In vitro digestibility of galactooligosaccharides: Effect of the structural features on their intestinal degradation

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Abstract

Small intestinal brush border membrane vesicles (BBMV) from pig were used to evaluate the digestibility of different mixtures of galactooligosaccharides from lactose (GOS), differing in the predominant glycosidic linkage, and from lactulose (OsLu). Dissimilar hydrolysis rates were detected after BBMV digestion. Predominant glycosidic linkages and monomeric composition showed to play a key role in the resistance to intestinal mammalian digestive enzymes. $\beta(1\rightarrow3)$ GOS mixture was the most susceptible to hydrolysis with 50.2 % of degradation after digestion, followed by $\beta(1\rightarrow4)$ with 34.9 % hydrolysis, whereas $\beta(1\rightarrow6)$ linkages showed to be highly resistant to digestion (27.1 %). Monomeric composition seems to provide a better resistance in $\beta(1\rightarrow6)$ oligosaccharides from lactulose (22.8 %) as compared to $\beta(1\rightarrow6)$-GOS (27.1 %). This was also observed in β-galactosyl-fructoses and β-galactosyl-glucoses disaccharides where the presence of fructose provided a higher resistance to digestion. Thus, the resistance to small intestinal digestive enzymes highly depends on structural characteristic and composition of prebiotic ingredients. Increasing knowledge on this regard could contribute to the future synthesis of new tailored prebiotic with specific functional properties.

Keywords: prebiotics, galactooligosaccharides, glycosidic linkages, *in vitro* digestion model, small intestine.

1. Introduction

Knowledge about the diversity of human microbiota and its relation to health has been largely gathered during last years. Moreover, there is a clear evidence suggesting that our microbiota is deeply implicated in a wide range of metabolic functions extending beyond the gut[1], such as, the regulation of the central nervous system homeostasis through immune, vagal and metabolic pathways [2,3,4] or the prevention of bone and respiratory diseases.[5,6] One of the most used strategies to modulate the composition and metabolic activity of microbiota is the use of prebiotics.[7]

Prebiotics definition refers to a "substrate that is selectively utilized by host microorganisms conferring a health benefit".[8] These compounds are characterized by the resistance to the digestion and acid conditions in the upper gastrointestinal tract and the ability to reach the colon without alteration in their structure.[9] To date, despite a considerable number of compounds have been proposed as potential prebiotics, all well-recognized prebiotics are carbohydrates, mainly inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS) and lactulose. Among these, GOS have attracted growing interest due to the presence of galactose-based oligosaccharides, similar to those in human milk oligosaccharides (HMOs).[10]

GOS are commonly obtained by enzymatic synthesis from lactose by β-galactosidases and they are constituted by a complex mixture of galactoses linked by different linkages $\beta(1\rightarrow1)$, $\beta(1\rightarrow2)$, $\beta(1\rightarrow3)$, $\beta(1\rightarrow4)$ and $\beta(1\rightarrow6)$ and can vary from 1 to 8 units and a terminal glucose.[11] Composition of the obtained GOS mixture is deeply affected by several factors such as, the enzyme source, lactose concentration, substrate composition and reaction conditions (temperature, time and pH).[12,11,13] Galactooligosaccharides derived from lactulose (OsLu) have been also proposed as emerging prebiotic compounds

since they might provide better prebiotic properties than GOS.[14,11] OsLu are obtained similarly to GOS using lactulose as substrate and are constituted by galactose units, linked by a variety of glycosidic linkages ($\beta(1\rightarrow6)$, $\beta(1\rightarrow1)$ and/or $\beta(1\rightarrow4)$) determined by the enzyme source, and a terminal fructose.[15]

The susceptibility of oligosaccharides to small intestinal digestion highly depends on their structure, compromising their absorption and digestion fate.[16] However, ever since prebiotics were first defined, most of the investigations have been carried out focusing on their effect on the gut microbiota composition and/or activity, and few efforts have been made towards the study of the resistance of these compounds to digestion in the small intestine. Moreover, the standardized official methods to determine the digestibility of carbohydrates present several limitations and, for instance, they do not take into consideration the disaccharidases that are present in the small intestinal brush border membrane vesicles in mammals.[17,18,19] Recently, the use of mammalian intestinal enzymes has been reported as an excellent alternative method to determine carbohydrate digestion.[20,21,22]

In vivo and *in vitro* studies have reported considerable digestion rates in the small intestine of different types of GOS in rats[20,23,14,24,25] questioning the general acceptance that these compounds reach intact the colon. These authors also have reported a different resistance to the upper gastrointestinal tract conditions as well as a different effect on microbiota depending on the main β-linkage in the mixture. Thus, $\beta(1\rightarrow6)$ linkages have been reported to be less prone to degradation by intestinal enzymes and to exert better prebiotic effect as compared to other β-linkages.

