  
243 concentration of lactose increases, the hydrolysis was decreasing and the GOS formation  
244 increasing. Similar results for other  $\beta$ -galactosidases enzymes were reported by many  
245 authors (Boon et al., 2000; Čurda et al., 2006; Martínez-Villaluenga et al., 2008b; Neri et  
246 al., 2009; Palai, Mitra, & Bhattacharya, 2012; Urrutia et al., 2013).

247

### 248 **3.2. Physicochemical parameters and microbiological counts of yogurt**

249 The contents of total solids, protein and fat (**Table 1**) were suitable as established by  
250 Argentinian Legislation (CAA, 2010). The addition of inulin in symbiotic yogurts produced  
251 an increase in the total solid content ( $P < 0.05$ ). No significant differences ( $P > 0.05$ ) in  
252 chemical composition of yogurts were observed by the inclusion of exogenous enzyme.

253 As expected, the pH sharply decreased during incubation process due to the metabolic  
254 activity of lactic acid bacteria. During the storage period, the pH continued to decline  
255 slightly in a similar way for all samples (the values at 7 days are shown in **Table 1**). No  
256 influence of the enzyme on pH values was detected during fermentation, while significant  
257 differences ( $P < 0.05$ ) were found at 14 days for drinkable yogurts and at 7 days for stirred

258 ones; the hydrolyzed yogurts (**E** and **EP**) had the highest values. Addition of inulin and La-5  
259 did not have a significant influence on pH values ( $P > 0.05$ ).

260 The titratable acidity increased progressively through storage from 60 to 71 °D for  
261 drinkable yogurts and from 77 to 94 °D for stirred ones (**Table 2**). All values were in  
262 accordance with those established by Argentinian Legislation (60-150 °D) (CAA, 2010).  
263 For drinkable yogurts, TA was significantly ( $P < 0.05$ ) affected by the enzyme addition at  
264 14 days and by the addition of probiotic and prebiotic (La-5/inulin) at 14 and 21 days. For  
265 stirred yogurts, the influence of enzyme addition was significant ( $P < 0.05$ ) at 14 and 21  
266 days while the addition of La-5/inulin did not influence on TA values. In both varieties of  
267 yogurt the enzyme incorporation led to lower values of TA and the La-5/inulin addition to  
268 higher values of TA.

269 Regarding the lactic acid concentrations, no significant difference was observed ( $P >$   
270  $0.05$ ) (**Table 2**). The mean values were 580 and 740 mg/100 g at the end of manufacture,  
271 and 660 and 880 mg/100 g at 21 days, for drinkable and stirred yogurts, respectively.  
272 However, the pattern was similar to that found for TA; the hydrolyzed yogurts (**E** and **EP**)  
273 had lower values of lactic acid content than unhydrolyzed ones (**C** and **P**).

274 The viable cell counts of *L. acidophilus* was  $10^7$  CFU/g in symbiotic yogurts and the  
275 total LAB counts in all yogurts were about  $10^9$  CFU/g, throughout the whole period of  
276 storage. They were in accordance with those fixed by Argentinian Legislation (LAB counts  
277  $> 10^7$  CFU/g; probiotic counts  $> 10^6$  CFU/g) (CAA, 2010; CAA, 2013). Similar levels of  
278 viable counts of La-5 were found by Özer, Akin, and Özer (2005) and Mazloomi,  
279 Shekarforoush, Edrahimnejad, and Sajedianfard (2011), which were maintained throughout  
280 14 days of storage in symbiotic yogurts. Likewise, they found that the probiotic addition  
281 did not affect the values of pH, TA and lactic acid. On the other hand, Ng, Yeung, and

282 Tong (2011) and Mazloomi et al. (2011) reported a reduction of approximately 1 log in the  
283 counts of *L. acidophilus* during storage of yogurts prepared without inulin.

