

1 **Production of lactulose oligosaccharides by isomerization of transgalactosylated**  
2 **cheese whey permeate obtained by  $\beta$ -galactosidases from dairy *Kluyveromyces***

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20 Running title: **Lactulose oligosaccharides obtained from cheese whey**

21

22 **Abstract**

23  $\beta$ -Galactosidases from *Kluyveromyces lactis* and *Kluyveromyces marxianus* isolated  
24 from artisanal ewes' milk cheeses, were used to transgalactosylate lactose from cheese  
25 whey permeate (WP). The content of galactooligosaccharides (GOS) obtained by  
26 transgalactosylation was comparable with that formed using pure lactose as substrate. In  
27 order to obtain a mixture with higher prebiotic oligosaccharide content, isomerization of  
28 the transgalactosylated WP was carried out using sodium aluminate as catalyst. The  
29 transgalactosylated mixtures at 6 hours of reaction contained amounts of prebiotic  
30 carbohydrates (tagatose, lactulose, GOS and oligosaccharides derived from lactulose,  
31 OsLu) close to 50 g/100 g of total carbohydrates for all the strains tested, corresponding  
32 to 322 g prebiotics/kg whey permeate. Thus, the suitability of this methodology to  
33 produce mixtures of dietary non-digestible carbohydrates with prebiotic properties from  
34 WP has been demonstrated, which is interesting for the food industry since it increases  
35 the value and the applicability of this by-product from cheese manufacture.

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39 *Keywords:* cheese whey permeate, transgalactosylation, isomerization, *Kluyveromyces*,  
40 prebiotic oligosaccharides.

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## 45 **Introduction**

46 Nowadays, the development of new bioactive oligosaccharides is gaining attention for  
47 their potential use as prebiotic compounds (Figuroa-González *et al.* 2011).  
48 Galactooligosaccharides (GOS) and lactulose are recognized as prebiotic carbohydrates  
49 and they are widely used in Japan, Europe and the United States (Tuohy *et al.* 2005).  
50 GOS are usually produced by transgalactosylation of lactose using microbial  $\beta$ -  
51 galactosidases, and in addition to their prebiotic character, other health benefits such as  
52 improvement of mineral absorption, prevention of intestinal infections and enhancement  
53 of immune function among others have been described (Pérez-Conesa *et al.* 2006;  
54 Arslanoglu *et al.* 2008; Vulevic *et al.* 2008; Ebersbach *et al.* 2010). Lactulose, a  
55 synthetic disaccharide manufactured by lactose isomerization in basic media, was the  
56 first carbohydrate commercialised with recognized beneficial effects on gut  
57 bifidobacteria (Méndez & Olano, 1979; Rycroft *et al.* 2001). This disaccharide has also  
58 been proposed as an enzymatic substrate to synthesize prebiotic oligosaccharides  
59 (OsLu) (Cardelle-Cobas *et al.* 2008a; Martínez-Villaluenga *et al.* 2008; Cardelle-Cobas  
60 *et al.* 2011). Another strategy for OsLu synthesis is the isomerization of GOS reaction  
61 mixtures obtained from transglycosylation of lactose solutions using commercial  $\beta$ -  
62 galactosidases (Cardelle-Cobas *et al.* 2008b).

63 Whey is the major by-product of the cheese making industry and presents  
64 important environmental problems since its disposal is highly contaminating (Gänzle *et al.*  
65 *et al.* 2008). Ultrafiltration of cheese whey yields whey protein concentrate used in the  
66 food industry, and whey permeate (WP), comprising mainly lactose and salts, with low  
67 market value. Thus, the possibility of using lactose from a waste material, such as WP,  
68 to obtain GOS is particularly interesting for the food industry (Lamsal, 2012).

69 In different studies, the feasibility of commercial yeast  $\beta$ -galactosidases to  
70 produce GOS from WP has been described (Pocedičová *et al.* 2010; Klein *et al.* 2013;  
71 Lorenzen *et al.* 2013). On the other hand, a new methodology to obtain mixtures of  
72 GOS and OsLu from WP by a combination of two reactions, isomerization using basic  
73 catalysts and transgalactosylation using commercial *Bacillus circulans*  $\beta$ -galactosidases,  
74 has been recently proposed (Corzo-Martínez *et al.* 2013). The use of both reactions is a  
75 feasible strategy to obtain a mixture of prebiotic carbohydrates with a wide diversity of  
76 structural features.

77 The potential use of  $\beta$ -galactosidases from *Kluyveromyces lactis* and *K.*  
78 *marxianus* strains isolated from artisanal cheeses (Padilla *et al.* 2014), to  
79 transgalactosylate buffered solutions of pure lactose and lactulose has been  
80 demonstrated (Padilla *et al.* 2012). Reaction mixtures with different levels of individual  
81 oligosaccharides were obtained. However, oligosaccharide production from WP using  
82 these  $\beta$ -galactosidases was not assayed and it is known that permeate ingredients such  
83 as mineral salts may hamper transgalactosylation reactions.

84 Therefore, in the present work, the feasibility of the above mentioned  $\beta$ -  
85 galactosidases from *K. lactis* and *K. marxianus* to produce prebiotic oligosaccharides  
86 from WP was explored. First, WP was submitted to transgalactosylation by  
87 *Kluyveromyces*  $\beta$ -galactosidases to obtain GOS mixtures, and in a second step  
88 transgalactosylated WP was isomerized using a basic catalyst with the aim of obtaining  
89 reaction mixtures of prebiotic carbohydrates with a wide diversity of structural features  
90 (GOS and OsLu). The use of different experimental conditions to obtain prebiotic  
91 carbohydrates may provide new ingredients with improved functionalities.

