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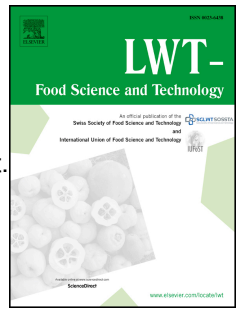

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GALACTO-OLIGOSACCHARIDES FORMATION DURING MANUFACTURE OF DIFFERENT VARIETIES OF YOGURT. STABILITY THROUGH STORAGE.

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Abstract

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

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

Keywords: Galacto-oligosaccharides, β-galactosidase, *L. acidophilus*, inulin, yogurt.

1. Introduction

Currently, galacto-oligosaccharides have attracted particular interest for research and applications in the field of food, due to their recognized functional properties. GOS are non-digestible and non-cariogenic carbohydrates that modulate the colonic microbiota, promoting the healthy balance (prebiotic effect), among other positive health effects (Caselato de Sousa, Freitas dos Santos, & Sgarbieri, 2011; Mussatto & Mancilha, 2007). These compounds are comprised of a variable number of galactose units and, in some cases, a terminal glucose unit, joined by glycosidic bonds. They are produced from lactose (or other galactoside) by enzymatic *via* with β-galactosidases. The first step involves the formation of the galactosyl-enzyme complex and release of the glucose unit. After that, two reactions can concomitantly occur, hydrolysis and transgalactosylation, depending on the galactosyl-moiety acceptor present in the reaction medium. When the acceptor is water, the hydrolysis takes place and lactose is split into glucose and galactose; while, when the acceptor is galactose (or potentially any sugar), the galactosyl transfer happens and a complex mixture of GOS is formed (Gosling, Stevens, Barber, Kentish, & Gras, 2010; Otieno, 2010). The predominance of the GOS synthesis over the hydrolysis, and the yield and composition of the GOS mixture obtained are significantly affected by the origin of β-galactosidase enzyme and the operating conditions (lactose concentration, dose of enzyme, temperature/time and pH) (Boon, Janssen, & van´t Riet, 2000; Gosling et al., 2010).

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

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

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

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

2. Materials and methods

2.1. Enzymatic hydrolysis/transgalactosylation from lactose in buffer

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

Lactose monohydrate (Sigma-Aldrich, Saint Louise, USA) solutions (100 mL) of 5, 10 and 20 g/100 mL were prepared in 100 mmol/L potassium phosphate buffer (pH 6.8) (Sigma-Aldrich, Saint Louise, USA) containing 1 mmol/L $MgCl_2$ (Sigma-Aldrich, Saint Louise, USA). The enzyme was added at different doses, 0.16, 0.25 and 0.40 g/L (equivalent to 8000, 12500 and 20000 units, respectively), and the reaction mixtures were incubated in a water bath at 42 ± 1 °C for 3 h. At different times (40, 60, 100, 140 and 180 min), aliquots (4 mL) were withdrawn and immediately immersed in a boiled water bath for 8 min to deactivate the enzyme. The samples were stored at -18 °C for carbohydrates analysis. The incubation experiences were carried out in duplicate.

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

2.2 Yogurt manufacture

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

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

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

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

placing in screw cap glass flasks (500 mL). Finally, the yogurts were stored at 5 ± 1 ºC for 21 days.

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

2.4. Carbohydrates and lactic acid analysis by HPLC

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

The analysis of GOS, lactose, glucose and galactose in the incubation experiences of lactose solution with the β-galactosidase enzyme were made on an Aminex HPX-87N column (300 x 7.8 mm) equipped with a cation Na^+ microguard cartridge (Bio-Rad Laboratories, Norwalk, USA). Chromatographic separation was performed using HPLC water as mobile phase at a flow rate of 0.3 mL/min, maintaining the column at 85 ºC. Aliquots of reaction mixtures were appropriately diluted with distilled water, filtered through 0.45 μm membranes (Millex, Millipore, São Paulo, Brazil) and injected into the chromatograph, using a loop of 20 μL.

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^+ 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 ºD = 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***

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

3. Results and discussion

3.1. Enzymatic hydrolysis/transgalactosylation from lactose in buffer solution

Lactose hydrolysis and transgalactosylation reactions by the commercial β-galactosidase enzyme YNL-2 in the incubation experiences were followed by HPLC-IR analyses of carbohydrate profiles.

