Effect of milk protein glycation and gastrointestinal digestion on the growth of bifidobacteria and lactic acid bacteria

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Abstract

In this paper, β-lactoglobulin (β-Lg) and sodium caseinate (SC) have been glycated via Maillard reaction with galactose and lactose and, subsequently, the effect of glycoconjugates hydrolyzed under simulated gastrointestinal digestion on the growth of pure culture of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* has been investigated. Glycopeptides were added to the growth media as the sole carbon source. None of the bacterial strains was able to grow in hydrolysates of native and control heated β-Lg and SC. However, glycopeptides were fermented, in different degree, by *Lactobacillus* and *Bifidobacterium* and hardly any effect was detected on the growth of *Streptococcus*. Digested β-Lg glycoconjugates showed a strain-dependent effect whereas growth profiles of bacteria when hydrolysates of SC glycoconjugates were used as substrates were very similar, regardless of the strain. A general preference towards peptides from β-Lg/SC glycated with galactose, particularly at the state of the reaction in which the highest content in the Amadori compound tagatosyl-lysine is present, was observed. SC glycoconjugates were quickly fermented by some strains, promoting their growth in a greater extent than β-Lg complexes or even glucose. Therefore, from the results obtained in this work it can be concluded that conjugation of both milk proteins with galactose and lactose via the Maillard reaction could be an efficient method to obtain novel food ingredients with a potential prebiotic character.

Keywords: Tagatosyl-lysine; Lactulosyl-lysine; β-lactoglobulin; Sodium caseinate; Bifidobacteria; Lactic acid bacteria.

1. Introduction

Nowadays, it is possible to dispose of products which afford not only the basic nutritional value but also a wide range of benefits that can contribute to improve consumer well-being. In this respect, due to the fact that severe health disorders can be related to the gastrointestinal function, some of the functional ingredients more demanded by consumers are those with a positive effect on gut microbiota (Saaraela et al., 2002). These ingredients are non-digestible carbohydrates with well-known properties, being lactulose (Tuohy et al., 2002), tagatose (Laerke and Jensen, 1999; Laerke et al., 2000; Jensen et al., 2001), fructooligosaccharides (FOS) and galactooligosaccharides (GOS) (MacFarlane et al., 2008) some of the main oligosaccharides considered as prebiotics.

On the other hand, deliberated glycosylation via the Maillard reaction (MR) or glycation has been largely investigated during the last years as a promising approach to alter the functional properties of proteins for food purposes (Oliver et al., 2006a). Concretely, it has been demonstrated that milk proteins (caseins and whey proteins), usually obtained as by-products in the dairy industry, can increase their degree of applicability by means of glycation. Thus, controlling the reaction conditions it is possible to obtain neoglycoconjugates with different glycation and aggregation degrees, being preferred, in general, the early steps of the reaction (Jimenez-Castaño et al., 2005a, 2005b; Kato, 2002; Oliver et al., 2006a). Particularly interesting could be the case of the conjugation of milk proteins with galactose and lactose, which are isomerised to tagatose and lactulose, respectively, giving rise to the corresponding Amadori compounds, tagatosyl- and lactulosyl-lysine. Taking into account the prebiotic properties of tagatose and lactulose, it could be expectable some effect of these glycoconjugates on gut microbiota.

In spite of the number of works on protein glycation, any information about intestinal absorption and endogenous metabolisation of Amadori compounds is still fragmentary. According to several authors, it seems that these initial products of the MR are scarcely digested and excreted, being fermented in the distal colon (Erbersdobler and Faist, 2001; Finot, 1973; Finot, 2005; Lee and Erbersdobler, 1994; Sanz et al., 2007). In this sense, the study of the effect of the Amadori compounds tagatosyl- and lactulosyl-lysine could be of great interest, since such compounds might reach more distal areas of gut than tagatose and lactulose and be fermented by the present microbiota. This is particularly relevant since the incidence of certain chronic gut disorders is much higher in the descending colon and rectum than in the first sections of the large gut (Gibson et al., 2004). Mills et al. (2008), using *in vitro* assays with faecal slurries, pointed out that glycated protein bovine serum albumin modulated the colonic microbiota of volunteers with ulcerative colitis towards a more detrimental composition with significant increases in putatively harmful bacteria and decreases in dominant and putatively beneficial bacterial group. This trend was not completely confirmed in non-ulcerative colitis volunteers.

Thus, the purpose of this work has been to study the effect of glycoconjugates obtained by glycosylation via the Maillard reaction of milk proteins (β-lactoglobulin and sodium caseinate) with galactose and lactose, and subsequently subjected to simulated gastrointestinal digestion, on the growth of twelve potential probiotic strains of *Lactobacillus, Streptococcus* and *Bifidobacterium* to broaden the knowledge on the role of Amadori compounds in the modulation of the human gut microbiota.

2. Materials and Methods

2.1 Materials

Glucose (Glu), galactose (Gal), lactose (Lac), tagatose (Tag), lactulose (Lu) and bovine β-lactoglobulin (β-Lg) (mixture of A and B variants) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and sodium caseinate (SC) (Rovita FN 5) was obtained from Proveedora Hispano Holandesa, S.A. (Barcelona, Spain).

Streptococcus salivarius ZL50-7, *Lactobacillus reuteri* R13, *Lactobacillus plantarum* CLC17, *Lactobacillus delbrueckii* ZL96-27, *Lactobacillus brevis* CLC23, *Lactobacillus gasseri* Lc9 and *Bifidobacterium breve* 26M2 belonged to the culture collection of the Nutrition, Bromatology and Food Technology Department of the Faculty of Veterinary Science (Universidad Complutense de Madrid). *Streptococcus thermophilus* STY-31, *Lactobacillus acidophilus* LA-5, *Bifidobacterium lactis* BB12 and *Lactobacillus casei* LC-01 strains had been previously purified from a commercial symbiotic product (Simbiotic Drink; Priégola, Madrid, Spain) at our laboratory (Tabasco et al., 2007). *Lactobacillus plantarum* IFPL722, isolated from cheese, was taken from our IFPL culture collection. All cultures were maintained at −80 °C in MRS broth (Pronadisa, Madrid, Spain) or, in the case of *S. salivarius* ZL50 and *S. thermophilus* STY-31, in M17 broth (Pronadisa), both supplemented with glycerol (40% v/v) and subcultured in MRS or M17 broth before use in experiments.