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93

94 **Materials and methods**

95 *Chemicals*

96 Lactose was obtained from Scharlau (Barcelona, Spain). D-Galactose, D-  
97 glucose, D-fructose, lactulose, raffinose, 6-galactobiose, phenyl- $\beta$ -D-glucoside and *o*-  
98 nitrophenyl  $\beta$ -D-galactopyranoside (*o*NPG) were purchased from Sigma-Aldrich Co.  
99 (Steinheim, Germany). D-Glucose and lactose for yeast culture media were obtained  
100 from Panreac (Barcelona, Spain), bacteriological peptone was acquired from Cultimed  
101 (Barcelona, Spain) and yeast extract and agar were purchased from Pronadisa (Madrid,  
102 Spain). Ultrapure water (18.2 M  $\Omega$ -cm, with levels of 1–5 ng/mL total organic carbon  
103 and <0.001 EU/mL pyrogen) produced in-house with a laboratory water purification  
104 system (Milli-Q Synthesis A10, Millipore, Billerica, MA, USA) was used throughout.

105

106 *Yeast strains*

107 Two yeast strains belonging to *K. lactis* and *K. marxianus* species (CECT 13121  
108 and CECT 13122, respectively) were isolated from artisanal ewes' milk cheeses  
109 produced in Cheese Company "Los Corrales" from rural Castelló province (Spain)  
110 (Padilla *et al.*, 2014). In addition, *K. lactis* CECT 1961<sup>T</sup> was obtained from the Spanish  
111 Type Culture Collection and was included in the study as a control.

112

113 *Kluyveromyces crude cell extracts (CCEs)*

114 Yeasts were grown overnight in medium GPY (glucose 2%, peptone 0.5% and  
115 yeast extract 0.5%) at 28°C. Afterwards, yeast cells were transferred to LPY medium  
116 (lactose 2%, peptone 0.5% and yeast extract 0.5%) and incubated overnight at 28°C.  
117 CCEs preparation was performed as described elsewhere (Padilla *et al.* 2012).

118

119 *Oligosaccharide synthesis from cheese whey permeate (WP)*

120 Industrial bovine cheese WP powder with a lactose content of 81.6 % (w/w dry  
121 matter) was kindly supplied by the dairy company Reny Picot (Navia, Spain). Physical  
122 and chemical composition of this WP was determined in a previous work (Díez-  
123 Municio et al., 2012). WP was reconstituted with ultrapure water at a lactose  
124 concentration of 250 g/L. The pH was measured using a pH meter (MP 230, Mettler-  
125 Toledo, Barcelona, Spain).

126 A solution of the reconstituted WP powder was prepared for transgalactosylation  
127 reaction. Enzymatic synthesis of oligosaccharides from cheese WP using different  
128 *Kluyveromyces* CCEs was performed under the defined reaction conditions of 250 g/L  
129 substrate at pH 6.5, temperature of 50 °C and 6 U  $\beta$ -galactosidase activity/mL (Padilla  
130 et al., 2012). Enzymatic reactions were performed in duplicate in a final volume of 10  
131 mL and were incubated under agitation. After 4 h, the reaction was stopped by  
132 immersing the reaction mixture in boiling water for 5 min to inactivate the enzyme. An  
133 aliquot of 600  $\mu$ L was withdrawn and stored at -20 °C until further analysis and the rest  
134 of the sample was submitted to isomerization reaction.

135

136 *Isomerization reaction of transglycosylated WP*

137 Isomerization assays (in duplicate) were carried out in cheese WP  
138 transgalactosylation mixtures containing 1 g carbohydrates. Sodium aluminate (0.7 g)  
139 was added as catalyst and then samples were diluted to 10 mL with Milli-Q water.  
140 Afterwards, samples were immersed into a water bath adjusted to the required  
141 temperature (40 °C) and maintained for a time period of 24 h (Cardelle-Cobas et al.  
142 2008b). Aliquots of 2 mL were withdrawn from the reaction mixtures at 0, 2, 4, 6, and  
143 24 h.

144 The reaction was stopped by placing the tubes in an ice bath and then adding a  
145 few drops of H<sub>2</sub>SO<sub>4</sub> (25%) to decrease the pH up to 3.5-4.5. In order to assist the  
146 precipitation of the formed salts, CaCO<sub>3</sub> (40%) was added until pH increased to 6.5-7.5.  
147 Then, sample was centrifuged at 7000 x g for 6 min and the supernatant was collected,  
148 filtered using a 0.45 µm syringe filter (Symta, Madrid, Spain) and diluted to a final  
149 volume of 10 mL with water. All assays were performed in duplicate.

150

#### 151 *Chromatographic determination of carbohydrates*

152 Carbohydrates in reaction mixtures were analysed by gas chromatography (GC).  
153 A volume of 300 µL of supernatant was added to 0.4 mL of internal standard (IS)  
154 solution, containing 0.5 mg/mL of phenyl-β-D-glucoside. The mixture was dried at 38-  
155 40 °C in a rotatory evaporator (Büchi Labortechnik AG, Falwil, Switzerland).

