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26 **Abstract**

27 The behaviour of oligosaccharides from lactulose (OsLu) included with milk was  
28 examined during in vitro gastrointestinal digestion using the Infogest protocol as well as  
29 some small intestine rat extract. The digestion was compared with commercial  
30 prebiotics GOS and Duphalac<sup>®</sup>. Electrophoretic analysis demonstrated that the prebiotic  
31 carbohydrates did not modify the gastric digestion of dairy proteins. Similarly, no  
32 significant effect of gastrointestinal digestion was shown on the prebiotic studied. In  
33 contrast, under the intestinal conditions using a rat extract, the oligosaccharides  
34 presented in OsLu samples were less digested (< 15%) than in GOS (35%). Moreover,  
35 lactulose was more prone to digestion than their corresponding trisaccharides. These  
36 results demonstrate the limited digestion of OsLu and their availability to reach the  
37 large intestine as prebiotic.

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41

42 **Keywords:** lactulose oligosaccharides, prebiotics, digestion, milk, galacto-  
43 oligosaccharides

## 44 **1. Introduction**

45 Prebiotics can reach the distal portions of the colon to selectively stimulate the  
46 growth of bifidobacteria and lactobacilli, providing important benefits to health (Gibson  
47 et al., 2004). The most relevant compounds are oligosaccharides. These prebiotics may  
48 exert other bioactive properties such as improving mineral absorption and metabolic  
49 disorders and slow gastric emptying, among other effects (Moreno et al., 2014).

50 Several commercial preparations of galactooligosaccharides (GOS) and  
51 fructooligosaccharides (FOS) are used as prebiotic ingredients in some foods such as  
52 infant formula and dairy products (Sabater et al., 2016). Lactulose (i.e. lactose isomer)  
53 is also a recognized prebiotic for the treatment of constipation and systemic portal  
54 encephalopathy (Corzo-Martínez et al., 2013). Given the huge interest in recent years  
55 towards the gastrointestinal function and new structures with improved properties, new  
56 routes to obtain a second-generation of prebiotic oligosaccharides are being explored  
57 (Moreno et al., 2017). This is the case of the oligosaccharides derived from lactulose  
58 (OsLu). These prebiotic mixtures, obtained by enzymatic synthesis using  $\beta$ -  
59 galactosidases from microbial origin, might impart better prebiotic properties than  
60 commercial GOS (Moreno et al., 2014).

61 One of the requirements for oligosaccharides to be considered as prebiotics is their  
62 resistance to digestion in the upper gastrointestinal tract. The susceptibility of prebiotic  
63 oligosaccharides to hydrolysis during their passage through the gastrointestinal tract is  
64 largely affected by the chemical structure and can impact their final state when they  
65 reach the colon to be fermented by the microbiota. Ohtsuka et al. (1990) found that the  
66 trisaccharide 4'-galactosyl-lactose was hardly digested in vitro with a homogenate of  
67 intestinal mucosa of rats. According to Torres et al. (2010), more than 90% of GOS are  
68 stable to digestive enzymes and can reach the colon to exert their positive effect.

69 Carbohydrate analysis before and after exposure to certain protocols of in vitro  
70 digestion have shown that xylo-oligosaccharides, palatinose condensates, commercial  
71 GOS and lactulose were very resistant to hydrolysis, In contrast, lactosucrose, gentio-  
72 oligosaccharides, soybean oligosaccharides, fructo-oligosaccharide and inulin were  
73 slightly hydrolysed under such conditions (Playne and Crittenden, 2009).

74 To our knowledge, limited studies have been carried out on the digestibility of  
75 OsLu. Hernandez-Hernandez et al. (2012) pointed out in in vivo assays a higher  
76 resistance of OsLu compared to GOS during gastrointestinal digestion. This was  
77 ascribed to the presence of fructose in  $\beta(1\rightarrow4)$  linkage with galactose at the reducing  
78 end of the OsLu molecules. However, there is a lack of studies on the susceptibility of  
79 OsLu to the gastrointestinal digestion when they are added in a food matrix and the  
80 impact of these compounds on the digestion of other food components. These  
81 considerations are important since standards would be more prone to changes as they  
82 are not protected in a food medium. Establishing the digestibility of prebiotic  
83 carbohydrates is of great practical application, since this influence the final dose of  
84 substrate that reaches the distal portions of gut to exert its prebiotic effect. Thus, the aim  
85 of this work has been to study the effect of the OsLu inclusion in milk on the digestion  
86 of proteins and the changes in the carbohydrate fraction using standardised in vitro  
87 digestive conditions with a more physiological relevant gastric digestion approach. A  
88 subsequent treatment with a rat small intestine extract has been included to study the  
89 effect of intestinal enzymes from mammals. The commercial prebiotics GOS and  
90 Duphalac<sup>®</sup> were also employed for comparison purposes.