2.2 Preparation and purification of glycoconjugates

Carbohydrates, Gal or Lac, and β-Lg in a weight ratio of 1:1 or 2:1, respectively, were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 (Merck, Darmstadt, Germany), and lyophilized. The β-Lg-Gal powders were kept at 40 and 50 °C for 24

126 and 48 h, respectively (Corzo-Martínez et al., 2008), whilst the β-Lg-Lac powders were
127 kept at 60 °C for 8 and 48 h (Table 1) (Fenaille et al., 2004), under vacuum in a
128 desiccator equilibrated at an a_w of 0.44, achieved with a saturated K_2CO_3 solution
129 (Merck). In addition, control experiments were performed with β-Lg stored at 40, 50
130 and 60 °C without reducing sugars during the same periods (control heated β-Lg).
131 Incubations were performed in duplicate, and all analytical determinations were
132 performed at least in duplicate.

133 Carbohydrates, Gal or Lac, and sodium caseinate (SC) in a weight ratio of 0.2:1
134 were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 (Merck) and lyophilized
135 (Corzo-Martinez et al., 2010b). The SC-Gal powders were kept at 60 ºC and 50 ºC for 4
136 and 72 h, respectively, whilst the SC-Lac powders were kept at 60 ºC for 8 and 24 h
137 (Table 1), under vacuum in a desiccator equilibrated at an a_w of 0.67 (Oliver et al.,
138 2006b), achieved with a saturated solution of $CuCl_2$ (Sigma-Aldrich). In addition,
139 control experiments were performed with SC stored at 50 and 60 ºC without reducing
140 sugars during the same periods (control heated SC). Incubations were performed in
141 duplicate, and all analytical determinations were performed at least in duplicate.

142 After incubation, the products were reconstituted in distilled water to a protein
143 concentration of 1 mg/mL. To remove free carbohydrate, 2 mL portions were
144 ultrafiltered through hydrophilic 3 kDa cut-off membranes (Centricon YM-3, Millipore
145 Corp., Bedford, MA) by centrifugation at 1548*g* for 2 h. After removal of free Gal or
146 Lac, samples were reconstituted in distilled water at a concentration of 2 mg/mL for
147 further analysis (Corzo-Martínez et al., 2008).

148 Taking into account the analyses of structural characterization previously carried
149 out in our laboratory (Corzo-Martínez et al., 2008; Corzo-Martínez et al., 2010a;
150 Laparra et al., 2011), two types of glycoconjugates were obtained at different stages of

151 the Maillard reaction after incubation of every combination of carbohydrate and protein
152 under conditions indicated above (Table 1). One of them, in early stages of the MR (β-
153 Lg:Gal [24 h, 40 ºC], β-Lg:Lac [8 h, 60 ºC], SC:Gal [4 h, 60 ºC] and SC:Lac [8 h, 60
154 ºC]), consisted primarily of complexes with a high content of the Amadori compound
155 tagatosyl- or lactulosyl-lysine and a low aggregation level, while those glycoconjugates
156 incubated under more severe conditions, in advanced stages of the MR (β-Lg:Gal [48 h,
157 50 ºC], β-Lg:Lac [48 h, 60 ºC], SC:Gal [72 h, 50 ºC] and SC:Lac [24 h, 60 ºC]),
158 exhibited a high glycation degree, considerable amounts of advanced glycation products
159 (AGEs and melanoidins), and a high aggregation level.
160
161 *2.3* In vitro *gastrointestinal digestion*

162 All SC and β-Lg glycoconjugates, as well as the control heated SC/β-Lg samples
163 were digested *in vitro* by following the simplified procedure described by Moreno et al.
164 (2005). This digestion model was based on *in vivo* data obtained by gastric and
165 duodenal aspiration and from collection of effluent from ileostomy volunteers at the
166 Institute of Food Research (Norwich, UK).

167 For the gastric digestion step, glycoconjugates (3 mg) were dissolved in 1 mL of
168 simulated gastric fluid (SGF, 0.15 M NaCl, pH 2.5). The pH was adjusted to 2.5 with 1
169 M HCl if necessary. A solution of 0.32% (w:v) porcine pepsin (EC 3.4.23.1) in SGF
170 (pH 2.5) (Sigma, activity of 3,300 units/mg of protein) was added at an approximately
171 physiological ratio of enzyme to substrate (1:20, w:w). The digestion was performed at
172 37 °C for 2 h. For the intestinal digestion step, the pH was increased to 7.5 with 40 mM
173 NH_4CO_3 (Panreac, Barcelona, Spain) dropwise to inactivate pepsin, and the following
174 was added to adjust the pH to 6.5 and simulate a duodenal environment: (i) a bile salt
175 mixture containing equimolar quantities (0.125 M) of sodium taurocholate (Sigma) and

glycodeoxycholic acid (Sigma), (ii) 1 M $CaCl_2$ (Panreac), and (iii) 0.25 M Bis-Tris (pH 6.5) (Sigma). Solutions of porcine trypsin (EC 3.4.21.4; 0.05%, w:v, Sigma, type IX-S, activity of 14,300 units/mg of protein) and bovine α-chymotrypsin (EC 3.4.21.1; 0.1%, w:v, Sigma, type I-S, activity of 62 units/mg of protein) in water were prepared and added at approximately physiological protein:trypsin:chymotrypsin ratios [1:(1/400):(1/100) (w:w:w)]. Simulated intestinal digestion of β-Lg was carried out at 37 °C for 15 min. After protein hydrolysis, trypsin and chymotrypsin were inactivated by heating at 80 ºC for 5 min. Digestions were performed without any derivatization of the sulfhydryl groups of cysteine residues in order to remain as close as possible to physiological conditions.

2.4 Analysis of digested samples

Analysis of digested samples was carried out using a Beckman HPLC system equipped with a Phenomenex Jupiter Proteo column (250 mm × 4.6 mm, 4 mm particle size, 90 Å pore size). Samples were eluted using 0.1% (v/v) TFA in double-distilled water as solvent A and 0.1% (v/v) TFA in double-distilled water and acetonitrile of HPLC grade (Scharlau Chemie, Barcelona, Spain) (1:9, v/v) as solvent B, following the method described by Moreno et al. (2004).