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

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

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

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

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

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

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

3.3. GOS and lactose concentrations in yogurts

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

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

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

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

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

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

On the other hand, the absence of GOS in unhydrolyzed yogurts (**C** and **P**) indicates that the β-galactosidases from YF-L811 and La-5 cultures were unable to produce these compounds under the conditions employed. Variable results were reported in relation to the ability of starter and probiotic cultures to produce GOS in fermented milks. Toba et al. (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 Chirdo 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 β-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 β-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 β-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 β-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 ± standard deviation; $n = 3$).

Yogurt		Total solids	Fat	Protein	pH
Drinkable	**C**	17.9 ± 0.2	2.8 ± 0.2	3.01 ± 0.06	4.42 ± 0.10
	E	17.5 ± 0.3	3.0 ± 0.1	3.00 ± 0.02	4.49 ± 0.06
	P	18.5 ± 0.2	2.5 ± 0.2	3.03 ± 0.05	4.43 ± 0.04
	EP	18.6 ± 0.1	2.6 ± □0.1	3.03 ± 0.05	4.51 ± 0.08
Significance of treatment effect					
Enzyme		NS	NS	NS	NS
La-5/inulin		*	NS	NS	NS
Stirred	**C**	20.4 ± □0.2	2.2 ± 0.2	4.20 ± 0.03	4.46 ± 0.04
	E	20.6 ± 0.1	2.2 ± 0.2	4.13 ± 0.07	4.51 ± 0.04
	P	21.4 ± 0.1	2.6 ± 0.1	4.24 ± 0.07	4.46 ± 0.04
	EP	21.4 ± 0.1	2.6 ± 0.2	4.22 ± 0.01	4.57 ± 0.05
Significance of treatment effect					
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 (ºDornic) 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
Drinkable	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
Significance of treatment effect							
Enzyme		NS	NS	*	NS	NS	NS
La-5/inulin		NS	NS	*	*	NS	NS
Stirred	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
Significance of treatment effect							
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
GOS								
Enzyme	*	*	*	*	*	*	*	*
Probiotic/prebiotic	NS	NS	NS	NS	NS	NS	NS	*
Lactose								
Enzyme	*	*	*	*	*	*	*	*
Probiotic/prebiotic	NS	NS	NS	*	NS	NS	NS	NS

End: pH 4.7.

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

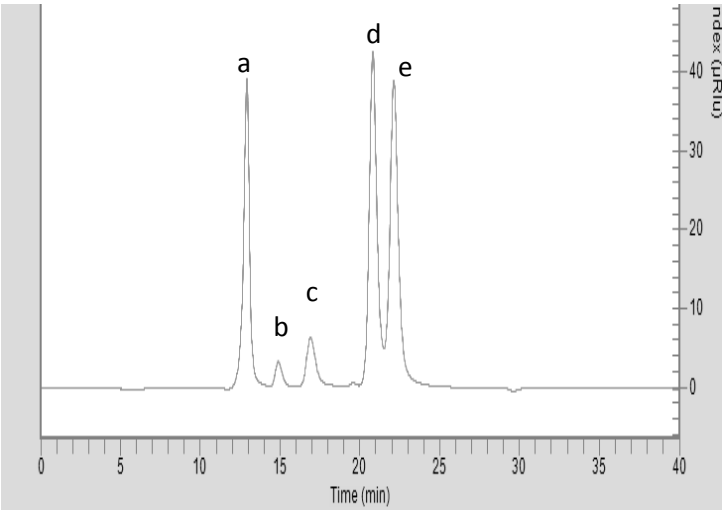

Fig. 1. HPLC-IR carbohydrate profile obtained from lactose hydrolysis with YNL-2 GODO *K. lactis* β-galactosidase enzyme. The chromatogram corresponds to the reaction mixture with 5 g/100 mL of initial lactose and 0.25 g/L of enzyme, at 180 min of incubation. a) unretained compounds, b) GOS, c) lactose,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