284

### 285 **3.3. GOS and lactose concentrations in yogurts**

286 The evolution of lactose concentration during manufacture and storage for drinkable  
287 and stirred hydrolyzed and unhydrolyzed yogurts is shown in **Fig. 4**. In turn, **Fig. 5**  
288 illustrates the GOS concentration of hydrolyzed yogurts (**E** and **EP**), as these compounds  
289 were not detected in unhydrolyzed ones (**C** and **P**). **Table 3** shows the significance of  
290 treatment effects on lactose and GOS concentrations.

291 Enzyme addition had a significant effect on lactose and GOS contents. La-5/inulin  
292 addition was significant on GOS concentration only for stirred products at 21 days; the  
293 symbiotic yogurts had the highest values. Meanwhile, the lactose content in drinkable  
294 symbiotic yogurts at 21 days was slightly lower ( $P < 0.05$ ) than the products without La-  
295 5/inulin.

296 The lactose values were lower in hydrolyzed yogurts compared to unhydrolyzed ones,  
297 for all sampling times. Residual lactose concentration in freshly made hydrolyzed yogurts  
298 was 1.26 and 1.52 g/100 g, for drinkable and stirred yogurts, respectively, compared to 4.08  
299 and 5.55 g/100 g for unhydrolyzed ones. The presence of GOS was already evident at 45  
300 min of fermentation, when the greatest decrease of lactose was obtained; then, GOS  
301 concentration slightly increased towards the end of fermentation. Mean values were 0.62  
302 and 0.36 g/100 g, for stirred and drinkable hydrolyzed yogurts, respectively. The difference  
303 found between both yogurt varieties is due to the higher content of lactose in the milk base  
304 and level of enzyme used in stirred yogurts in comparison to drinkable ones, which

305 improves the transgalactosylation reaction. This fact is consistent with the data obtained in  
306 the preliminary experiences of hydrolysis/transgalactosylation from lactose solutions.

307 In addition, no changes in the contents of GOS were observed through the refrigerated  
308 storage period ( $P > 0.05$ ), which states that the GOS formed were stable in the different  
309 yogurt matrices. Even though we observed a diminution in the amount of GOS after  
310 reaching a maximum in some preliminary experiences of incubation of lactose solutions,  
311 this behavior was not found in yogurts. This fact could be due that the enzyme employed  
312 was inactivated at the pH of yogurts, while in the reaction mixtures the pH was maintained  
313 at the optimal for the enzyme activity (pH 6-8).

314 Limited information is available about the GOS formation during the manufacture of  
315 hydrolyzed yogurts and their stability on storage. In this sense, Toba, Arihara, and Adachi  
316 (1986) found the maximum content of oligosaccharides at 2 h of incubation (approximately  
317 1.2%) during yogurt making with the inclusion of  $\beta$ -galactosidase from *Aspergillus orizae*.  
318 After that, the GOS level dropped to half toward the end of fermentation (8 h) and they  
319 continued to decline even more in the storage period (10 d, 5 °C). The authors indicated that  
320 the exogenous enzyme could have hydrolyzed the GOS formed. Recently, Martins, Manera,  
321 Monteiro, Burkert, and Burkert (2011) studied the GOS production by Lactomax Flex  
322 enzyme (composed by  $\beta$ -galactosidases from *K. lactis* and *Aspergillus niger*) in probiotic  
323 yogurts; they found 0.27 and 0.42 g GOS/100 mL.

324 On the other hand, the absence of GOS in unhydrolyzed yogurts (**C** and **P**) indicates  
325 that the  $\beta$ -galactosidases from YF-L811 and La-5 cultures were unable to produce these  
326 compounds under the conditions employed. Variable results were reported in relation to the  
327 ability of starter and probiotic cultures to produce GOS in fermented milks. Toba et al.  
328 (1986) reported GOS values of 0.09% in traditional yogurts. Lamoureux et al. (2002) found