For SDS-PAGE analysis, 32.5 mL of the samples taken at different stages of the digestion were added to 12.5 mL of 4× NuPAGE® LDS Sample buffer (Invitrogen, CA, USA) and 5 mL of 0.5 M dithiothreitol (DTT, Sigma-Aldrich), and heated at 70 ºC for 10 min. Samples (20 mL) were loaded onto a 12% polyacrylamide NuPAGE® Novex Bis Tris pre-cast gel and a continuous 2-(N-morpholino)ethanesulfonic acid (MES) SDS

199 running buffer was used. Gels were run for 40 min at 120 $mAgel^{-1}$ and 200 V and
200 stained using the Colloidal Blue Staining Kit (Invitrogen).
201
202 *2.5 Bacterial growth on digested glycoconjugates*
203 For experiments, strains were grown into MRS fermentation broth (Pronadisa),
204 which does not contain either Glu or meat extract (De Man et al., 1960), enriched with
205 0.2% Tween 80, 0.8% casein acid hydrolysate and 0.05% cysteine, or ESTY broth
206 (Pronadisa), without any carbon source. These media were supplemented with digested
207 β-Lg and SC glycoconjugates at a final concentration of 0.3% (w/v) of the sugar moiety
208 of the molecules. Glu, Tag, Lu, bovine native β-Lg and SC, and β-Lg and SC heated
209 without reducing sugars were used as controls, at the same final concentration that
210 corresponding glycoconjugates. Each substrate was weighed into sterile tubes, then the
211 corresponding autoclaved basic medium was added, and the mixtures were sterilized by
212 filtration (0.22 µm).
213 Bacterial strains were cultured overnight at 1% in MRS or M17 broth and grown
214 cultures were diluted 1:10 in MRS fermentation broth or in ESTY broth without any
215 carbon source. Culture dilutions were used as inocula to evaluate the growth of bacterial
216 strains on galactosylated and lactosylated peptides derived from *in vitro* gastrointestinal
217 digestion and on controls. Basic media plus the tested substrates were inoculated with
218 1% of diluted inocula of each strain and pipetted into 300 µl wells of sterile 96-well
219 microplates with lid (Sarstedt Inc., Newton, USA). All strains were grown in aerobic
220 conditions at 37 ºC for 24 h, in triplicate, excepting *L. acidophilus* LA-5, *L. gasseri* Lc9,
221 *B. breve* 26M2 and *B. lactis* BB12, which were incubated under anaerobic conditions
222 (Gas-Pack, Anaerogen; Oxoid Ltd., Hampshire, England), also in triplicate. The optical
223 densities (OD_{600}) of the aerobic strains were recorded at 20-60 min intervals with an

automated microplate reader (Varioskan Flash, Thermo Electron Corporation, Vantaa, Finland) at 600 nm. Maximum growth rates (μ_{max}) and lag parameters (lag) were calculated by fitting the curves to a sigmoid model using the Microsoft Excel add-in DMfit v. 2.1 (Barayni and Roberts, 1994) (available at http://www.ifr.ac.uk/safety/DMfit/default.html). In the case of the anaerobic strains, OD_{600} was recorded at 0 h and 24 h. Blanks with medium and carbon source added but without bacteria were carried out.

2.6. Statistical analysis

Statistical analysis was performed using the Statgraphic 5.1 Program (Statistical Graphics Corporation, Rockville, MD, USA) for Windows. One-way analysis of variance (ANOVA) (least significant difference, LSD, test) was used for the statistical evaluation of results derived from the measurement of maximum optical density (OD_{600}) reached for each bacterial culture with each substrate studied. Differences were considered significant when $P< 0.05$.

3. Results and Discussion

3.1 Characterization of hydrolysates obtained after in vitro gastrointestinal digestion of sodium caseinate and β-lactoglobulin glycoconjugates

To simulate the physiological conditions, glycoconjugates were subjected to an *in vitro* gastrointestinal digestion process consisting of a first stage of gastric digestion with pepsin for 2 h at 37 ºC and a second stage of duodenal digestion with trypsin/chymotrypsin for 15 min at 37 ºC; then, the digested glycoconjugates were analysed by SDS-PAGE. As observed in Figure 1, control heated β-Lg (lanes a and c)

249 was rapidly broken down during simulated duodenal digestion, and only traces of intact
250 protein could be observed after 15 min of incubation with trypsin/chymotrypsin.
251 However, in the glycated protein, particularly in β-Lg:Gal [48 h, 50 ºC], two bands
252 corresponding to its monomeric and dimeric forms could be clearly visualized after
253 digestion. Glycation with Lac had a similar influence on β-Lg digestibility (data not
254 shown). These results confirmed that glycation and glycation-induced aggregation of β-
255 Lg during the advanced stages of the MR, protects β-Lg from proteolysis during *in vitro*
256 gastrointestinal digestion. In fact, in a previous work carried out in our laboratory
257 (Laparra et al., 2011), by means of SEC analysing of digested β-Lg:Gal/Lac conjugates,
258 we observed an increase in the area of the less retained peaks (corresponding to the
259 formation of aggregated and larger peptides) after incubation under more severe
260 conditions, i.e. 48 h at 50 ºC (in the case of glycation with Gal) and 48 h at 60 ºC (in the
261 case of glycation with Lac).

262 SC glycoconjugates were more efficiently digested than complexes derived from
263 β-Lg, regardless the employed glycation conditions (Figure 2). This result can be
264 attributed to the fact that caseins have a flexible and linear conformation rather than a
265 rigid and compact structure (Kaminogawa, 2000), facilitating their enzymatic digestion.
266 In contrast, β-lactoglobulin presents a high structural stability at acid pH, having its
267 peptic cleavage sites (hydrophobic or aromatic amino acid side chains) buried inside its
268 characteristic β-barrel structure, forming a strong hydrophobic core and preventing
269 hydrolysis (Dalgalarrondo et al., 1995; Reddy et al., 1988). Thus, as observed in the
270 chromatographic profiles shown in Figure 2, both unglycated and glycated SC were
271 quickly proteolyzed, even in the case of SC glycated under the conditions that promote
272 high protein aggregation levels. Nevertheless, SEC analysis also indicated the higher
273 size of peptides contained in hydrolysates of SC glycoconjugates obtained under more

274 severe conditions, particularly when SC was glycated with Gal at 50 ºC for 72 h
275 (Laparra et al., 2011).
276
277 *3.2 Bacterial growth on digested β-lactoglobulin:galactose/lactose conjugates*
278 Growth curves for representative strains grown aerobically on peptides from
279 native, control heated and glycated β-Lg, and on Tag, Lu and Glu as sole carbon sources
280 are shown in Figure 3. None of the bacterial strains was able to grow in hydrolysates of
281 native and control heated β-Lg (data not shown). However, in general, the sugar moiety
282 from the digested glycoconjugates was fermented, in different degree, by all
283 microorganisms assayed, except for *S. thermophilus* STY-31 and *S. salivarius* ZL50-7.
284 As observed in Figure 3, the strains showed very different metabolic activity for the
285 same substrate (behaviour strain-dependent), giving raise to different growth profiles. A
286 general preference towards peptides from β-Lg glycated with Gal, particularly at 40 ºC
287 for 24 h, over those from β-Lg:Lac conjugates was observed, the former being
288 efficiently metabolized by all strains of *Lactobacillus*.
289 In order to relate the bacterial growth to substrate utilization, we calculated the
290 maxima growth rates (μ_{max}) for each of the samples studied. These values and lag times
291 (lag) are shown in Table 2. Likewise, the corresponding values of maximum DO_{600} are
292 also shown in Table 3.
293 *L. casei* LC-01 was able to grow in all the tested substrates, reaching a μ_{max} of
294 0.142 h^{-1} with Lu as carbon source (Table 2), this being similar to that achieved with
295 Glu and Tag. When *L. casei* LC-01 was grown with the conjugate β-Lg:Gal [24 h, 40
296 ºC] digested, it achieved cell densities similar to Glu and Tag, with maximum OD_{600} of
297 0.9 (Figure 3 and Table 3). In addition, although *L. casei* LC-01 growth on digested β-