91

## 92 2. Materials and methods

### 93 2.1. Chemicals and reagents

94 Galactose, D-glucose, fructose, lactose, lactulose, raffinose, stachyose, phenyl- $\beta$ -  
95 glucoside and Intestinal acetone powders from rat (rat intestine extract) from Sigma-  
96 Aldrich chemical Company (St Louis, MO).

## 97 2.2. Obtainment of prebiotic ingredients

98 OsLu were obtained at pilot scale by Innaves S.A. (Vigo, Spain) following the  
99 method described by Anadón et al. (2013). In brief, OsLu were synthesised using a  
100 commercial lactulose preparation (670 g/L; Duphalac®, Abbott Biologicals B.V., Olst,  
101 The Netherlands), diluted with water to 350 g/L and pH adjusted to 6.7 with KOH, and  
102  $\beta$ -galactosidase from *Aspergillus oryzae* (16 U/mL; Sigma), selected by its high yield  
103 for synthesis of OsLu (Cardelle-Cobas et. al., 2016). Enzymatic reactions were carried  
104 out at 50 °C in an orbital shaker at 300 rpm for 24 h. Afterwards, samples were  
105 immediately immersed in boiling water for 10 min to inactivate the enzyme. The  
106 mixture of oligosaccharides (20% [w/v]) was treated with fresh *Saccharomyces*  
107 *cerevisiae* (1.5% [w/v]; Levital, Paniberica de Levadura S.A., Valladolid, Spain) at  
108 30°C and aeration at 20 L/min, to decrease the monosaccharides content (Sanz et al.,  
109 2005). Finally, the samples were vacuum concentrated at 40 °C in a rotary evaporator  
110 (Büchi Labortechnik AG, Flawil, Switzerland). GOS syrup was kindly provided by  
111 Friesland Campina Domo (Hanzeplein, The Netherlands).

112

## 113 2.3. Milk samples

114 Skim Milk Powder (low-heat organic, protein 42.34%, fat 0.89%, lactose 49.8%  
115 (w/w) (SMP) was kindly provided by Fonterra NZ. The SMP was reconstituted at 10%  
116 with distilled water and, subsequently, lactulose (Duphalac®), GOS or OsLu were added  
117 at 5% (w/w), taking into account previous recommendations for prebiotic doses (3.3 g  
118 of prebiotic carbohydrates/100 mL) (Walton et al., 2012; Whisner et al., 2013; Lopez-

119 Sanz et al., 2015). The samples were labeled as SMP+Duphalac<sup>®</sup>, SMP+GOS and  
120 SMP+OsLu and were kept refrigerated until subsequent assays.

121

#### 122 2.4. In vitro gastrointestinal digestion

123 The solutions (see Figure 1) used for the simulation of the oral and gastric phases  
124 were based on the standardized static digestion protocol Infogest (Minekus et al., 2014).  
125 5 mL of sample was placed into a 70 mL glass v-form vessel thermostated at 37 °C. To  
126 simulate the oral phase, 4 mL of Simulated Salivary Fluid (SSF, Table 1S, Verhoeckx et  
127 al., 2015), 25 µL 0.3 M CaCl<sub>2</sub>(H<sub>2</sub>O) and 0.975 mL Milli-Q water were added and mixed  
128 for approximately 2 min using a 3D action shaker (Mini-gyro rocker-SSM3-Stuart,  
129 Barloworld Scientific limited, UK) at 35 rpm. The simulation of the gastric phase was  
130 conducted using a semi-dynamic model described by Mulet-Cabero et al., (2017). The  
131 gastric fluids and enzyme solution were added gradually. Two solutions were added at a  
132 constant rate for 2 h: (1) 9 mL of a mixture consisted of 88.9% Simulated Gastric Fluid  
133 (SGF), 0.06% 0.3 M CaCl<sub>2</sub>(H<sub>2</sub>O), 4.4% Milli-Q water and 6.7% 2 M HCl was added  
134 using the dosing device of an autotitrator (836 Titrande-Metrohm, Switzerland) and (2)  
135 1 mL of pepsin (3,214 U/mg solid, using haemoglobin as substrate) solution (in water)  
136 was added to reach the protease activity of 2,000 U/mL in the final digestion mixture.  
137 This enzyme solution was added using a syringe pump (Harvard apparatus, PHD ultra,  
138 USA). The system was agitated using the 3D action shaker at 35 rpm during the  
139 digestion time.