156 Previous to GC analysis of carbohydrates, oximes of trimethylsilyl derivatives  
157 (TMSO) must be prepared (Brobst & Lott, 1966). First, oximes were obtained by  
158 addition of 250 µL of a solution of 2.5% hydroxylamine chloride in pyridine to the  
159 carbohydrate mixture after 30 min at 70 °C. Subsequently, the oximes were silylated  
160 with hexamethyldisilazane (250 µL) and trifluoroacetic acid (25 µL) at 50 °C for 30  
161 min. Then, reaction mixtures were centrifuged at 10000 x g for 2 min. This  
162 derivatization procedure gives rise to a single chromatographic peak for non-reducing  
163 sugars, corresponding to their trimethylsilyl ethers, whereas two peaks are detected for  
164 reducing sugars, corresponding to their *syn*- (*E*) and *anti*- (*Z*) oxime isomers.

165 GC analysis of derivatized samples was carried out using an Agilent  
166 Technologies 7890A gas chromatograph (Wilmington, DE, USA) equipped with a with  
167 a flame ionization detector (FID). A commercial fused silica capillary column SPB-17,  
168 crosslinked phase (50% diphenyl / 50% dimethylsiloxane; 30 m × 0.32 mm *i.d.* × 0.5

169  $\mu\text{m}$  film thickness) (Supelco, Bellefonte, PA, USA) was used. The initial oven  
170 temperature was 200 °C, increasing to 230 °C at a rate of 4 °C/min, and finally  
171 increased to 290 °C at 2 °C/min and held for 25 min. The injector and detector  
172 temperatures were set at 280 °C and 290 °C, respectively. Injections were carried out in  
173 split mode (1:30) using nitrogen at 1 mL/min as carrier gas. Data acquisition and  
174 integration were performed using Agilent ChemStation Rev. B.03.01 software.

175 Quantitative analysis was carried out through the IS method. Response factors  
176 relative to IS (phenyl- $\beta$ -D-glucoside) were calculated from the analysis of standard  
177 solutions containing tagatose, fructose, glucose, galactose, lactose and lactulose,  
178 prepared over the expected concentration range in the samples. Also, raffinose was used  
179 as a standard to quantify trisaccharides. The identities of oligosaccharides produced  
180 after transglycosylation and isomerization of WP were confirmed by comparison with  
181 relative retention times of standards previously synthesized, purified and characterized  
182 in our laboratory (Cardelle-Cobas *et al.* 2008b; Cardelle-Cobas *et al.* 2008c; Martinez-  
183 Villaluenga *et al.* 2008; Cardelle-Cobas *et al.* 2009; Cardelle-Cobas, 2009). The  
184 amounts of lactose, lactulose, glucose, galactose, tagatose, fructose and other sugars  
185 remaining in the transgalactosylation and isomerization mixtures were calculated as  
186 grams per 100 g of the total carbohydrate content. All analyses were performed in  
187 duplicate

188

### 189 *Statistical Analysis*

190 Fisher's Least Significant Difference (LSD) test was used for mean comparison at  
191 95% confidence level (StatGraphics Plus 5.1, StatPoint, Herndon, VA).

192

193



194 **Results and discussion**

195 *Transgalactosylation of lactose from WP*

196 In this study, the feasibility of dairy *Kluyveromyces* CCEs to hydrolyze and  
197 transgalactosylate lactose present in cheese WP to produce GOS was evaluated. The  
198 conditions used to hydrolyze lactose from cheese WP were selected taking into account  
199 previous reported results, where the optimal production of GOS from pure lactose  
200 solutions employing CCEs from dairy *Kluyveromyces* was reached after 4 h of reaction  
201 (pH 6.5, 50 °C) (Padilla *et al.* 2012). **Figure 1** shows the chromatographic profile of  
202 carbohydrates found in the transgalactosylated reaction mixture of lactose in cheese WP  
203 by  $\beta$ -galactosidase activity of *K. lactis* CECT 13121. It can be observed the presence of  
204 released monosaccharides (galactose and glucose, peaks 1 and 2) as well as unreacted  
205 lactose (peaks 3 and 4). Moreover, the formation of GOS (di- and trisaccharides)  
206 obtained by transgalactosylation reaction was also detected. Allolactose ( $\beta$ -1-6-  
207 galactosyl glucose, peaks 5 and 7),  $\beta$ -1,6-galactobiose (peaks 6 and 8), 4'-galactosyl  
208 lactose (peak 9) and 6'-galactosyl lactose (peaks 10 and 11) could be identified. These  
209 assignments were made by comparing relative retention times to those of authentic  
210 standards and to those found in previous studies (Cardelle-Cobas *et al.* 2009). Different  
211 unknown di- and trisaccharides were also detected (labelled with an asterisk in **Figure**  
212 **1**). For the other two studied strains the GC profiles obtained were very similar.

213 Quantitative composition of the reaction mixtures originated by  $\beta$ -galactosidase  
214 activity of the three studied strains after 4 h of reaction is depicted in **Table 1**. During  
215 the production of GOS from lactose, significant amounts of free glucose and galactose  
216 were released as a consequence of lactose hydrolysis although considerable lactose  
217 content remained unaltered. GOS yield (consisting of di- and trisaccharides) above 30

218 g/100 g total carbohydrates for the three CCEs tested was found, in agreement with  
219 previous results using pure lactose solutions as substrate (Padilla *et al.* 2012) and  
220 commercial  $\beta$ -galactosidase from *K. lactis* (Martínez-Villaluenga *et al.* 2008). These  
221 results indicate that the salts present in WP did not seem to have an effect on  
222 transgalactosylation reactions. Regarding other experiments conducted with cheese WP  
223 and commercial *K. lactis*  $\beta$ -galactosidases, final GOS yields are difficult to compare, as  
224 reaction conditions are highly variable among different reported studies. Lisboa *et al.*  
225 (2012) found a similar maximum yield using WP and Lactozym 3000 L from *K. lactis*.