298 Lg:Gal [24 h, 40 ºC] was slower (0.048 h^{-1}) than on Glu and Tag (0.135 and 0.129 h^{-1},
299 respectively), the lag time of growth on glycoconjugate was zero, whilst on Glu and Tag
300 it was 7 h, approximately (Table 2). Regarding peptides from β-Lg:Gal [48 h, 50 ºC], β-
301 Lg:Lac [8 h, 60 ºC] and β-Lg:Lac [48 h, 60 ºC], they presented lower cell densities,
302 with OD_{600} values below 0.7 (Table 3), and slower growth than Glu, Tag and Lu,
303 although their lag time was reduced, except in the case of β-Lg:Lac [48 h, 60 ºC].

304 *L. reuteri* R13, *L. plantarum* IFPL722 and *L. plantarum* CLC17 showed a similar
305 behaviour. Tagatose was not efficiently used as carbon source by any of the three
306 strains. However, they were able to grow with Glu and Lu, reaching cell densities
307 particularly high with Lu (OD_{600} ~ 1.2) in the case of *L. plantarum* IFPL722, and with
308 Glu (OD_{600} ~ 1) in *L. reuteri* R13. When digested β-Lg:Gal [24 h, 40 ºC] was used as
309 carbon source, OD_{600} and μ_{max} values of these three strains were similar to those
310 observed with Glu (Figure 3, Tables 2 and 3). In addition, the lag time of growth on this
311 compound was reduced to zero. Likewise, although to a lesser extent than peptides from
312 β-Lg:Gal [24 h, 40 ºC], hydrolysate of β-Lg glycated with Gal at 50 ºC for 48 h also
313 promoted the growth of these bacterial strains. In contrast, incubation with peptides
314 from β-Lg:Lac [8 and 48 h, 60 ºC] conjugates as substrates resulted in a poor growth of
315 *L. reuteri* R13, *L. plantarum* IFPL722 and *L. plantarum* CLC17, reaching OD_{600} and
316 μ_{max} values markedly lower than with Lu.

317 *L. delbrueckii* ZL95-27 and *L. brevis* CLC23 did not utilize Tag or Lu as carbon
318 source. Both strains reached the highest cell densities and the fastest growth rates with
319 digested β-Lg:Gal [24 h, 40 ºC], displaying no-lag time period (Table 2). This was
320 particularly noticeable in the case of *L. brevis* CLC23, which fermented this compound
321 even faster than Glu, giving rise to significantly differences in the maximum DO_{600}
322 values (Table 3). Digested β-Lg:Gal [48 h, 50 ºC] was also efficiently metabolized by

both strains, although its fermentation gave rise to lower cell densities and μ_{max} values than that of digested β-Lg:Gal [24 h, 40 ºC]. Regarding to digested β-Lg:Lac conjugates, as Lu, they did not promote the growth of *L. delbrueckii* ZL95-27 and *L. brevis* CLC23.

Unlike the previous mentioned strains of *Lactobacillus*, *S. salivarius* ZL50-7 and *S. thermophilus* did not efficiently utilize any of the studied β-Lg glycoconjugates or Tag, only growing on Glu and Lu, being especially noteworthy the fast growth rate of *S. salivarius* ZL50-7 with Glu (μ_{max} = 0.747, the highest μ_{max} of all strains and substrates studied).

With respect to the growth of the anaerobic strains, OD_{600} values for the carbon sources under study are shown in Table 4. *B. breve* 26M2 achieved higher and significantly different cell densities with Lu than with Glu as carbon source. However, no growth of *B. lactis* BB-12 was observed with Lu as substrate after 24 h of incubation. Likewise, none of digested β-Lg:Lac conjugates under study was metabolized by *B. breve* 26M2 and *B. lactis* BB12. However, it is noteworthy the ability of both strains of bifidobacteria to show some growth after 24 h of incubation with β-Lg:Gal conjugates, reaching OD_{600} values of up to 0.38, unlike Tag, which was not used as substrate. *L. gasseri* Lc9 was not able to grow with Tag, Lu or β-Lg:Lac conjugates as carbon sources. The maximum growth of this strain was recorded on hydrolysates of β-Lg galactosylated, particularly at 40 ºC for 24 h, with an OD_{600} of 0.522. Finally, *L. acidophilus* LA-5 stood out by its ability to utilize all the digested β-Lg:Gal and β-Lg:Lac conjugates studied, unlike Lu, which was not used as carbon source.

In summary, the bifidobacteria and lactic acid bacteria studied did not ferment efficiently the hydrolysates of β-Lg:Lac conjugates. In contrast, all bacterial strains assayed, excepting streptococci, grew to some extent on hydrolysates of β-Lg:Gal

conjugates, both [24 h, 40 ºC] and [48 h, 50 ºC], observing high μ_{max} values and no-lag time periods. This was particularly notable when it was used as substrate the hydrolysate derived from galactosylated β-Lg at 40 ºC for 24 h, with the highest content in the Amadori compound tagatosyl-lysine, suggesting that it has a positive effect on the strains studied, which even might be higher than that of Tag. However, other strains should be tested in further studies to confirm these results. In addition, the comparation with other prebiotics could also be interesting.

On the other hand, the lower bacterial growth observed with the hydrolysate of galactosylated β-Lg at 50 ºC for 48 h could be attributed to its higher aggregation degree, lower digestibility (Figure 1), and, consequently, the higher difficulty to bacteria to metabolize peptides obtained after its digestion, with a higher size and aggregation degree than those derived from β-Lg:Gal [24 h, 40 ºC] (Laparra et al., 2011).

3.3 Bacterial growth on digested sodium caseinate:galactose/lactose conjugates

Figure 3 also depicts growth curves for strains grown in aerobic conditions on peptides from digested SC glycoconjugates as sole carbon sources. Maxima growth rates (μ_{max}), lag times (lag) and maxima DO_{600} values for each of substrates studied are also shown in Tables 2 and 3, respectively.