140 The pH was recorded throughout the procedure. Samples (0.5 mL) were taken after  
141 0, 1 and 2 h of digestion and the pepsin activity was stopped with 100 µL of 1 M  
142 NaHCO<sub>3</sub> for a subsequent analysis of the protein fraction and the rest of the sample with  
143 150 µL of 5 M NaOH for the following intestinal digestion. This last sample was

144 labelled as GPhase sample. After gastric digestion two different procedures for small  
145 intestinal digestion were carried out:

146 i) 2 mL of GPhase was freeze-dried and kept at -20°C until used for intestinal  
147 digestion assays with a crude enzyme of rat small intestine extract (RSIE). 5 mg  
148 of GPhase was mixed with 100 mg of RSIE and 1 mL distilled water. The  
149 mixture was incubated at 37° for 2 h, taking samples after 0, 0.5, 1 and 2 h.  
150 These samples were centrifuged at 10,000 rpm for 2 min and 100 µL of the  
151 supernatant was taken for carbohydrate analysis.

152 ii) The rest of the liquid GPhase (~ 16.5 mL) was subjected to the small intestine  
153 conditions following the Infogest Protocol (Minekus et al., 2014). The digestion  
154 was carried out at 37°C for 2 h. Samples (5 mL) were taken at 0, 1 and 2 h of  
155 small intestinal digestion, which were respectively labelled as 0-IPhase, 1-  
156 IPhase and 2-IPhase. They were freeze-dried until further analysis.

157

## 158 2.5. Protein determination

159 The changes in the protein fraction during gastric digestion of milk containing  
160 prebiotic ingredients (GPhase 0, 1 and 2 h) were followed by SDS-PAGE. 65 µL of  
161 sample was mixed with 25 µL of 4X NuPAGE LSD sample buffer (Invitrogen,  
162 Carlsbad, California, USA) and 10 µL of 8% dithiothreitol. The mixture was heated  
163 at 70 °C for 10 min. 20 µL of mixture was loaded on a 12% polyacrylamide  
164 NuPAGE Novex Bis-Tris precast gel (Invitrogen, Carlsbad, California, USA) and  
165 RunBlue Precast SDS-PAGE gel cassette (Expedeon Ltd., Cambridgeshire, United  
166 Kingdom). SDS-PAGE was performed according to the manufacture's instructions.  
167 Mark 12 Unstained Standard (Invitrogen) was used as a molecular weight marker  
168 (ranging from 2.5 to 200 kDa).



169

## 170 2.6. Carbohydrate analysis by GC-FID

171 Trimethyl silylated oximes (TMSO) of carbohydrates (mono-, di- and  
172 trisaccharides) present in samples were determined by Gas Chromatography following  
173 the method described by Montilla et al. (2009). Samples corresponding to 0.5 mg of  
174 saccharides were added to 0.2 mL of Internal Standard (I.S.) solution which contained  
175 0.5 mg/mL of phenyl- $\beta$ -glucoside. Response factors respect to I.S. were calculated after  
176 the duplicate analysis of standard solutions (fructose, galactose, glucose, lactose,  
177 lactulose, sucrose, raffinose and stachyose), at different concentrations ranging from  
178 0.005 to 4 mg/mL.

179

## 180 2.9. Statistical analysis

181 All digestions were carried out in duplicate and analyses were also performed in  
182 duplicate (n=4). The comparison of means was carried out using one-way analysis of  
183 variance (Tukey HSD Multiple Range Test). Statistical analyses were performed using  
184 the SPSS statistical package (Inc., Chicago, Il). The differences were considered  
185 significant when  $P < 0.05$ .

186

## 187 **3. Results and discussion**

### 188 3.1. Effect on protein digestion

189 Figure 1S (complementary material) shows the pH profile of the different samples  
190 of SMP with the addition of prebiotic ingredients (Table 2S, carbohydrate composition  
191 analysed by GC-FID) during their digestion in the semi-dynamic gastric model. The  
192 initial pH values were close to 7 in all cases and gradually decreased to 1.8 at the end of

193 the gastric digestion. In general, the profiles of the milk samples with prebiotic  
194 ingredients were similar to that of the SMP (no prebiotic ingredient added). The gradual  
195 lowering of pH enables the restructuring of the proteins due to acid induced coagulation  
196 to be simulated and is based on typical pH profiles measured in vivo (Malagelada et al.  
197 1979).

198 The electrophoretic profile of proteins corresponding to samples 0, 1 and 2 h of  
199 gastric digestion are illustrated in Figures 2 and 3. These figures show bands of pepsin,  
200 caseins, BSA,  $\beta$ -lactoglobulin ( $\beta$ -Lg) and  $\alpha$ -lactalbumin ( $\alpha$ -La). In the case of mixtures  
201 with OsLu and GOS at 0 h (Figure 2) more intense bands appeared in the area  
202 corresponding to  $\alpha$ -La, probably due to the formation of complexes between the protein  
203 and carbohydrates, which disappeared during the digestion. In general, after 2 h of  
204 gastric digestion, the bands corresponding to undigested proteins from both SMP and  
205 SMP with added prebiotics were not detected with the exception of  $\beta$ -Lg which has  
206 been shown to be more resistant to pepsin hydrolysis (Mandalari et al. 2009). Figure 3  
207 shows some diffuse, low molecular weight bands in samples corresponding to 1 and 2 h  
208 of digestion which could be related to small molecular weight peptides formed after  
209 milk protein digestion (lanes 5-12). The intensity of these bands was estimated by the  
210 Quantity One software. This showed an increase of intensity with digestion time  
211 obtaining values of 0.54 at 0.62 after 1 h and 0.64 at 0.75 after 2 h, with the lowest  
212 values corresponding to skim milk control.