#### 226 *Isomerization of transgalactosylated WP*

227 Galactose, glucose and unreacted lactose present in transgalactosylation reaction  
228 mixtures from WP do not have prebiotic properties because they are absorbed in the  
229 small intestine and are not selectively fermented by intestinal microbiota. Moreover,  
230 glucose in reaction mixtures increases the glycemic index. Isomerization reaction of  
231 lactose and galactose leads to lactulose and tagatose, respectively, which are  
232 carbohydrates considered as prebiotics (Bertelsen *et al.* 1999; Olano, & Corzo, 2009).  
233 Therefore, isomerization of transgalactosylated WP containing mono-, disaccharides  
234 and GOS (allolactose,  $\beta$ -1,6-galactobiose and; 4' and 6'-galactosyl lactose) to  
235 corresponding ketoses can contribute to enrich them in prebiotic carbohydrates.  
236 Additionally because glucose is converted into fructose, a decrease of glycemic index of  
237 the final product can occur. **Figure 2** shows mono-, di- and trisaccharide GC-FID  
238 profiles obtained before (0 h) and after isomerization reaction (6 and 24 h) of the  
239 transgalactosylated mixture from WP. In the monosaccharide region (**Fig.2A**), the  
240 products resulting from isomerization of glucose and galactose (peaks 4, 5 and 6) to  
241 fructose and tagatose (peaks 1, 2 and 3), respectively are observed. In the disaccharide  
242 region (**Fig. 2B**) after 6 and 24 h of reaction, besides the isomerization of lactose (peaks

243 8 and 9) to lactulose (peaks 7 and 8), two peaks corresponding to allolactulose can be  
244 observed (peaks 15 and 16). The occurrence of an unknown disaccharide (peak 17),  
245 probably derived from lactulose, was also detected. Moreover, during the isomerization  
246 the disappearance of some unknown peaks present in the sample at time 0 h (such as  
247 peaks 10, 11 and 12) could be observed. The trisaccharide region (**Fig. 2C**) at 6 and 24 h  
248 of isomerization shows the presence of 4'-galactosyl lactulose (peak 23), 6'-galactosyl  
249 lactulose (peaks 25 and 26) as well as other oligosaccharides which could be derived  
250 from lactulose (peaks 22, 29 and 30). Peaks corresponding to 4'- and 6'-galactosyl  
251 lactose (peaks 24 and 28) were not detected after 24 h of reaction, except the peak 26  
252 corresponding to 6' galactosyl lactulose, indicating a complete isomerization.

253 The time course of carbohydrate isomerization from transgalactosylated WP  
254 followed up to 24 h is depicted in **Figures 3 and 4**. **Figure 3** shows the evolution of the  
255 released glucose, galactose and unreacted lactose during transgalactosylation of WP as  
256 well as the formation of their corresponding isomerized carbohydrates. Lactose was  
257 rapidly isomerized (**Fig. 3A**) into lactulose (**Fig. 3B**) which levels increased during 6 h  
258 of reaction reaching concentrations ranging from 4 to 10 g/100 g total carbohydrates.  
259 The level of lactose found in mixtures from *K. marxianus* was lower than in the other  
260 two tested strains and, therefore, less lactulose was formed during isomerization.  
261 Additionally, glucose (**Fig. 3C**) and galactose (**Fig. 3E**) decreased over time since they  
262 were converted into fructose (**Fig. 3D**) and tagatose (**Fig. 3F**), respectively. The latter,  
263 increased during reaction achieving levels of approximately 20 to 30 g/100 g total  
264 carbohydrates, respectively.

265 In **Figure 4**, the evolution of GOS isomerization in transgalactosylated WP (di-  
266 and trisaccharides, **Fig. 4A** and **4C**, respectively) to form OsLu (di- and trisaccharides,  
267 **Fig. 4B** and **4D**, respectively) is represented. Total GOS content (**Fig. 4E**) decreased

268 during reaction time in all the mixtures while total OsLu content (**Fig. 4F**) increased  
269 during isomerization, reaching a maximum yield of trisaccharides after 6 h for the three  
270 CCEs tested. Levels of GOS and OsLu found in the isomerized mixtures after 6 h of  
271 reaction were in the range of 12-14 and 16-18 g/100 g total carbohydrates, respectively.  
272 It is important to remark that the initial mixture obtained by *K. marxianus* CCE  
273 contained less lactose and GOS and consequently, when the catalyst agent acts, less  
274 lactulose and OsLu were formed. The formation of prebiotic carbohydrates after 6 h of  
275 isomerization, taking into account tagatose, lactulose, GOS and OsLu, reached levels of  
276 44.4-50.4 g/100 g total carbohydrates (**Figure 4 E**).

277 Results obtained in the present study show that the combined reactions of  
278 transgalactosylation of lactose from cheese WP using  $\beta$ -galactosidase from dairy  
279 *Kluyveromyces* (*K. lactis* and *K. marxianus* from cheese origin) and subsequent  
280 isomerization lead to mixtures containing a high concentration of prebiotic  
281 carbohydrates (50 g/100 g total carbohydrates, resulting in a total of 322 g prebiotics/kg  
282 whey permeate). Cardelle-Cobas *et al.* (2008c), obtained similar results when  
283 transgalactosylation reaction was performed using pure lactose solutions and  
284 commercial  $\beta$ -galactosidase from *K. lactis* and subsequent isomerization using the same  
285 catalyst (sodium aluminate). Therefore, it has been demonstrated that all tested  
286 *Kluyveromyces* CCEs will be suitable for prebiotic synthesis, being *K. lactis* CCEs  
287 slightly best producers.