While none of the microorganisms assayed was able to grow on digests from native and control heated SC (data not shown), peptides from SC glycoconjugates were fermented in a strain-dependent way by all tested bacterial strain, in some cases even faster and leading to higher cell densities than Glu, and promoting bacterial growth in greater extent than peptides from digested β-Lg complexes. This is probably due to the better digestibility of SC glycoconjugates as compared to those of β-Lg, which allows

372 the formation of smaller peptides (Laparra et al., 2011) that could be, therefore, more
373 easily internalized and metabolized by bacteria than those derived from gastrointestinal
374 digestion of β-Lg glycoconjugates.

375 Moreover, unlike digested β-Lg glycoconjugates, which showed a strain-
376 dependent effect, growth profiles obtained when hydrolysates of SC glycoconjugates
377 were used as substrates were very similar, regardless of the strain. The growth of most
378 of the lactic acid bacteria assayed resulted particularly favoured in presence of digested
379 SC:Gal [4 h, 60 ºC] complex, showing no-lag time periods and reaching the highest
380 OD_{600} (ranging from 0.85 to 1.6) and μ_{max} values (ranging from 0.232 to 0.673 h^{-1}) of
381 all the substrates tested, including Tag (Tables 2 and 3). These results support that the
382 Amadori compound tagatosyl-lysine might promote the growth of gut microbiota in
383 greater extent than Tag, according to the previous results showed for β-Lg
384 glycoconjugates.

385 Moreover, although to a lesser extent than SC:Gal [4 h 60 ºC], digested SC:Gal
386 [72 h, 50 ºC] was also used more efficiently and faster than Tag as carbon source by all
387 the bacterial strains assayed, with the exception of streptococci. The ability of the latter
388 SC:Gal glycoconjugate to promote the bacterial growth lower than the former could be
389 attributed to the higher size of SC:Gal [72 h, 50 ºC] peptides generated after digestion
390 (Laparra et al., 2011), which could present a higher difficulty to be metabolized by
391 bacteria than SC:Gal [4 h, 60 °C] peptides.

392 On the other hand, all the bacterial strains utilized the digested SC:Lac conjugates
393 particularly that obtained after 8 h of incubation at 60 ºC, with the highest content in the
394 Amadori compound lactulosyl-lysine. Using this digested conjugate as substrate, all
395 lactobacilli reached higher maximum OD_{600} values (from 0.85 to 1.5) and faster growth
396 rates (from 0.192 to 0.474 h^{-1}) than with Lu, displaying no-lag time periods (Tables 2

and 3). These results suggest that the Amadori compound lactulosyl-lysine, derived from SC glycation with Lac, might have a greater beneficial effect than Lu on most of the pure cultures studied.

In good agreement with these results, cell densities of anaerobic strains (Table 4) reached higher OD_{600} values after 24 h of incubation with digested SC galactosylated, particularly at 60 ºC for 4 h, than with Tag, this being specially noticeable in the case of bifidobacteria, which were unable to grow with Tag as carbon source. Likewise, hydrolysates of lactose conjugates also led to high cell densities, giving rise to maximum OD_{600} values after 24 h of incubation much higher than with Lu. *L. gasseri* Lc9 and *L. acidophilus* LA-5 cell densities increased with SC:Gal [4 h, 60 °C], SC:Lac [8 h, 60 °C] and SC:Lac [24 h, 60 °C] as substrates considerably more than with Glu. Moreover, it is noteworthy that, with bifidobacteria, the highest OD_{600} values were achieved when hydrolysates of SC:Lac conjugates were used as substrates. This suggests that the Amadori compound lactulosyl-lysine, formed during the initial stages of the MR between SC and Lac, might possess a particularly positive effect on bifidobacteria growth.

4. Conclusions

The effect of digested β-Lg glycoconjugates on the growth of the potential probiotic strains assayed was strain-dependent, whereas growth profiles of bacteria when hydrolysates of SC glycoconjugates were used as substrates were very similar, regardless of the strain. Digests of β-Lg:Gal conjugates were notably fermented by all lactobacilli and bifidobacteria assayed, but not by streptococci, a higher bacterial growth than with tagatose being appreciated in most of the cases. In addition, such glycoconjugates promoted bacterial growth more efficiently than β-Lg:Lac complexes.

On the other hand, digests of SC glycated with Gal and Lac favoured in great extent the growth of all the bacterial strains studied, higher cell densities and faster growth with digested glycoconjugates than with tagatose and lactulose as substrates being observed.

Regarding to the glycation degree, a higher bacterial growth was generally detected with glycoconjugates at initial stages of the MR, with the highest content in the Amadori compound tagatosyl- or lactulosyl-lysine, than with those at advanced stages of the reaction.

Therefore, from the findings described in this work we can infer that conjugation of both milk proteins with galactose and lactose via the Maillard reaction could be an efficient method to obtain novel food ingredients with a potential prebiotic character. To corroborate these results, other probiotic strains should be tested in further studies, comparing, moreover, the effect of Maillard conjugates with that of other prebiotics .

In addition, future studies are needed to determine the effect of these Maillard complexes on mixed cultures from human faeces, this representing a model closer to what might occur *in vivo.*

Acknowledgments

This work has been funded by projects Consolider Ingenio 2010 FUN-c-FOOD CSD2007-00063 (MICINN), ALIBIRD S2009/AGR-1469 (CAM), AGL2009-13361-C02-02 (MICINN) and IBEROFUN 110AC0386 (CYTED). M. Corzo-Martínez thanks Danone Institute for a grant.

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531

532 **Tables**

533

534 **Table 1.** Structural features of glycoconjugates used for the bacterial growth study.

Glycoconjugates	Incubation conditions	Structural features	Reference
β-Lg:Gal	40 ºC, 24 h	Maximum content in the Amadori compound tagatosyl-lysine and non-aggregated	Corzo-Martínez et al. (2008)
	50 ºC, 48 h	Highly glycated and aggregated	Corzo-Martínez et al. (2008)
β-Lg:Lac	60 ºC, 8 h	Maximum content in the Amadori compound lactulosyl-lysine and non-aggregated	Laparra et al. (2011)
	60 ºC, 48 h	Highly glycated and aggregated	Laparra et al. (2011)
SC:Gal	60 ºC, 4 h	Maximum content in the Amadori compound tagatosyl-lysine and non-aggregated	Corzo-Martínez et al. (2010)
	50 ºC, 72 h	Highly glycated and aggregated	Corzo-Martínez et al. (2010)
SC:Lac	60 ºC, 8 h	Maximum content in the Amadori compound lactulosyl-lysine and non-aggregated	Corzo-Martínez et al. (2010)
	60 ºC, 24 h	Highly glycated and aggregated	Corzo-Martínez et al. (2010)

535

536

537 **Table 2.** Maxima growth rates (μ_{max}, h^{-1}) and lag parameters (lag, h) of bacteria growing aerobically on glucose, tagatose, lactulose, and

538 hydrolysates derived from *in vitro* gastrointestinal digestion of galactosylated and lactosylated β-Lg and SC as substrates.