329 levels of approximately 0.28% in freshly made yogurts, which increased to values between  
330 0.49 to 0.72% with the inclusion of different bifidobacteria species in the formulation.  
331 Martinez-Villaluenga et al. (2008a) informed GOS contents of about 0.23, 0.37 and 0.50%  
332 in commercial yogurts, in ready-to-drink yogurts containing *L. casei* and in yogurts  
333 containing bifidobacteria, respectively. In turn, Yadav et al. (2007) pointed out that the  
334 ability to produce GOS was different among strains/species, because they found values  
335 ranged from 0.33 to 0.53 g/100 mL in fermented milks made with *Lactococcus lactis*, *L.*  
336 *acidophilus* and *L. casei*. In all these studies no change in the GOS contents was observed  
337 during the storage of yogurts or fermented milks. Meanwhile, Martins et al. (2011) have not  
338 detected GOS in probiotic yogurts, indicating that the starter culture and *Bifidobacterium*  
339 *animalis* and *L. acidophilus* were not able to produce the compounds that being sought;  
340 these results are similar with those obtained in our work.

341 Finally, it is interesting to highlight that the GOS contents we have achieved in yogurts  
342 were comparable with those reported by Ruiz-Matute et al. (2012) for commercial lactose-  
343 free UHT milks and dairy drinks (0.10 to 0.44 g/100 mL) and by Chirido et al. (2011) for  
344 infant formulas from different brands (0.33 to 0.72 g/100 mL).

345

#### 346 **4. Conclusion**

347 The results obtained in our study indicate that the commercial  $\beta$ -galactosidase enzyme  
348 tested had ability to produce GOS during manufacturing of yogurts, while the starter and  
349 probiotic cultures did not show it. The presence of GOS was already evident at 45 min of  
350 fermentation in yogurts with addition of  $\beta$ -galactosidase, and then it slightly increased until  
351 the end of process and remained stable during the storage period of products.



352 On other hand, the enzyme produced a reduction in the lactose content, so the product  
353 obtained was beneficial for lactose intolerant people.

354 The stability of GOS during storage of the yogurts was probably due to the inability of  
355 cultures added to metabolize them and the inactivation of the  $\beta$ -galactosidase enzyme from  
356 *K. lactis* at the pH values of yogurts. This fact is important in order to grant consumers the  
357 beneficial effect of these compounds. However, the stability of GOS could be different in  
358 yogurts made with other cultures or with  $\beta$ -galactosidases enzymes with optimal pH acidic.

359 In the present work, we obtained different varieties of reduced-lactose yogurts enriched  
360 in galacto-oligosaccharides; the levels found were similar to those reported in commercial  
361 lactose-free milks and infant formulas. Furthermore, the presence of probiotic and prebiotic  
362 would increase the functional properties of yogurts.

363

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371

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**Table 1.** Composition (g/100 g) and pH of yogurts at 7 days of storage (mean  $\pm$  standard deviation;  $n = 3$ ).

Yogurt		Total solids	Fat	Protein	pH
<b>Drinkable</b>	<b>C</b>	17.9 $\pm$ 0.2	2.8 $\pm$ 0.2	3.01 $\pm$ 0.06	4.42 $\pm$ 0.10
	<b>E</b>	17.5 $\pm$ 0.3	3.0 $\pm$ 0.1	3.00 $\pm$ 0.02	4.49 $\pm$ 0.06
	<b>P</b>	18.5 $\pm$ 0.2	2.5 $\pm$ 0.2	3.03 $\pm$ 0.05	4.43 $\pm$ 0.04
	<b>EP</b>	18.6 $\pm$ 0.1	2.6 $\pm$ $\square$ 0.1	3.03 $\pm$ 0.05	4.51 $\pm$ 0.08
<i>Significance of treatment effect</i>					
Enzyme		NS	NS	NS	NS
La-5/inulin		*	NS	NS	NS
<b>Stirred</b>	<b>C</b>	20.4 $\pm$ $\square$ 0.2	2.2 $\pm$ 0.2	4.20 $\pm$ 0.03	4.46 $\pm$ 0.04
	<b>E</b>	20.6 $\pm$ 0.1	2.2 $\pm$ 0.2	4.13 $\pm$ 0.07	4.51 $\pm$ 0.04
	<b>P</b>	21.4 $\pm$ 0.1	2.6 $\pm$ 0.1	4.24 $\pm$ 0.07	4.46 $\pm$ 0.04
	<b>EP</b>	21.4 $\pm$ 0.1	2.6 $\pm$ 0.2	4.22 $\pm$ 0.01	4.57 $\pm$ 0.05
<i>Significance of treatment effect</i>					
Enzyme		NS	NS	NS	*
La-5/inulin		*	NS	NS	NS