213 These results show that the SDS-PAGE profile of milk with prebiotic  
214 carbohydrates was similar to that of milk without addition of these ingredients,  
215 indicating that the presence of these prebiotics in milk at the concentration required to  
216 achieve a prebiotic effect, did not modify the gastric digestion of dairy proteins.

217

### 218 3.2.Effect on carbohydrate fraction

219 The effect of gastrointestinal digestion on the three different prebiotics, Duphalac<sup>®</sup>,  
220 GOS and OsLu included in milk was investigated. For this purpose, the samples from  
221 the semi-dynamic gastric model were subjected to two different intestinal digestion  
222 protocols, as indicated above (Infogest protocol or RSIE). In the case of the Infogest  
223 method, Figure 2S (complementary material) illustrates, as an example, the  
224 chromatogram obtained by GC-FID of TMSO derivatives of carbohydrates present in  
225 the milk samples with OsLu after gastric digestion and the beginning of the intestinal  
226 phase (G+I 0 h). The peaks corresponding to carbohydrates with degree of  
227 polymerisation (DP) from 1 to 4 were found; among them galactose, lactulose and di-,  
228 tri- and tetrasaccharides derived from OsLu ingredient, and galactose, glucose and  
229 lactose from milk. Galactose was present in SMP with OsLu in higher proportion than  
230 in SMP with GOS (Table 1) in which the most abundant monosaccharide was glucose,  
231 due to their presence in the original prebiotic mixtures. In this respect, the addition of  
232 OsLu to milk or other products could be more interesting since OsLu presents lower  
233 proportion of caloric carbohydrates with lower glycaemic index than GOS (López-Sanz  
234 et al. 2015). As observed in Table 1, SMP+Duphalac<sup>®</sup> had higher concentration of  
235 lactulose than SMP+OsLu because lactulose is used as substrate during its enzymatic  
236 hydrolysis and transgalactosylation.

237 Limited modifications were observed in the carbohydrate fraction following  
238 digestion using the Infogest protocol. In spite of the fact that there was a slight decrease  
239 of OS and trisaccharides in SMP+GOS after 2 h of digestion, these differences were not  
240 statistically significant. None of the carbohydrates derived from the prebiotic  
241 ingredients provided any significant change, indicating their stability during this  
242 enzymatic digestion by pancreatic fluids and bile salts. Moreover, it seems to be clear

243 that the presence of other milk components did not impact the passage of GOS,  
244 Duphalac<sup>®</sup> and OsLu throughout the gastrointestinal digestion evaluated by the Infogest  
245 protocol.

246 In order to gain more insight in this subject and given that the Infogest protocol is  
247 mainly focus on the digestion of proteins, this study was completed with the evaluation  
248 of carbohydrate fraction of SMP with the three prebiotic ingredients after a subsequent  
249 digestion by means of an intestinal extract of from rats, labelled as RSIE, as indicated in  
250 Materials and Methods section. Figure 4 A, B, C, D illustrates the evolution of each  
251 carbohydrate fraction in the SMP added with Duphalac<sup>®</sup>, GOS and OsLu after their  
252 gastric and intestinal (Infogest) and with RSIE (0.5, 1 and 2 h) of digestion. Data are  
253 expressed as % of hydrolysis, for lactose, lactulose and oligosaccharides, and increase  
254 of monosaccharides, taking into account the control samples immediately taken after the  
255 addition of RSIE. The hydrolysis of compounds with  $DP \geq 2$  and mainly lactose  
256 increased with time of reaction, probably due to the presence of lactase ( $\beta$ -  
257 galactosidase) in the RSIE, in good agreement with the increase of the monosaccharide  
258 proportion.

259 In general, lactose was more hydrolysed than lactulose due to the presence of  
260 fructose instead of glucose in the  $\beta$  linkage of the latter (Olano and Corzo, 2009), being  
261 SMP+Duphalac<sup>®</sup> the sample with the highest degree of hydrolysis of lactose. In general,  
262 no significant differences ( $p > 0.05$ ) were found for SMP samples with OsLu and GOS.  
263 Lactulose was significantly less susceptible to hydrolysis in SMP+Duphalac<sup>®</sup> than in  
264 SMP+OsLu. Furthermore, lactulose present in OsLu and Duphalac<sup>®</sup> was more prone to  
265 degradation than OS, probably ascribed to its lower Mw, although the difference was  
266 only significant after 1 h of digestion. Finally, OS were significantly more hydrolysed in  
267 SMP+GOS than in SMP+OsLu reaching values of 35% and 15%, respectively after 2 h;

268 this was probably due to the more stable  $\beta(1-6)$  linkages in the OsLu mixture as  
269 compared to  $\beta(1-4)$  in GOS and the presence of fructose at the terminal end of molecule  
270 (Hernandez-Hernandez et al. 2012). These results indicate that OS ( $DP \geq 3$ ) present in  
271 OsLu were scarcely affected by the gastrointestinal digestion under the conditions used  
272 in the present work, being digested in a very low proportion in the small intestine which  
273 would favour the presence of a OS in the distal portions of colon to be fermented by  
274 beneficial bacteria.