288 It should be pointed out that isomerization reaction, apart from enriching the  
289 reaction mixtures in oligosaccharides of high polymerization degree, produced a  
290 decrease of lactose, glucose and galactose concentrations, lowering the final calorific  
291 value of the mixture and making the product suitable for diabetics or subjects with  
292 lactose intolerance.

293           Additionally, GOS as well as OsLu have been proved to be an excellent  
294 alternative to simple carbohydrates to promote the growth of *Bifidobacterium* and  
295 *Lactobacillus* (Cardelle-Cobas *et al.* 2011; Cardelle-Cobas *et al.* 2012; Hernández-  
296 Hernández *et al.* 2012; Marín-Manzano *et al.* 2013). Regarding tagatose, health benefits  
297 related to its consumption have been described, such as beneficial effects on  
298 postprandial hyperglycemia and hyperinsulinaemia as well as prebiotic and antioxidant  
299 activities (EFSA, 2010; Lu *et al.* 2008).

### 300           **Conclusions**

301           The results presented here demonstrate the feasibility of using  $\beta$ -galactosidases  
302 from *K. lactis* and *K. marxianus* isolated from ewe's milk cheese to transgalactosylate  
303 lactose from cheese WP and thus to increase the value of this by-product. The  
304 subsequent isomerization enhanced the diversity of potentially prebiotic carbohydrates  
305 present in the mixture (50 g/100 g total carbohydrates) composed of tagatose, lactulose,  
306 GOS and OsLu, suggesting the suitability of this method to produce novel mixtures of  
307 dietary non-digestible carbohydrates. Moreover, the procedure proposed here  
308 (transgalactosylation and isomerization of WP) yield 322 g prebiotics /kg whey  
309 permeate. Therefore, in this work a new strategy to obtain prebiotic oligosaccharides  
310 derived from lactulose using an inexpensive raw material such as cheese whey permeate  
311 has been proposed.

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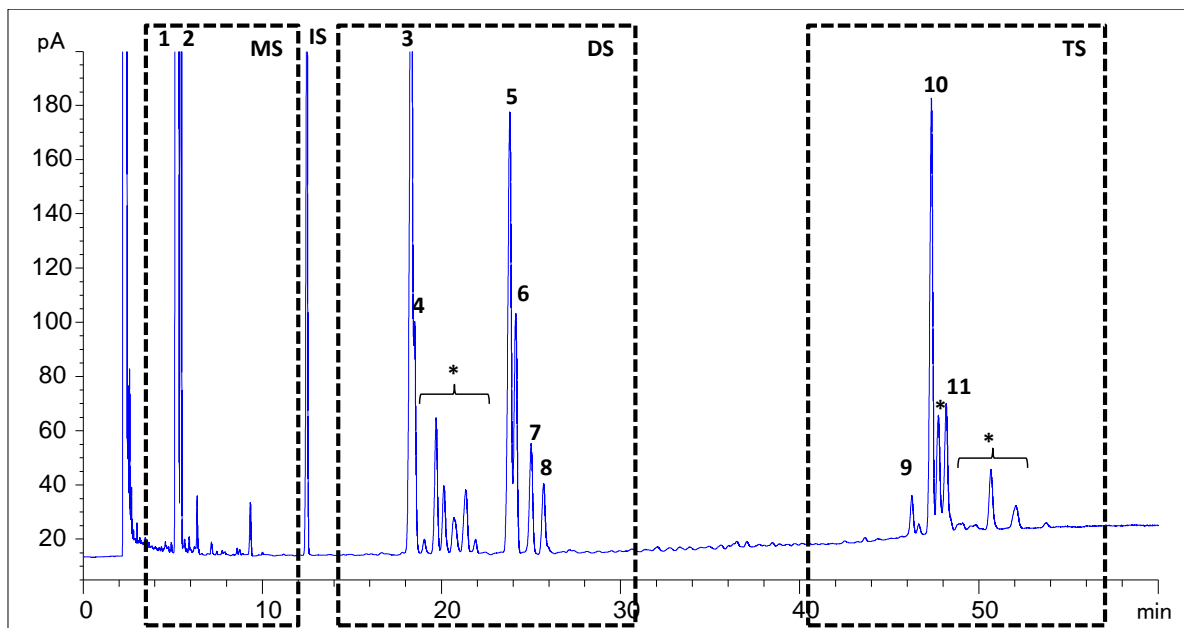
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432