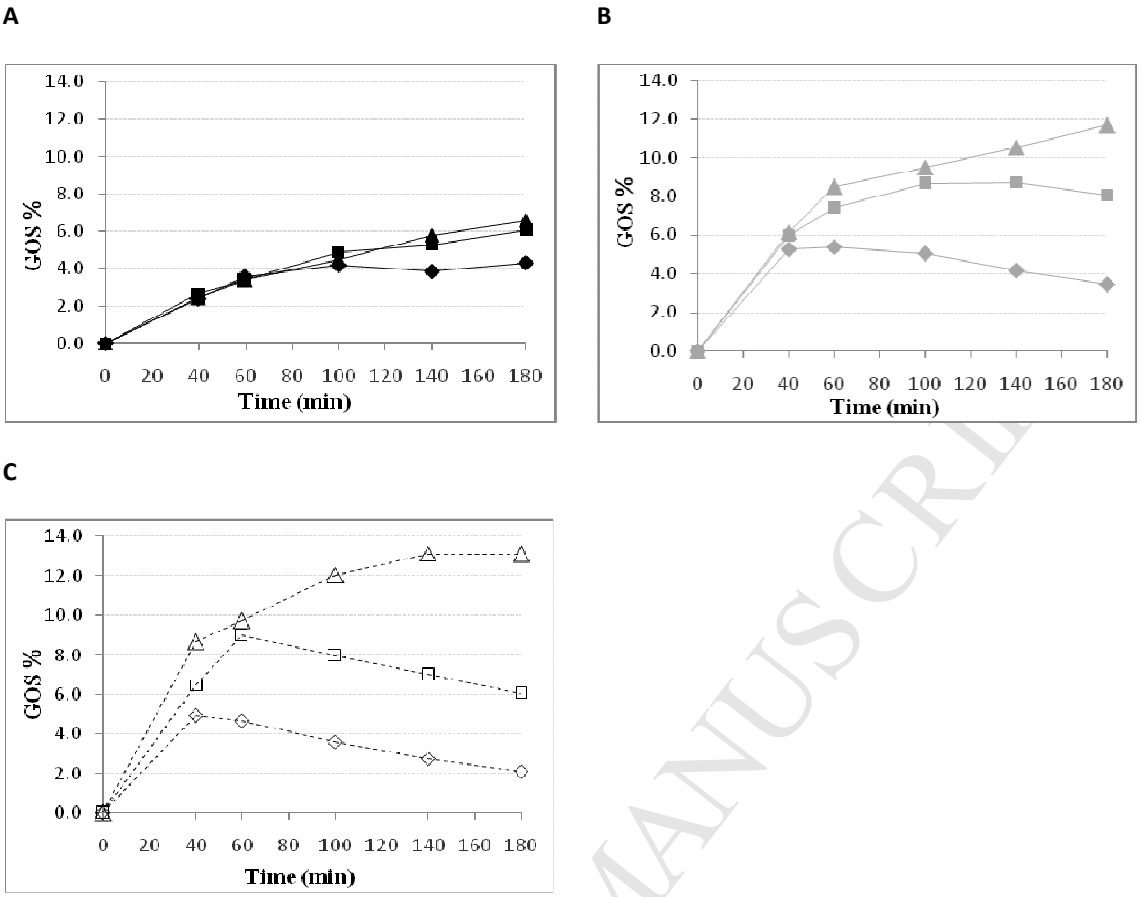

Fig. 2. Formation of galacto-oligosaccharides (expressed as percentage of total carbohydrates) by *K. lactis* β-galactosidase at different doses: 0.16, 0.25 and 0.40 g/L (A, B and C, respectively) performed at 42 °C for 3 h from different initial lactose concentrations: 5.0 (diamond symbol), 10.0 (square 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%.