**C:** unhydrolyzed yogurts; **P:** unhydrolyzed symbiotic yogurts; **E:** hydrolyzed yogurts; **EP:** hydrolyzed symbiotic yogurts.

Two-way ANOVA analysis; NS: Not significant; \*:  $P < 0.05$ .

**Table 2.** Titratable acidity (°Dornic) and lactic acid concentration (mg/100 g) in yogurts during storage (mean ± standard deviation;  $n = 3$ ).

Yogurt	Titratable acidity				Lactic acid		
	1 day	7 days	14 days	21 days	End (pH=4.7)	21 days	
<b>Drinkable</b>	C	62.9 ± 1.6	67.6 ± 1.2	69.1 ± 0.8	69.9 ± 0.6	598.8 ± 35.1	685.2 ± 77.7
	E	61.0 ± 1.2	64.7 ± 2.4	66.2 ± 1.5	65.8 ± 1.5	549.1 ± 49.9	675.1 ± 18.8
	P	60.3 ± 1.1	66.9 ± 1.6	70.6 ± 1.9	71.5 ± 2.7	615.9 ± 14.3	662.0 ± 79.3
	EP	61.9 ± 1.4	67.1 ± 0.7	69.2 ± 1.6	70.5 ± 2.8	549.4 ± 57.8	602.7 ± 50.2
<i>Significance of treatment effect</i>							
Enzyme	NS	NS	*	NS	NS	NS	
La-5/inulin	NS	NS	*	*	NS	NS	
<b>Stirred</b>	C	82.0 ± 2.0	89.4 ± 1.8	91.7 ± 0.7	93.6 ± 1.2	793.2 ± 55.3	986.9 ± 25.8
	E	81.6 ± 1.9	87.8 ± 3.4	88.2 ± 0.8	90.0 ± 1.8	743.1 ± 18.1	795.7 ± 12.2
	P	78.1 ± 2.3	89.3 ± 2.8	91.5 ± 1.8	94.1 ± 2.7	720.2 ± 56.4	876.8 ± 72.0
	EP	76.9 ± 1.6	85.2 ± 3.2	89.1 ± 3.2	91.9 ± 2.6	716.2 ± 85.3	862.6 ± 95.3
<i>Significance of treatment effect</i>							
Enzyme	NS	NS	*	*	NS	NS	
La-5/inulin	NS	NS	NS	NS	NS	NS	

**C:** unhydrolyzed yogurts; **P:** unhydrolyzed symbiotic yogurts; **E:** hydrolyzed yogurts; **EP:** hydrolyzed symbiotic yogurts.

Two-way ANOVA analysis; NS: Not significant; \*:  $P < 0.05$ .