433 **FIGURE CAPTIONS**

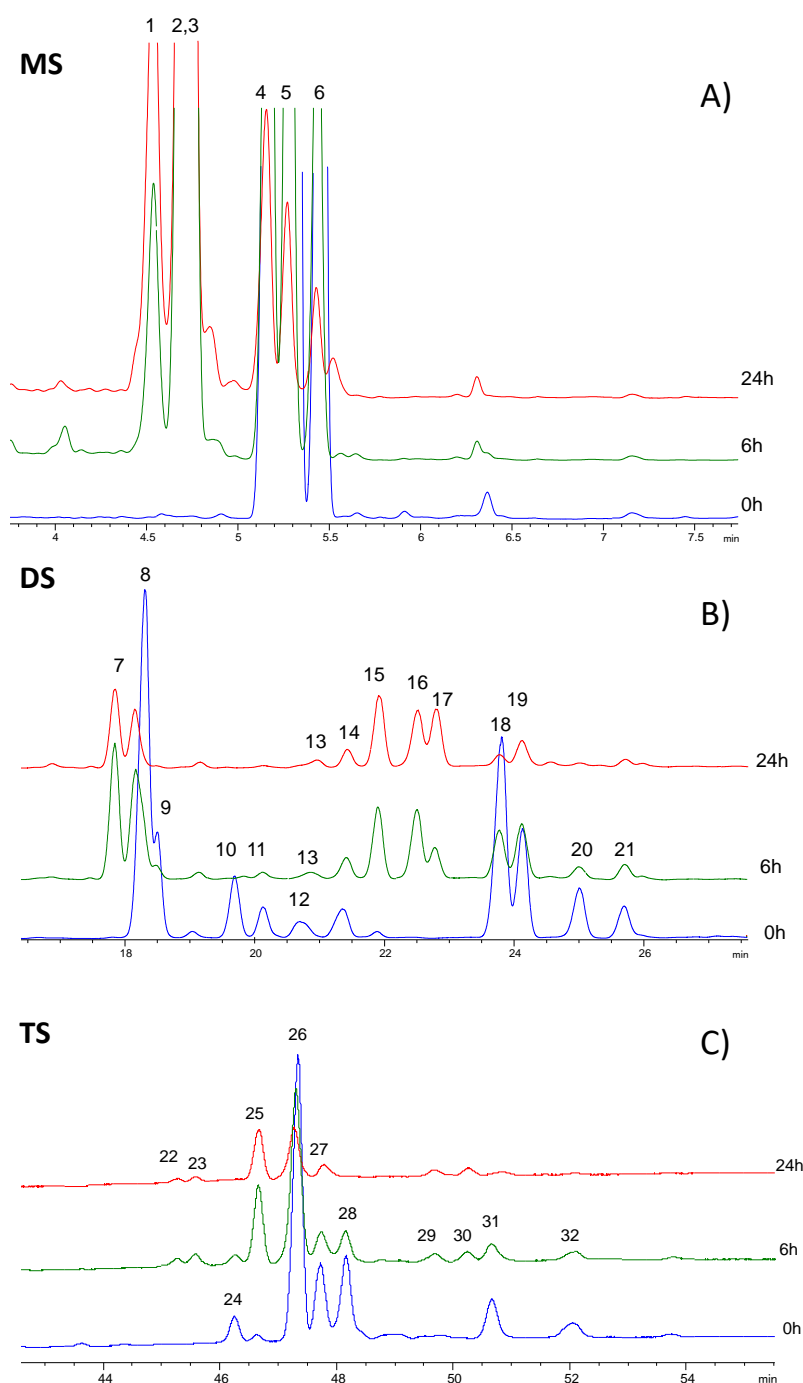
434 **Figure 1.** GC-FID profile obtained for the transgalactosylated reaction mixture of  
435 lactose from cheese WP by  $\beta$ -galactosidase activity of *K. lactis* CECT 13121 after 4h at  
436 pH 6.5, 50 °C. Peaks: **1)** galactose **2)** glucose, **3)** lactose *E*, **4)** lactose *Z*, **5)** allolactose  
437 *E*, **6)**  $\beta$ -1,6-galactobiose *E*, **7)** allolactose *Z*, **8)**  $\beta$ -1,6-galactobiose *Z*, **9)** 4'-galactosyl-  
438 lactose, **10)** 6'-galactosyl-lactose *E*, **11)** 6'-galactosyl-lactose *Z* and \*) unknown GOS.  
439 **MS:** monosaccharides; **DS:** disaccharides; **TS:** trisaccharides.