275 To the best of our knowledge this is the first in vitro study on the digestion of  
276 prebiotics derived from lactose and lactulose as ingredients in a real food. The results  
277 obtained underline those of Hernandez-Hernandez et al. (2012) who pointed out, in in  
278 vivo assays with rats, that mixtures of OsLu were less digested than GOS. Particularly,  
279 the trisaccharide fraction of the former was 13% digested in the ileum, whereas in the  
280 latter case digestion was close to 53%. In both cases, the studied samples were the  
281 corresponding enzymatic mixtures obtained by transglycosylation and the presence of  
282 other food components was not considered. The small differences found in the total  
283 hydrolysis values with respect of our results could be ascribed to the differences in the  
284 experimental conditions.

285

## 286 **Conclusions**

287 According to the results obtained is possible to conclude that the presence of  
288 prebiotic carbohydrates in milk, at prebiotic doses, did not affect the gastric digestion of  
289 milk proteins, following the Infogest protocol. Similarly, under the same gastrointestinal  
290 digestion method, hardly any change was detected in the carbohydrate fraction of milk  
291 with GOS, Duphalac<sup>®</sup> and OsLu after 2 h of digestion. This might indicate the  
292 resistance of the three prebiotic mixtures, including OsLu, to gastric and pancreatic

293 fluids and bile salts. However, when the digested samples of milk with prebiotics were  
294 subjected to intestinal digestion by a small gut intestinal extract of rat a dissimilar  
295 behaviour in the three cases was observed, OsLu samples being the most resistant to the  
296 action of enzymes present in the rat intestine extract, mainly in the case of OS fraction.  
297 These results highlight the possibility of OsLu to reach the large intestine, target organ,  
298 to exert their potential prebiotic effects.

299

300

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302

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306

307

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402

403 **Figure caption**

404 **Figure 1.** Scheme of the experimental procedure.

405 **Figure 2.** Electrophoretic profiles of milk protein fractions (caseins,  $\beta$ -Lg,  $\alpha$ -La, BSA)  
406 before and after 2 h of digestion (Bis-Tris-Gel, Novex, NuPage). M: Marker, 1: SMP 0  
407 h, 2: SMP 2 h, 3: SMP+OsLu 0 h, 4: SMP+OsLu 2 h, 5: SMP+ Duphalac 0 h, 6:  
408 SMP+Duphalac<sup>®</sup> 2 h, 7: SMP+GOS 0 h, 8: SMP + GOS 2 h, 9: blank

409 **Figure 3.** Electrophoretic profiles of milk protein fractions (caseins,  $\beta$ -Lg,  $\alpha$ -La, BSA)  
410 during 0, 1 and 2 h of digestion (RunBlue Precast gels). M: Marker; 1, 5 and 9 SMP; 2,  
411 6 and 10 SMP+OsLu; 3, 7 and 11 SMP+GOS; 4, 8 and 12 SMP+Duphalac. \*Optical  
412 density was measured in the maximum of the peak with the Software Quantity One.

413 **Figure 4.** Evolution of carbohydrates over time during the gastric and intestinal  
414 digestion with RSIE. Figure shows the results for each fraction analyzed A)  
415 Monosaccharides, B) Lactose, C) Lactulose and D) Oligosaccharides after 0.5, 1.0 and  
416 2.0 h of digestion. Grey bar represents SMP samples; Striped bar, SMP+Duphalac;  
417 Black bar, SMP+GOS and White bar, SMP+OsLu. The results are shown as percentage  
418 of increase (A) or hydrolysis (B, C, D) relatively to their respective controls. Results are  
419 presented as mean  $\pm$  SD (n=4). Bar with different lower-case letters (a–d) represent  
420 statistical significant differences between each carbohydrate fraction at the same  
421 digestion time for their mean values at the 95.0 % confidence.