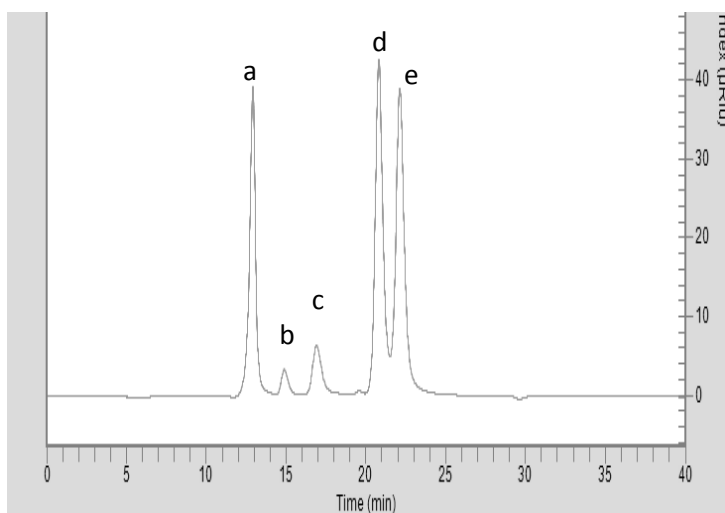


**Table 3.** Significance of treatment effect on GOS and lactose concentration.

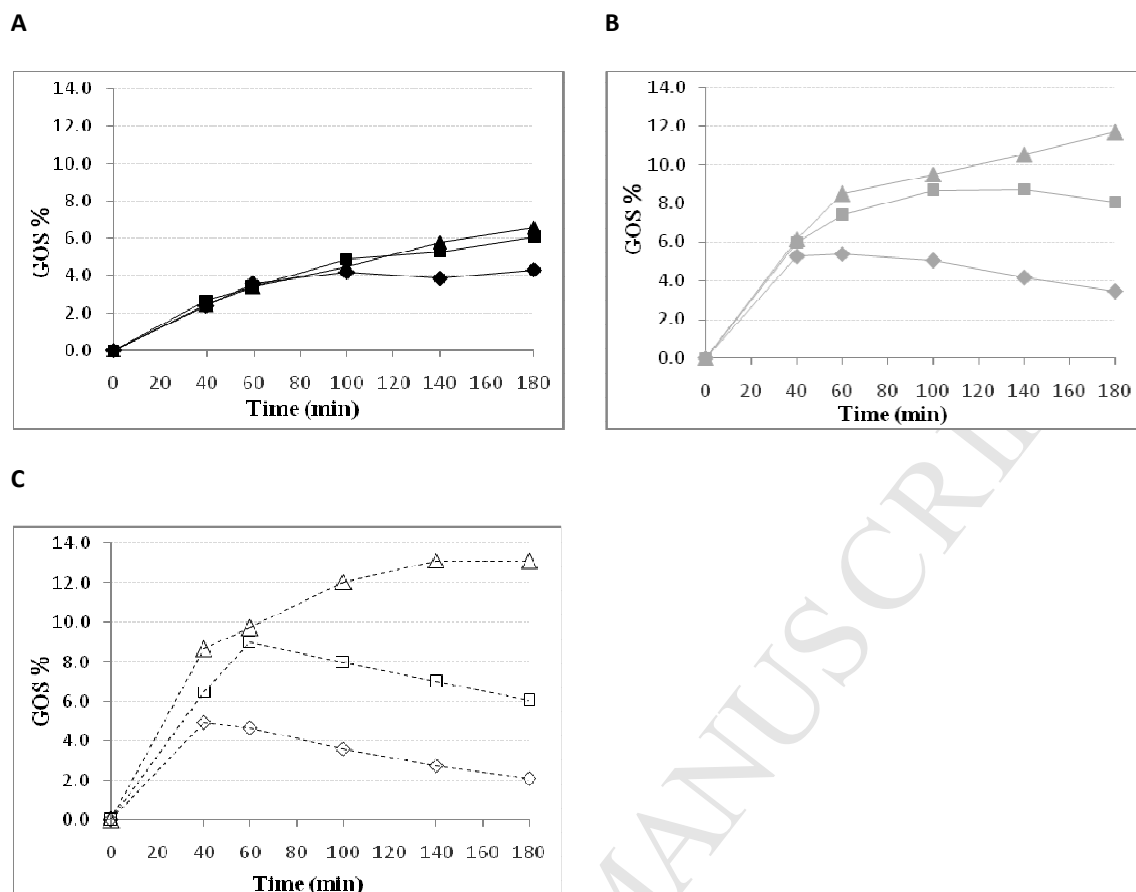
	Drinkable yogurt				Stirred yogurt			
	45 min	End	7 days	21 days	45 min	End	7 days	21 days
<i>GOS</i>								
<b>Enzyme</b>	*	*	*	*	*	*	*	*
<b>Probiotic/prebiotic</b>	NS	NS	NS	NS	NS	NS	NS	*
<i>Lactose</i>								
<b>Enzyme</b>	*	*	*	*	*	*	*	*
<b>Probiotic/prebiotic</b>	NS	NS	NS	*	NS	NS	NS	NS

End: pH 4.7.