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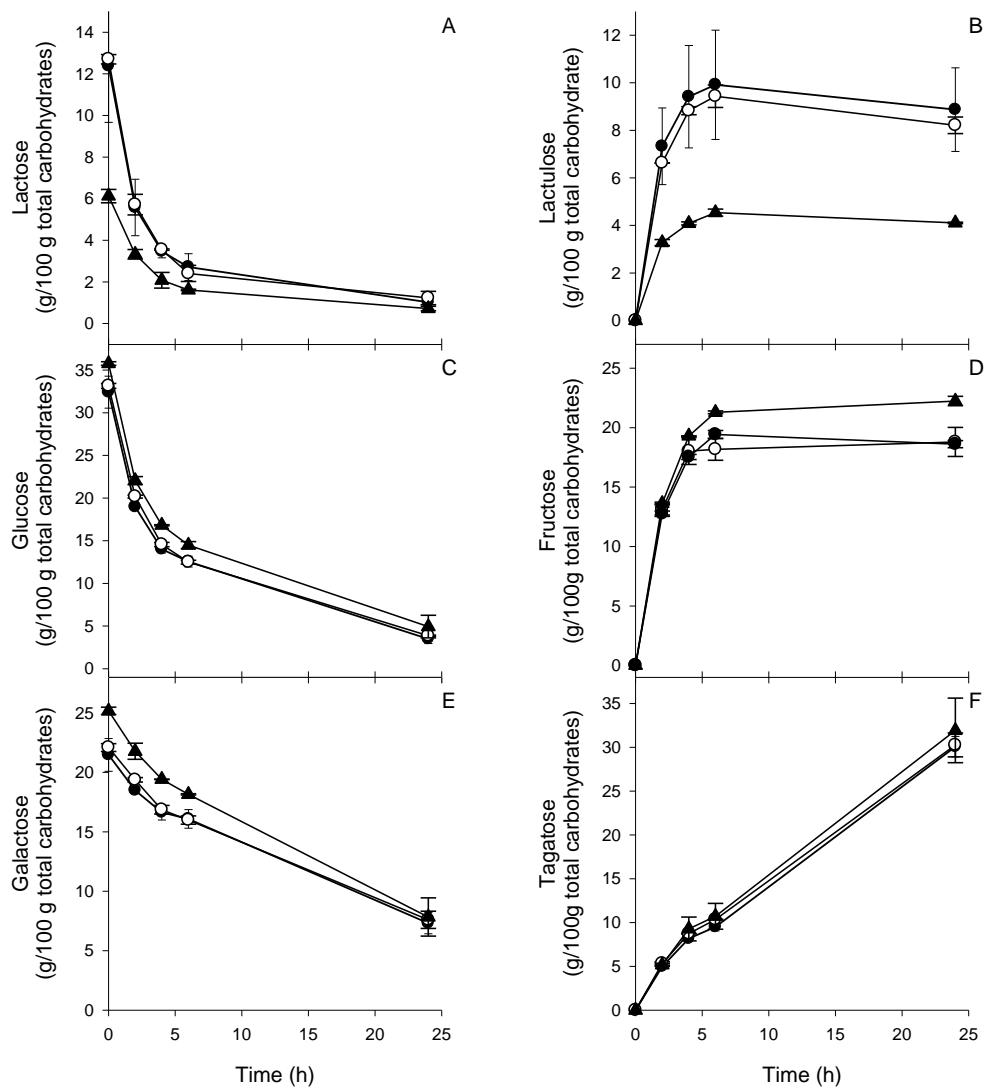
442 **Figure 2.** Mono- (A), di- (B) and trisaccharide (C) GC-FID profiles obtained before  
 443 (0h; blue line, *on-line version*) and after isomerization reaction (6h, green line *on-line*  
 444 *version*) and 24h, red line *on-line version*) of transgalactosylated WP. Peaks: *1*)  
 445 *tagatose 1*, *2*) *tagatose 2 + fructose 1*, *3*) *fructose 2*, *4*) *galactose E*, *5*) *glucose E*, *6*)  
 446 *galactose Z + glucose Z*, *7*) *lactulose 1*, *8*) *lactulose 2 + lactose E*, *9*) *lactose Z*, *10*, *11*,  
 447 *12*, *13* and *14*) *unknown galactosyl lactoses*, *15*) *allolactulose 1*, *16*) *allolactulose 2*, *17*)  
 448 *unknown galactosyl lactulose*, *18*) *allolactose E*, *19*)  $\beta$ -1,6-galactobiose *E*, *20*)  
 449 *allolactose Z*, *21*)  $\beta$ -1,6-galactobiose *Z*, *22*), *29*) and *30*) *unknown lactulose*  
 450 *trisaccharides*, *23*) *4'-galactosyl lactulose*, *24*) *4'-galactosyl lactose*, *25*) *6'-galactosyl*  
 451 *lactulose 1*, *26*) *6'-galactosyl lactulose 2 + 6'-galactosyl lactose E*, *27*), *31*) and *32*)  
 452 *unknown lactose trisaccharides*, *28*) *6'-galactosyl lactose Z*. *In italics: products*  
 453 *resulting from isomerization.*



454

455 **Figure 3.** Carbohydrate yields during isomerization with sodium aluminate at 40°C of  
 456 the transgalactosylated whey permeate (WP) (250 g/L carbohydrates) obtained by β-  
 457 galactosidase activity of *Kluyveromyces CCEs*: *K. lactis* CECT 1961<sup>T</sup> (—●—); *K. lactis*  
 458 CECT 13121 (—○—) and *K. marxianus* CECT 13122 (—▲—).