**Table 1 – Carbohydrate evolution of milk samples during Intestinal digestion (G+I Phase), according to Infogest Protocol.**

		Carbohydrate content (%)							
		Galactose	Glucose	Lactulose	Lactose	Other Disaccharides	Trisaccharides	Tetrasaccharides	Oligosaccharides*
SMP	0h	0.3 ± 0.1	0.4 ± 0.2	N.D.	99.4 ± 0.2	N.D.	N.D.	N.D.	N.D.
	1h	0.3 ± 0.1	0.5 ± 0.1	N.D.	99.2 ± 0.1	N.D.	N.D.	N.D.	N.D.
	2h	0.3 ± 0.0	0.4 ± 0.2	N.D.	99.4 ± 0.2	N.D.	N.D.	N.D.	N.D.
SMP + GOS	0h	0.5 ± 0.1	7.6 ± 1.0	N.D.	65.6 ± 3.7	11.0 ± 0.8	12.9 ± 1.8	2.4 ± 0.6	26.4 ± 3.1
	1h	0.5 ± 0.0	7.7 ± 1.5	N.D.	66.3 ± 3.3	12.0 ± 2.2	12.3 ± 1.4	3.3 ± 0.7	27.6 ± 4.2
	2h	0.5 ± 0.0	6.9 ± 0.2	N.D.	68.4 ± 1.4	10.8 ± 1.3	10.9 ± 0.7	2.4 ± 1.7	24.1 ± 1.5
SMP + Duphalac <sup>®</sup>	0h	3.6 ± 0.4	0.4 ± 0.4	22.0 ± 5,1	73.6 ± 4.9	N.D.	N.D.	N.D.	N.D.
	1h	3.4 ± 0.8	0.2 ± 0.2	20.6 ± 1,1	76.5 ± 1.1	N.D.	N.D.	N.D.	N.D.
	2h	3.1 ± 0.2	0.4 ± 0.2	21.6 ± 1,9	75.6 ± 1.7	N.D.	N.D.	N.D.	N.D.
SMP + OsLu	0h	5.0 ± 0.3	0.3 ± 0.1	6.3 ± 2.1	68.4 ± 1.7	9.8 ± 0.3	9.3 ± 0.2	0.9 ± 0.2	20.1 ± 0.6
	1h	5.0 ± 0.1	0.4 ± 0.2	7.1 ± 1.4	67.4 ± 1.3	9.8 ± 0.4	9.5 ± 0.4	0.8 ± 0.3	20.1 ± 0.3
	2h	5.3 ± 0.3	0.3 ± 0.0	6.0 ± 0.4	69.0 ± 1.1	10.2 ± 0.5	8.6 ± 1.0	0.8 ± 0.6	19.6 ± 1.6

The data are expressed as the mean ± SD (p>0.05). No statistical difference was determinates between 0, 1 and 2 h samples in all compounds using a one-way analysis of variance (ANOVA) (n=4). N.D. No detected.

\*Oligosaccharides: Values represent the sum of di-, tri- and tetrasaccharides.