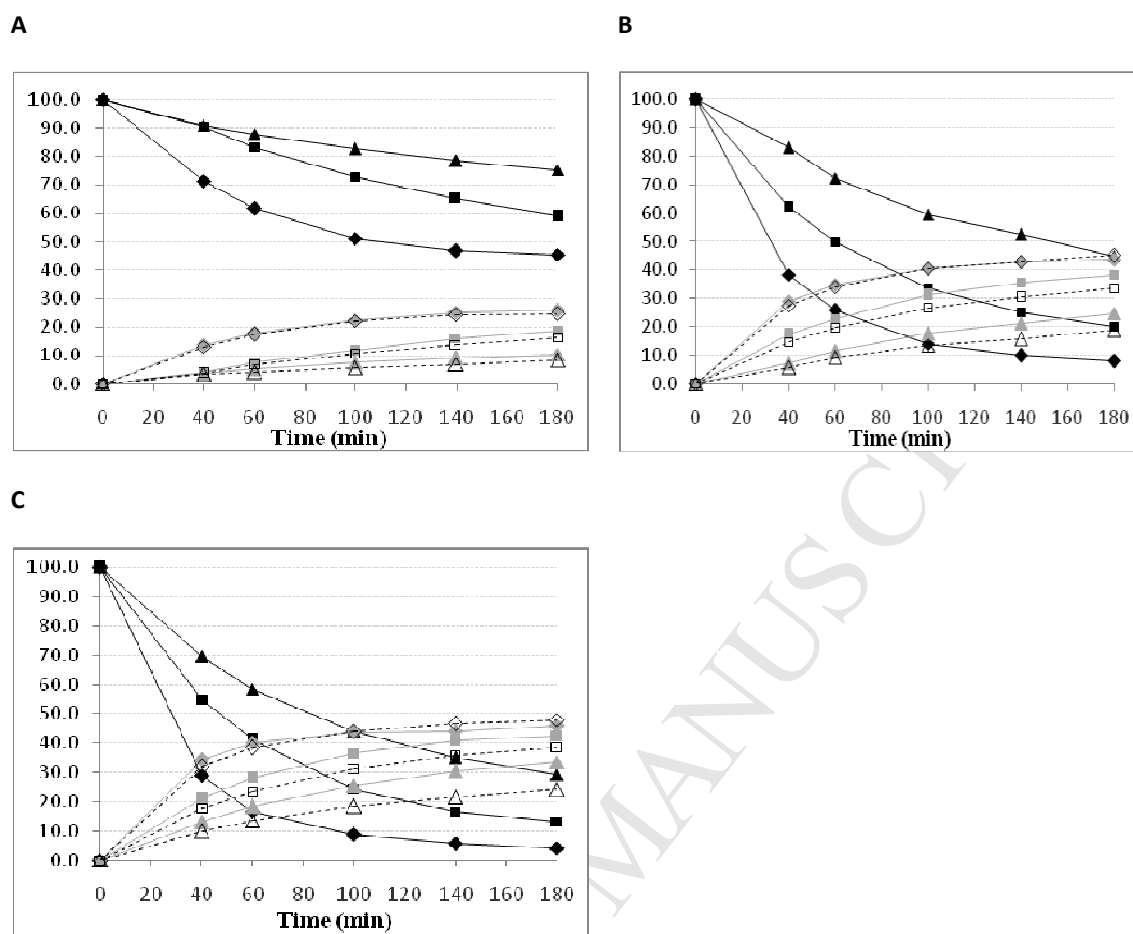
Two-way ANOVA analysis; NS: Not significant; \*:  $P < 0.05$ .