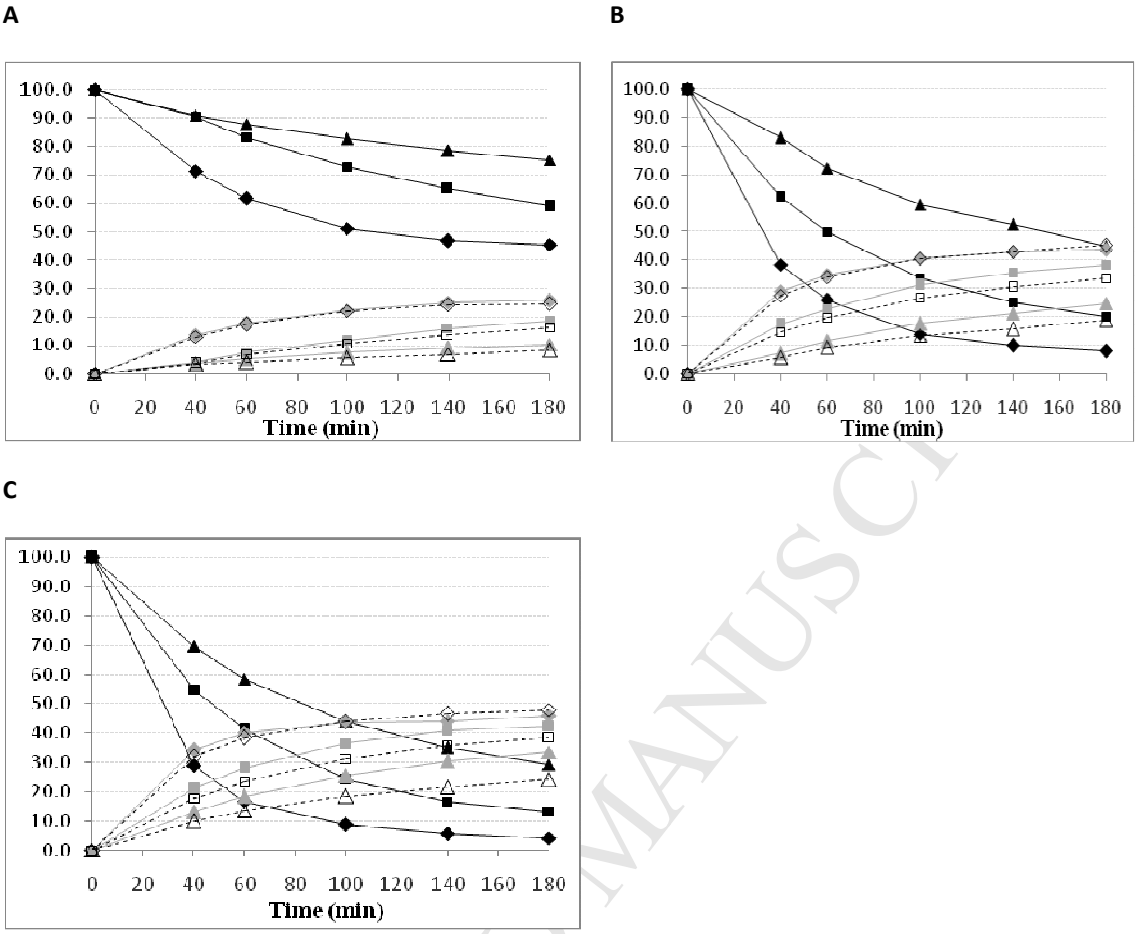

Fig. 3. Changes in residual lactose (black line), glucose (grey line) and galactose (dashed line) (expressed as percentage of total carbohydrates) by *K. lactis* β-galactosidase at different doses: 0.16, 0.25 and 0.40 g/L (A, B and C, respectively) performed at 42 °C for 3 h from different initial lactose 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.