Figure 3

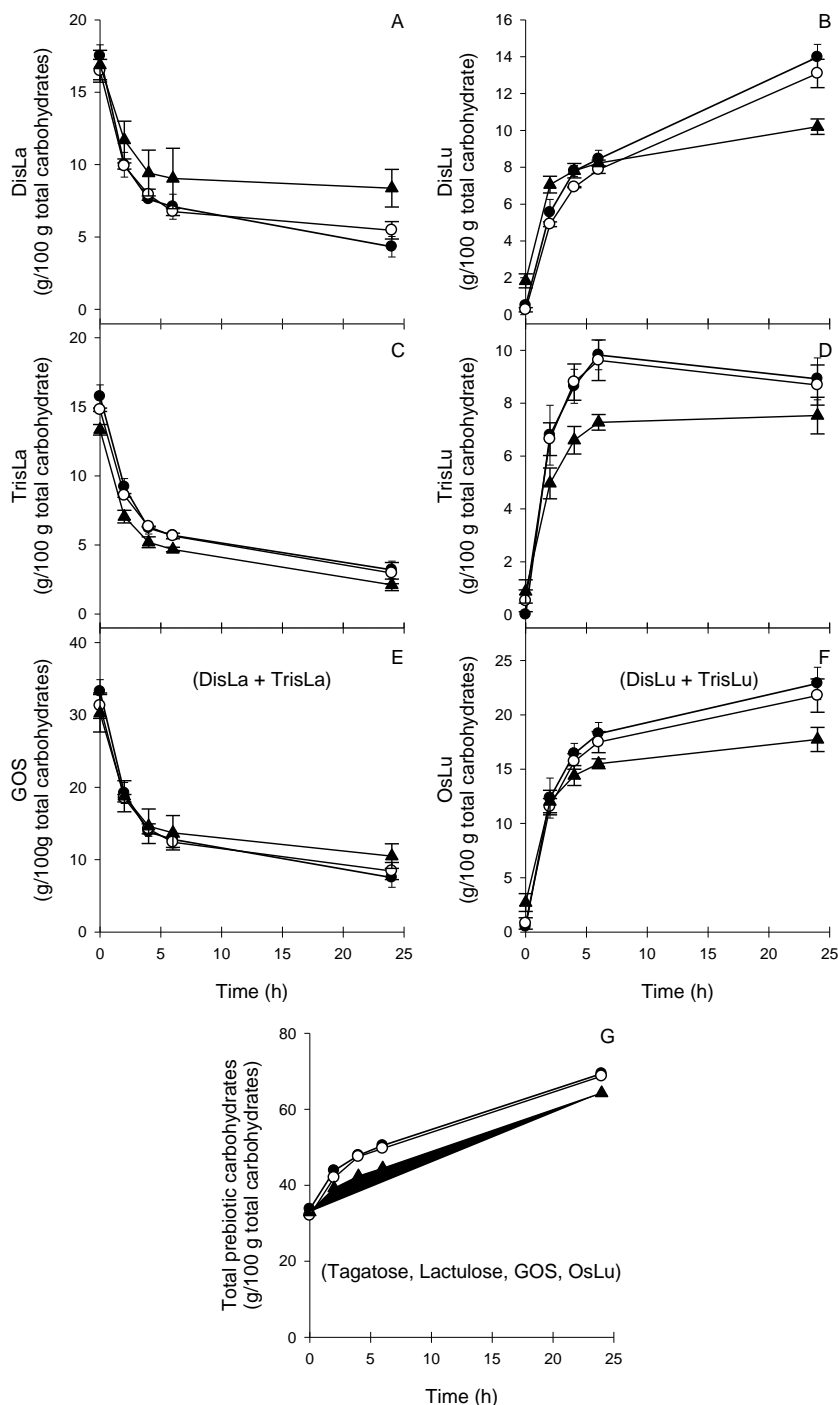


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460

461 **Figure 4.** Oligosaccharide yields during isomerization with sodium aluminate at 40°C of  
 462 the transgalactosylated whey permeate (WP) (250 g/L of carbohydrates) obtained by  $\beta$ -  
 463 galactosidase activity of *Kluyveromyces CCEs*: *K. lactis* CECT 1961<sup>T</sup> (●—); *K. lactis*  
 464 CECT 13121 (○—) and *K. marxianus* CECT 13122 (▲—) GOS: oligosaccharides  
 465 derived from lactose. Dis La: allolactose, 6-galactobiose and other unknown  
 466 disaccharides. Tris La: 4' and 6' galactosyl lactose and other unknown trisaccharides.  
 467 OsLu: oligosaccharides derived from lactulose. Dis Lu: allolactulose and unknown  
 468 disaccharides; Tris Lu: 6' galactosyl lactulose and unknown trisaccharides. Total  
 469 prebiotic oligosaccharides: tagatose, lactulose, GOS and OsLu.

Figure 4



470

471 **Table 1.** Carbohydrate composition (g/100 g of total carbohydrates) of the transgalactosylated reaction mixtures of cheese WP by *Kluyveromyces*  
 472  $\beta$ -galactosidases after 4 h at pH 6.5, 50 °C.

Strains	Monosaccharides			Disaccharides			Trisaccharides		Total GOS**
	Galactose	Glucose	Lactose	Unknown galactose derivatives	Allolactose	6-Galactobiose	Unknown galactose derivatives	6' Galactosyl lactose	
<i>K. lactis</i> CECT 1961 <sup>T</sup>	21.5 ± 1.4 <sup>a*</sup>	32.4 ± 1.9 <sup>a</sup>	12.4 ± 2.7 <sup>b</sup>	2.5 ± 0.3 <sup>a</sup>	9.8 ± 0.2 <sup>b</sup>	5.2 ± 0.0 <sup>b</sup>	5.2 ± 0.1 <sup>b</sup>	10.5 ± 0.3 <sup>a</sup>	33.2 ± 0.5 <sup>b</sup>
<i>K. lactis</i> (Kl)	22.1 ± 0.3 <sup>a</sup>	33.1 ± 0.3 <sup>a</sup>	12.7 ± 0.2 <sup>b</sup>	2.1 ± 0.2 <sup>a</sup>	9.4 ± 0.0 <sup>a</sup>	5.0 ± 0.0 <sup>a</sup>	5.1 ± 0.1 <sup>ab</sup>	9.8 ± 0.6 <sup>ab</sup>	31.3 ± 0.9 <sup>ab</sup>
<i>K. marxianus</i> (Km)	25.2 ± 0.3 <sup>b</sup>	35.8 ± 0.2 <sup>a</sup>	6.1 ± 0.3 <sup>a</sup>	2.1 ± 0.2 <sup>a</sup>	9.5 ± 0.0 <sup>a</sup>	5.4 ± 0.0 <sup>c</sup>	4.9 ± 0.5 <sup>a</sup>	8.5 ± 0.5 <sup>c</sup>	30.3 ± 1.6 <sup>a</sup>

473 \*Different letters indicate significant differences for the carbohydrate group (LSD test; p < 0.05).

474 \*\* These values include: disaccharides (unknown galactose derivatives, allolactose, 6-galactobiose) and trisaccharides (unknown lactose derivatives and 6' galactosyl-lactose).  
 475

476