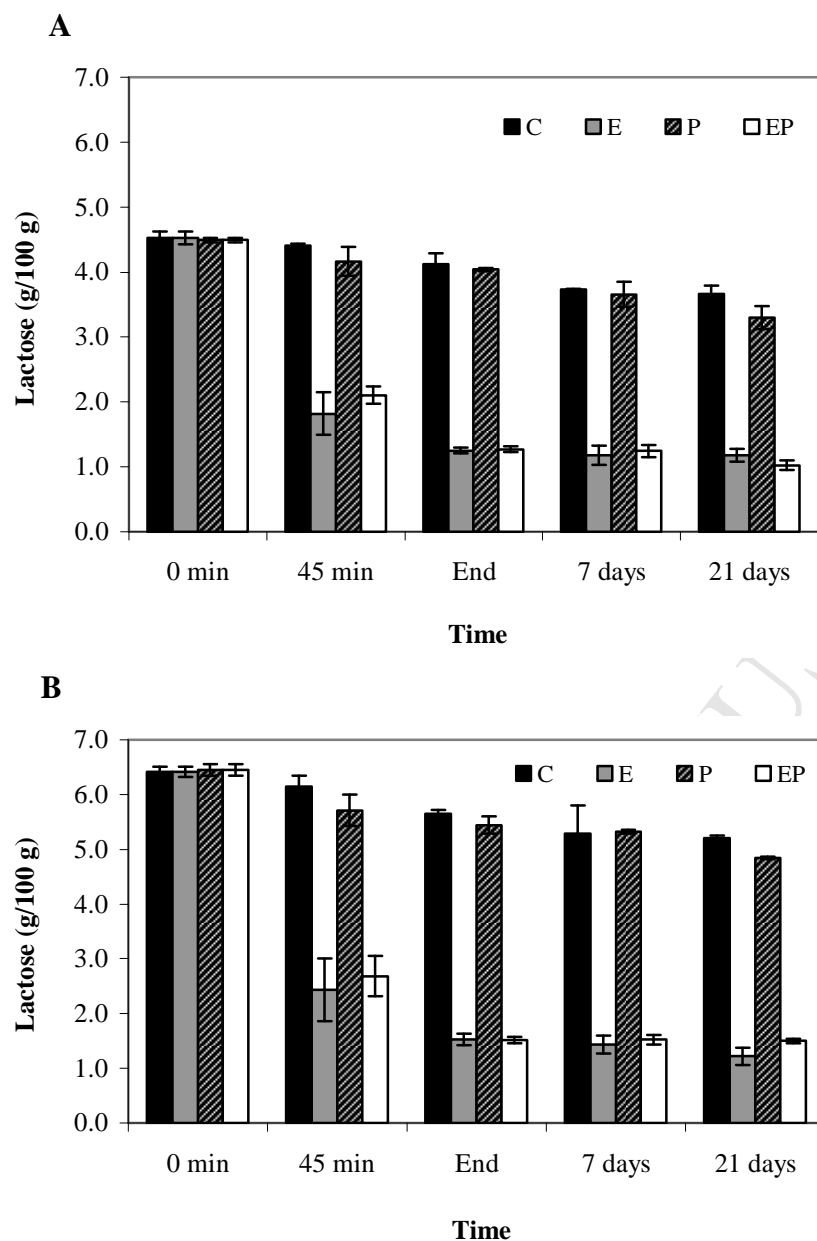
**Fig. 1.** HPLC-IR carbohydrate profile obtained from lactose hydrolysis with YNL-2 *GODO K. lactis*  $\beta$ -galactosidase enzyme. The chromatogram corresponds to the reaction mixture with 5 g/100 mL of initial lactose and 0.25 g/L of enzyme, at 180 min of incubation. a) unretained compounds, b) GOS, c) lactose, d) glucose, e) galactose.