539
540

Sample			*L. casei* LC-01		*L. reuteri* R13		*L. plantarum* IFPL722		*L. plantarum* CLC17		*L. delbrueckii* ZL95-27		*L. brevis* CLC23		*S. thermophilus*		*S. salivarius* ZL50-7	
Conjugate	**t (h)**	**T[a] (°C)**	**μ_{max}**	**lag**	**μ_{max}**	**lag**	**μ_{max}**	**lag**	**μ_{max}**	**lag**	**μ_{max}**	**lag**	**μ_{max}**	**lag**	**μ_{max}**	**lag**	**μ_{max}**	**lag**
Glucose	0	0	0.135	6.97	0.532	3.25	0.162	7.04	0.167	7.34	0.246	9.45	0.057	4.66	0.112	7.00	0.747	6.46
Tagatose	0	0	0.129	7.13	NG*		NG		NG		NG		NG		NG		NG	
Lactulose	0	0	0.142	7.52	0.210	4.17	0.177	7.95	0.113	7.91	NG		NG		0.177	8.96	0.492	6.60
β-Lg:Gal	24	40	0.048	0	0.132	0	0.191	0	0.102	0	0.102	0	0.114	0	NG		NG	
	48	50	0.048	3.47	0.043	0.44	0.056	0	0.038	0	0.045	0	0.044	0.44	NG		NG	
β-Lg:Lac	8	60	0.020	1.21	NG		NG		NG		NG		NG		NG		NG	
	48	60	0.037	8.79	NG		NG		NG		NG		NG		NG		NG	
SC:Gal	4	60	0.541	0	0.516	0	0.539	0	0.591	0	0.536	0	0.673	0.31	0.232	0	0.253	0
	72	50	0.103	0	0.130	2.84	0.118	0	0.103	0	0.140	1.61	0.135	3.18	NG		NG	
SC:Lac	8	60	0.445	0	0.410	0	0.410	0	0.428	0	0.474	0	0.323	0	0.192	0	0.373	0
	24	60	0.232	0	0.254	0.83	0.198	0	0.258	0.51	0.251	0	0.265	1.06	0.088	0	0.091	0

541 *NG: no growth (OD_{600} < 0.2)

542

543 **Table 3**. Values of maximum DO_{600} reached by aerobic strains incubated with glucose, tagatose, lactulose, and hydrolysates derived from *in*

544 *vitro* gastrointestinal digestion of galactosylated and lactosylated β-Lg and SC as substrates.

Sample			*L. casei* LC-01	*L. reuteri* R13	*L. plantarum* IFPL722	*L. plantarum* CLC17	*L. delbrueckii* ZL95-27	*L. brevis* CLC23	*S. thermophilus*	*S. salivarius* ZL50-7
Conjugate	**T (h)**	**T[a] (ºC)**								
Glucose	0	0	0.881 [a]	1.076 [a]	0.916 [a]	0.884 [a]	0.912 [a]	0.793 [a]	0.663 [a]	1.304 [a]
Tagatose	0	0	0.902 [a]	NG	NG	NG	NG	NG	NG	NG
Lactulose	0	0	0.997 [b]	0.974 [b]	1.195 [b]	0.895 [a]	NG	NG	0.986 [b]	1.314 [a]
β-Lg:Gal	24	40	0.875 [a]	0.833 [c]	0.962 [c]	0.886 [a]	1.068 [ae]	0.946 [b]	NG	NG
	48	50	0.691 [c]	0.678 [d]	0.695 [d]	0.663 [b]	0.682 [b]	0.642 [c]	NG	NG
β-Lg:Lac	8	60	0.390 [d]	NG	NG	NG	NG	NG	NG	NG
	48	60	0.412 [d]	NG	NG	NG	NG	NG	NG	NG
SC:Gal	4	60	1.622 [e]	1.528 [e]	1.592 [e]	1.537 [c]	1.573 [c]	1.493 [d]	0.857 [c]	0.851 [b]
	72	50	1.229 [f]	0.886 [bc]	1.201 [b]	0.967 [ae]	0.964 [ae]	0.741 [e]	NG	NG
SC:Lac	8	60	1.467 [g]	1.419 [f]	1.410 [f]	1.238 [d]	1.376 [d]	1.336 [f]	0.857 [d]	0.946 [c]
	24	60	1.254 [f]	0.919 [bc]	1.296 [g]	1.036 [e]	1.110 [e]	0.878 [g]	0.707 [e]	0.738 [d]

545 *NG: no growth (OD_{600} < 0.2)

546 [a-g] Different case letters indicate statistically significant (P<0.05) differences between substrates for each bacterial strain.

Table 4. Values of DO_{600} after 24 h of incubation of anaerobic strains with glucose, tagatose, lactulose, and hydrolysates derived from *in vitro* gastrointestinal digestion of galactosylated and lactosylated β-Lg and SC as substrates.

Sample			*B. lactis* BB12	*B. breve* 26M2	*L. gasseri* Lc9	*L. acidophilus* LA-5
Conjugate	**T (h)**	**T[a] (ºC)**				
Glucose	0	0	1.049 [a]	0.624 [a]	0.799 [a]	0.790 [a]
Tagatose	0	0	NG*	NG	NG	0.703 [b]
Lactulose	0	0	NG	0.914 [b]	NG	NG
β-Lg:Gal	24	40	0.382 [b]	0.388 [c]	0.522 [b]	0.522 [c]
	48	50	0.208 [c]	0.169 [d]	0.206 [c]	0.206 [d]
β-Lg:Lac	8	60	NG	NG	NG	0.386 [e]
	48	60	NG	NG	NG	0.386 [e]
SC:Gal	4	60	0.714 [d]	0.700 [e]	1.259 [d]	1.292 [f]
	72	50	0.568 [e]	0.440 [f]	0.673 [e]	0.588 [g]
SC:Lac	8	60	1.100 [f]	1.081 [g]	0.989 [f]	1.233 [h]
	24	60	0.993 [a]	1.136 [h]	1.103 [g]	1.200 [i]

*NG: no growth (OD_{600} < 0.2)

[a-i] Different case letters indicate statistically significant (P<0.05) differences between substrates for each bacterial strain.