**Figure 1.**

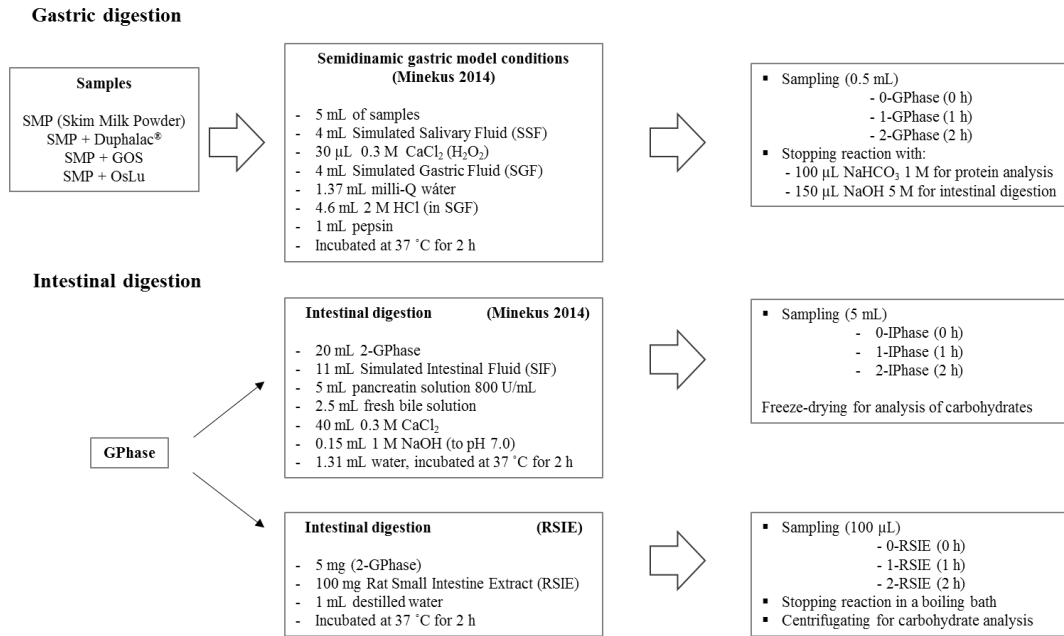
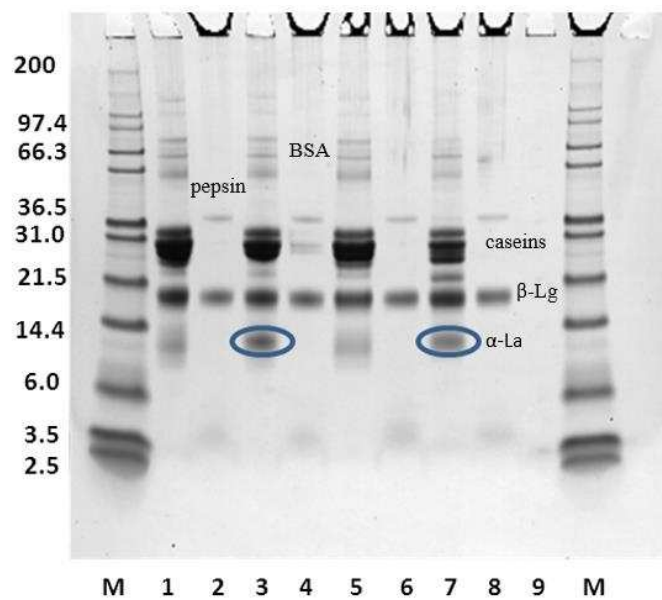
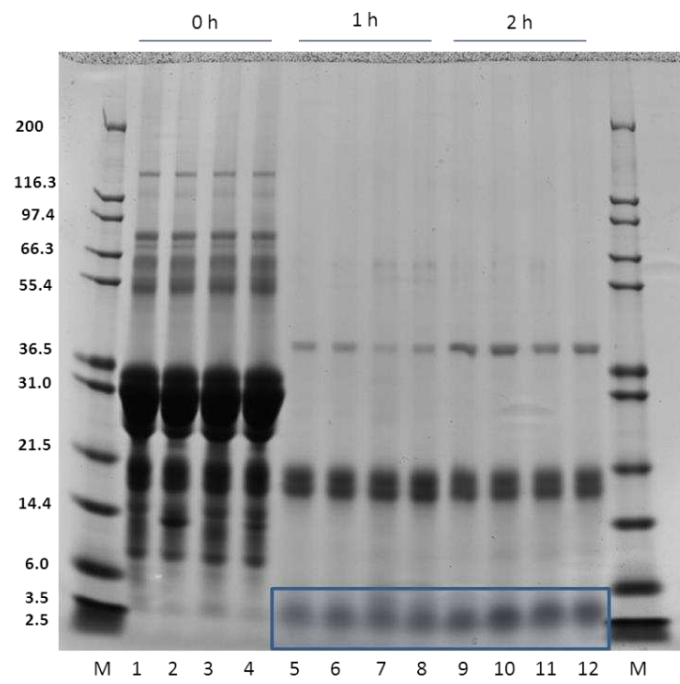


Figure 2.



**Figure 3.**



1 **Figure 4.**

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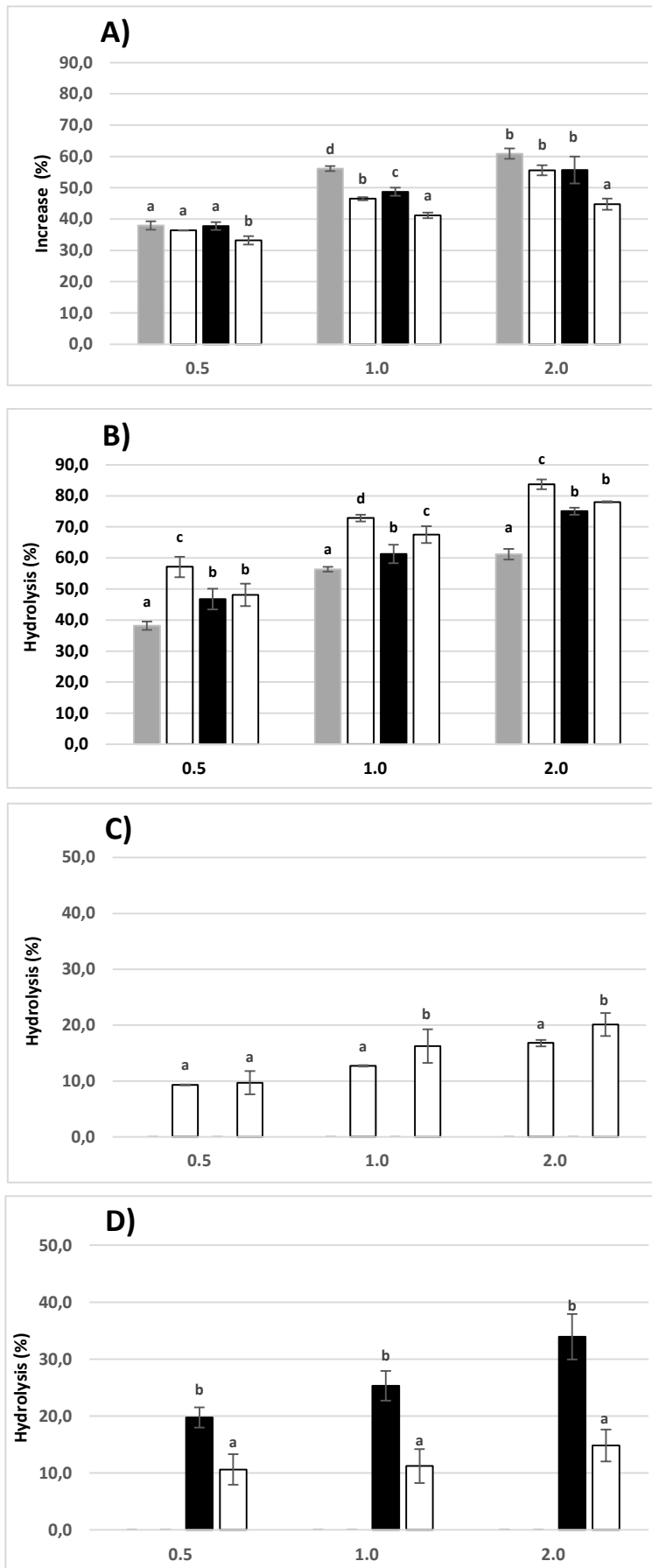
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- Prebiotic carbohydrates added to milk do not modify the gastric digestion of proteins
- Carbohydrates keep stable at enzymatic digestion by pancreatic fluid and bile salts
- Lactulose was more prone to digestion than their corresponding trisaccharides
- Oligosaccharides derived from lactulose were less digested than those from lactose