**Fig. 2.** Formation of galacto-oligosaccharides (expressed as percentage of total carbohydrates) by *K. lactis*  $\beta$ -galactosidase at different doses: 0.16, 0.25 and 0.40 g/L (A, B and C, respectively) performed at 42 °C for 3 h from different initial lactose concentrations: 5.0 (diamond symbol), 10.0 (square symbol) and 20.0 (triangle symbol), g/100 mL. Values are the means of the results ( $n = 2$ ); the coefficients of variation were between 2.0 and 6.3%.

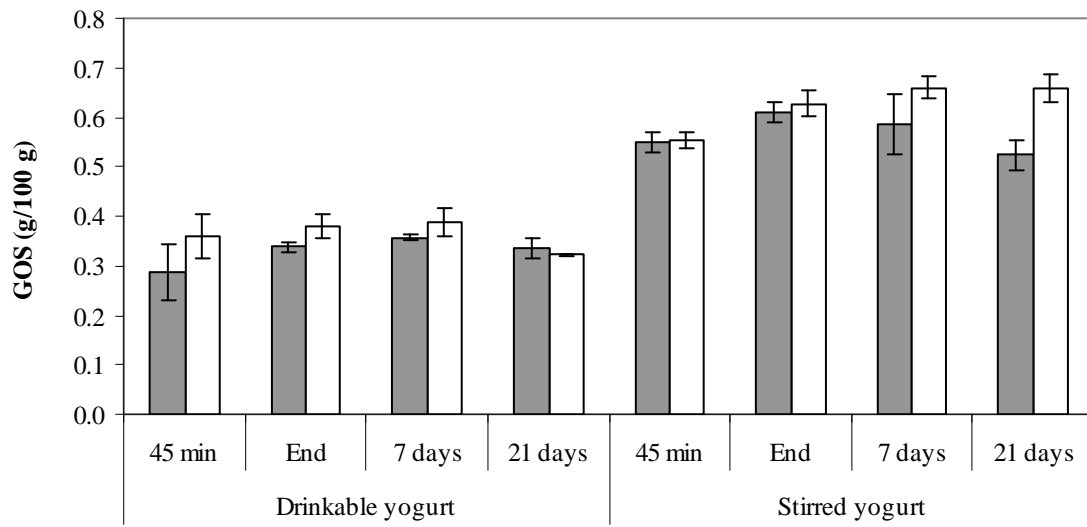


**Fig. 3.** Changes in residual lactose (black line), glucose (grey line) and galactose (dashed line) (expressed as percentage of total carbohydrates) by *K. lactis*  $\beta$ -galactosidase at different doses: 0.16, 0.25 and 0.40 g/L (A, B and C, respectively) performed at 42 °C for 3 h from different initial lactose concentrations: 5.0 (diamond symbol), 10.0 (square symbol) and 20.0 (triangle symbol), g/100 mL. Values are the means of the results ( $n = 2$ ); the range of coefficients of variation were 1.0-5.6% for lactose, 1.4-3.8% for glucose and 1.7-2.9% for galactose.



**Fig. 4.** Lactose concentration during manufacture and storage for drinkable (A) and stirred (B) yogurts. Values are means ( $n = 3$ ).

**C:** unhydrolyzed yogurts (■); **P:** unhydrolyzed symbiotic yogurts (▨); **E:** hydrolyzed yogurts (■); **EP:** hydrolyzed symbiotic yogurts (□). End: pH 4.7.



**Fig. 5.** GOS concentration during manufacture and storage for drinkable and stirred yogurts.

Values are means ( $n = 3$ ).

**E:** hydrolyzed yogurts (■); **EP:** hydrolyzed symbiotic yogurts (□). End: pH 4.7.

**Highlights**

- $\beta$ -galactosidase YNL-2 GODO can synthesize galacto-oligosaccharides (GOS) in lactose solution and yogurt.
- Varieties of reduced-lactose yogurts enriched in GOS were obtained.
- Small changes in quality parameters were produced in yogurts by enzyme and *Lactobacillus acidophilus*/inulin addition.
- GOS formed were stable throughout the storage period of yogurts.
- GOS contents were similar to that found in infant formulas and other dairy foods.