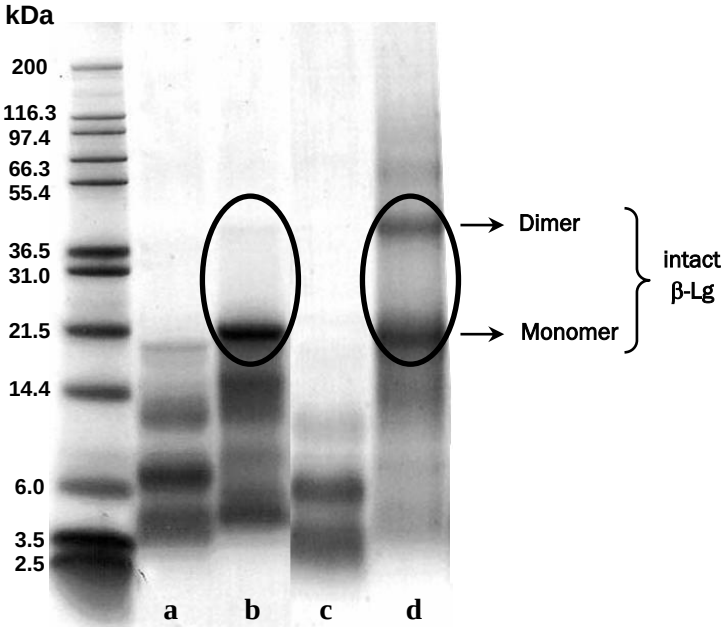
Figure captions

Figure 1. SDS-PAGE analysis of hydrolysates of control β-Lg incubated for 24 h at 40ºC (a) and 48 h at 50ºC (c) and β-Lg glycated with Gal for 24 h at 40ºC (b) and 48 h at 50ºC (d) after *in vitro* gastrointestinal digestion. M: M*w* markers.

Figure 2. Chromatographic profiles obtained by RP-LC-UV of: (**A**) native SC non-digested and control heated SC [4 h, 60 ºC] and [72 h, 50 ºC] subjected to *in vitro* gastrointestinal digestion, and (**B**) native SC non-digested and SC glycated with Gal at 60 ºC for 4 h and at 50 ºC for 72 h subjected to *in vitro* gastrointestinal digestion.

Figure 3. Growth curves of aerobic strains of lactic acid bacteria assayed with ⊣▲⊢ glucose, ⊣△⊢ tagatose, ⎯⎯ lactulose and hydrolysates derived from *in vitro* gastrointestinal digestion of galactosylated (⊣◆⊢ 24 h at 40 ºC and ⊣◇⊢ 48 h at 50 ºC) and lactosylated (⊣■⊢ 8 and ⊣□⊢ 48 h at 60 ºC) β-Lg and galactosylated (⊣✳⊢ 4 h at 60 ºC and ⊣×⊢ 72 h at 50 ºC) and lactosylated (⊣●⊢ 8 and ⊣○⊢ 24 h at 60 ºC) SC as substrates.

Figure 1.


kDa
200
116.3
97.4
66.3
55.4
36.5
31.0
21.5
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3.5
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a
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c
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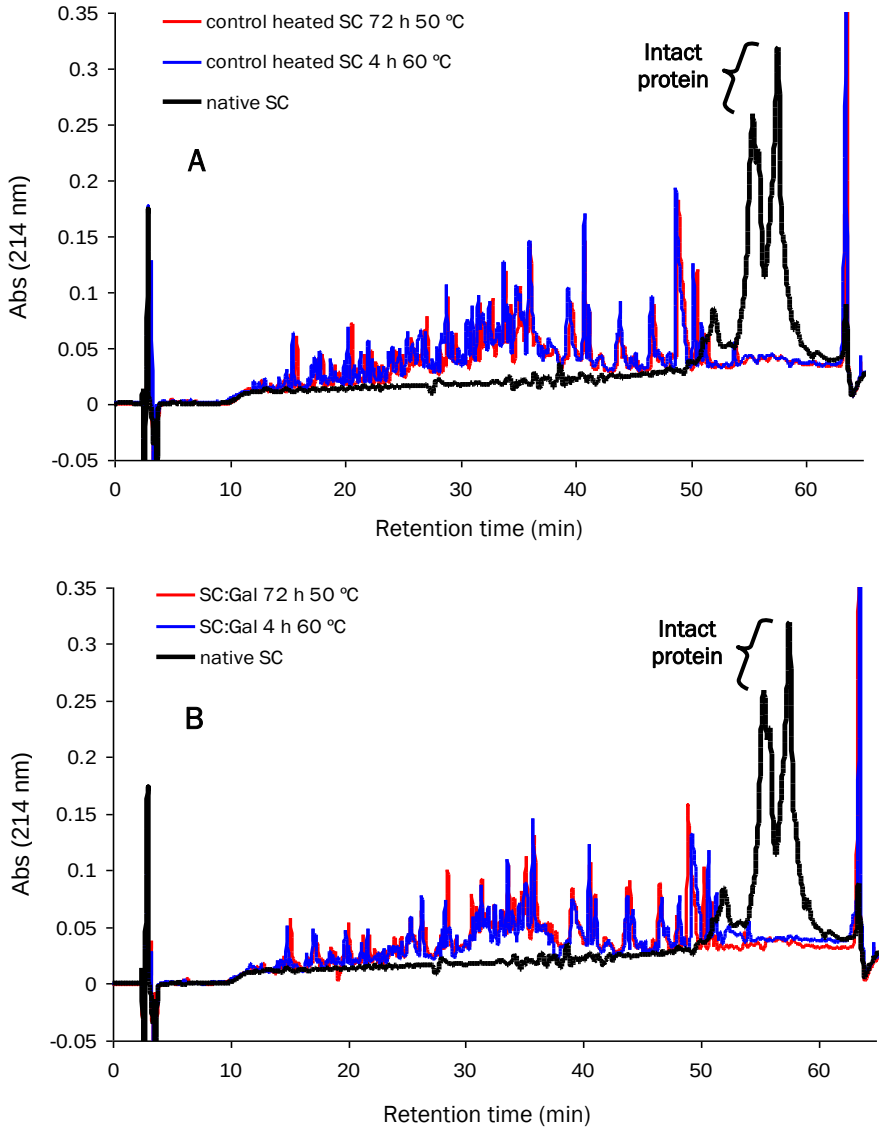
Dimer
Monomer
intact
β-Lg

Figure 2.


control heated SC 72 h 50 ℃
control heated SC 4 h 60 ℃
native SC
A
Intact protein
Abs (214 nm)
Retention time (min)
SC:Gal 72 h 50 ℃
SC:Gal 4 h 60 ℃
native SC
B
Intact protein
Abs (214 nm)
Retention time (min)