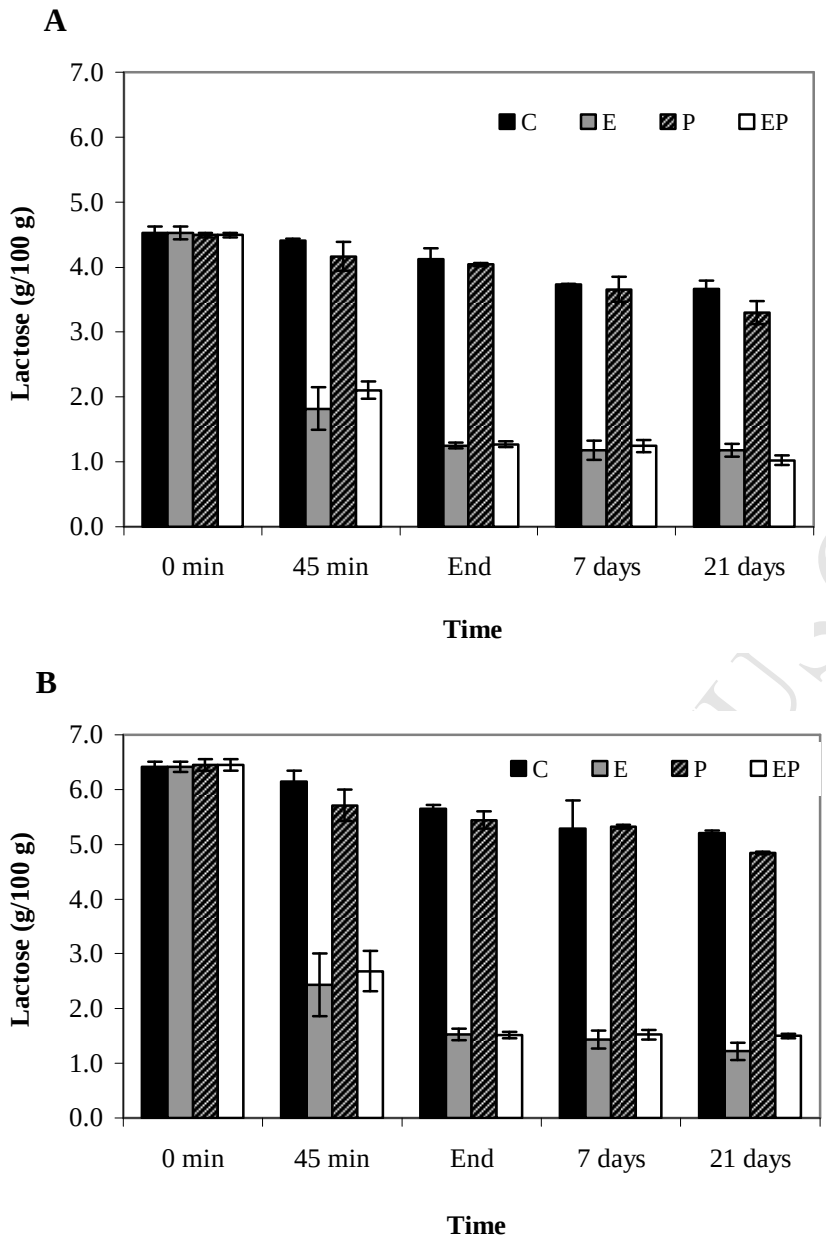

Fig. 4. Lactose concentration during manufacture and storage for drinkable (A) and stirred (B) yogurts. Values are means (n = 3).

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

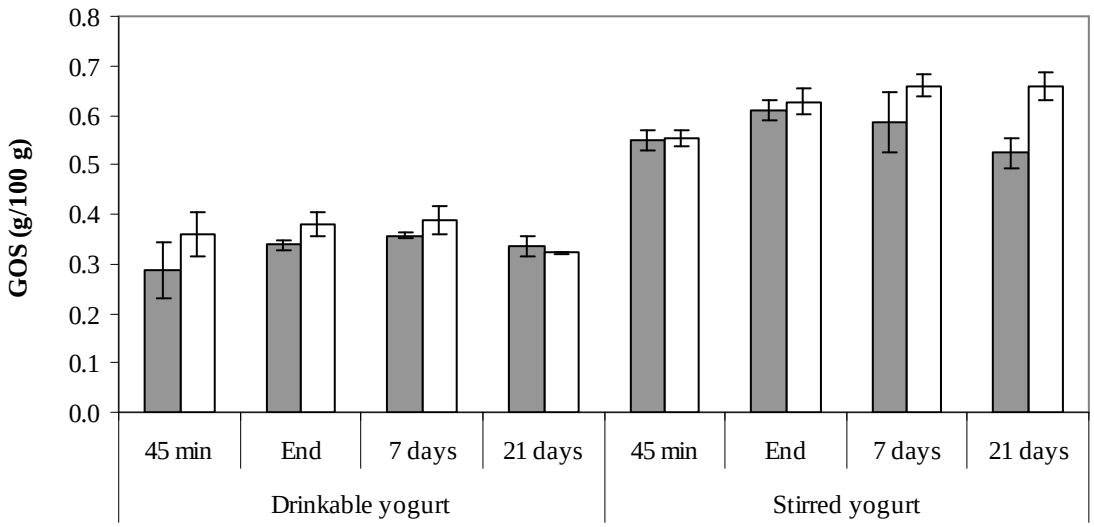

Fig. 5. GOS concentration during manufacture and storage for drinkable and stirred yogurts. Values are means (n = 3).

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

Highlights

- β-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.