**Table 1S. Composition of simulated salivary fluid (SSF)**

Constituent	SSF (pH 7) /mmol/L
K <sup>+</sup>	18.8
Na <sup>+</sup>	13.6
Cl <sup>-</sup>	19.5
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	3.7
HCO <sub>3</sub> <sup>-</sup> , CO <sub>3</sub> <sup>2-</sup>	13.7
Mg <sup>2+</sup>	0.15
NH <sub>4</sub> <sup>+</sup>	0.12
Ca <sup>2+</sup>	1.2

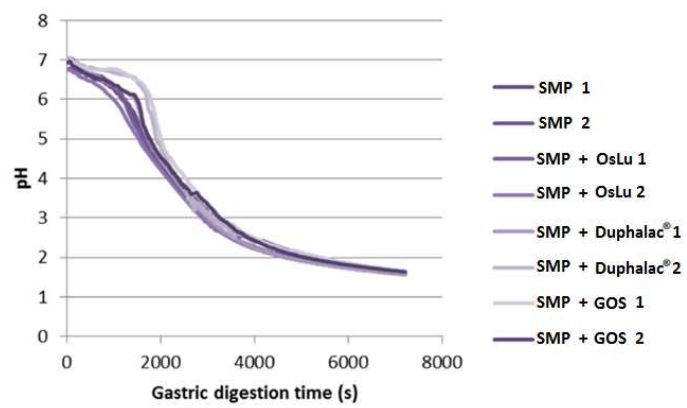
$\alpha$ -amilase at 150 units per mL of SSF ([Verhoeckx et al., 2015](#))

**Table 2S. Carbohydrate composition (% of total carbohydrates) of OsLu, Vivinal®GOS and Duphalac®.**

Samples	Glucose	Fructose	Galactose	Other Disaccharides	Lactose	Lactulose	Trisaccharides	Tetrasaccharides	Pentasaccharides	Hexasaccharides
OsLu	-	-	14.1 (1.0)	21.1 (1.1)	N.D.	26.1 (1.2)	25.6 (0.7)	9.7 (0.7)	2.6 (0.6)	0.2 (0.1)
Vivinal®GOS	20.7 (2.1)	-	1.4 (0.1)	20.5 (0.6)	18.0 (0.2)	-	21.0 (0.7)	13.1 (0.8)	4.8 (0.6)	0.7 (0.4)
Duphalac®	0.3 (0.0)	-	7.9 (0.7)	-	3.2 (0.2)	88.7 (0.6)	-	-	-	-

Data are expressed as the mean (SD) (p>0.05).  
N.D. No detected.

**Figure 1S.** pH profile of milk samples with the prebiotic ingredients during gastric digestion.



**Figure 2S.** GC-FID profile of TMSO derivatives of carbohydrates present in milk samples with OsLu after 1 h of gastric digestion. Peak 1 Galactose; 2 Glucose; 3 Galactose + Glucose; I.S. Internal Standard; 4 Lactose; 5 Other disaccharides. \* Matrix effect, DP: Degree of Polymerisation.