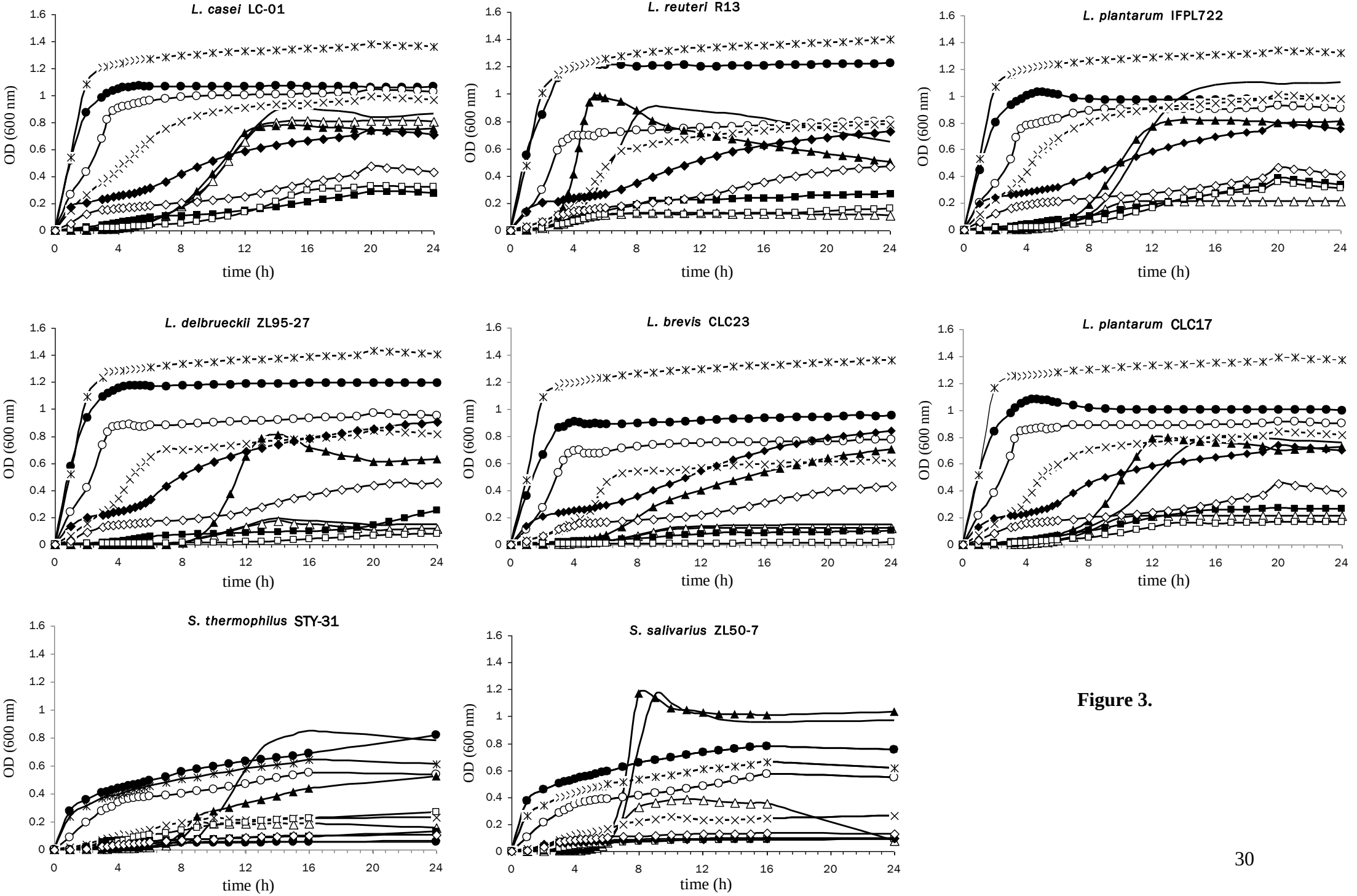

L. casei LC-01
L. reuteri R13
L. plantarum IFPL722
L. delbrueckii ZL95-27
L. brevis CLC23
L. plantarum CLC17
S. thermophilus STY-31
S. salivarius ZL50-7
OD (600 nm)
time (h)

Figure 3.

1

Effect of milk protein glycation and gastrointestinal digestion on the growth of bifidobacteria and lactic acid bacteria
Corzo-Martínez et al.

Reviewers' comments:

Reviewer #1:

The paper is interesting. Few but important recommendations are needed and they should take in account by the authors.

1. Introduction: it seems to long. I think the authors should decrease it.

According to the referee indication, we have decreased the section of Introduction of the revised version of the paper.

2. Material and Methods:

2.1. An important remark is it takes clear for the reader the strain used. Please, add some reference where the commercial starters are used in probiotic dairy food processing. Preferentially, recent IFJM references. Item 2.3 - please, add a reference.
The reference Tabasco et al. 2007 has been added in line 116 to indicate the origin of the commercial starter strains.
Item 2.3: reference Moreno et al. (2005) in line 163 was added for previous description of substrates digestion.

2.2. Include a section of statistical analysis.

Following the referee's suggestion, we have included a section of statistical analysis (Section 2.6, see lines 232-238 in the new version of the manuscript).

3. Results and discussion: I think prudent to include this behaviour is strain-dependent and other strains should be tested in further studies. Additional, the comparation with other prebiotics is interesting. This recommendation should be include in this part and emphasized in the conclusion section.

We are in complete agreement with the reviewer. Thus, we have included in the text that the behaviour observed is strain-dependent (see lines 285 and 368 in the new version of the manuscript) and referee's recommendations (see lines 352-354 in the new version of the manuscript).

4. Conclusion: some recommendation should be included.

Referee's recommendation has been included in the section of Conclusion of the revised version of the paper (see lines 415-418 and 431-433).

Reviewer #2:

The results of the paper with title" Effect of milk protein glycation and gastrointestinal digestion on the growth of bifidobacteria and lactic acid bacteria" demonstrated that conjugation of both milk proteins with galactose and lactose via the Maillard reaction could be an efficient method to obtain novel food ingredients with a potential prebiotic character. The manuscript is well organised and clearly written. It contains a sufficient number of experiments.

For better understand of results I recommend to apply statistical interpretation for Table 2 and 3.

Following the referee's suggestion and also according to reviewer 1, we have carried out the statistical analysis of data (see new tables 3 and 4 of the revised manuscript). In the case of aerobic cultures (Table 2), we have included the statistical analysis in another table (new table 3) with the values of maximum optical density (OD_{600}), since the statistical analysis was unpractical for μmax and lag data (table 2).