Bearing that in mind, the aim of the present study was to evaluate the digestibility of recognized prebiotics such as GOS, with predominant $\beta(1\rightarrow3)$, $\beta(1\rightarrow4)$ or $\beta(1\rightarrow6)$

88 linkages, as well as emerging prebiotic candidates derived from lactulose (OsLu, *β*(1→6))
89 using small intestinal brush border membrane vesicles from pig.

90

91 ## 2. Materials and methods

92 ### *2.1 Chemical and reagents*

93 D-Galactose (Gal), D-glucose (Glc), sucrose (*β*-D-Fru(2→1)-α-D-Glc), trehalose (α-D-
94 Glc(1→1)-α-D-Glc), lactulose (*β*-D-Gal(1→4)-D-Fru), phenyl-*β*-glucoside, *o*-nitrophenyl
95 (*o*-NP), p-nitrophenyl (*p*-NP), *o*-nitrophenyl-*β*-D-glucopyranoside (*o*-NPG) and *p*-
96 nitrophenyl-α-glucopyranoside (*p*-NPG) standards were obtained from Sigma-Aldrich
97 (St Louis, MO). Lactose (*β*-D-Gal(1→4)-D-Glc) was obtained from ACROS organics
98 (Geel, Belgium) and fructose was obtained from Fluka analytical (St. Gallen,
99 Switzerland). All standard carbohydrates were of analytical grade (purity ≥ 95%).

100 *Kluyveromyces marxianus* cells were kindly provided by Professor Robert Rastall from
101 The University of Reading (United Kingdom). Nutritive medium (peptone, lactose and
102 yeast extract) were supplied by Sigma-Aldrich.

103

104 ### *2.2 Small intestinal brush border membrane vesicles (BBMV) preparation*

105 Small intestinal brush border vesicles from six post-weaned pigs (7-10 months old)
106 were obtained following methodology previously reported.[26,22] Briefly, three pig small
107 intestines, from the duodenum to the ileum, were obtained from a local slaughterhouse
108 (Coca, Segovia, Spain). Immediately after sacrifice, the samples were kept at 4 ºC and
109 transferred to the laboratory in less than 2 h. The small intestines were rinsed with cold
110 phosphate buffered saline solution (PBS) (pH 7.3 – Oxoid; Basingstoke, UK), then slit

111 open and scrapped with a glass slide. The mucose scrapped was suspended (1:1, w/v) in
112 50 mM mannitol dissolved in PBS at 4 ºC, homogenized during 10 min using a Ultra-
113 Turrax® (IKA T18 Basic), adjusted with $CaCl_2$ to a final concentration of 10 mM and
114 centrifuged at 3,000 *g* during 30 min. The supernatant was centrifuged at 27,000 *g* during
115 40 min and the resulting pellet, containing the BBMV, was re-suspended in buffer maleate
116 (50 mM) pH 6.0 containing $CaCl_2$ (2 mM) and sodium azide (0.02%). Samples were
117 lyophilized and kept at -80ºC.

118

119 *2.3 Prebiotic oligosaccharides*

120 OsLu were obtained at pilot plant scale by Innaves S.A. (Vigo, Spain) following the
121 method described by López-Sanz et al. (2015).[27] Briefly, OsLu were synthesized using a
122 commercial lactulose preparation (670 g/L; Duphalac, Abbott Biologicals B.V., Olst, The
123 Netherlands), and a commercial preparation including *β*-galactosidase from *Aspergillus*
124 *oryzae* (16 U/mL; Sigma) at pH 6.5, 50 ºC and 350 rpm during 24 h. In addition, three
125 different commercially available GOS mixtures with predominant *β*(1→3) linkages GOS
126 (named GOS-1), predominant *β*(1→4) linkages GOS (named GOS-2) and predominant
127 *β*(1→6) GOS (named GOS-3), were tested.

128 2.3.1 Prebiotic oligosaccharides purification

129 Presence of low molecular weight of high glycaemic index is common in this type of
130 oligosaccharide mixture. Purification of prebiotic compounds was carried out by yeast
131 treatment with *K. marxianus*.

132 *K. marxianus* cells were growth in YPD (1 % (w/v) yeast extract, 2 % peptone and 2
133 % lactose) (500 mL) at 37 ºC during 48 h. Samples were then centrifuged at 4,000 *g* for
134 10 min and washed three times on PBS (500 mL), supernatant was discarded, and washed

135 samples were taken to incubation. Twenty-five mL of prebiotic ingredients (10% in PBS)
136 and *K. marxianus* yeast (equivalent to 25 mL YPD) were incubated at 37 ºC for 48 h.
137 Samples were then centrifuged at 4,000 x*g* for 20 min, filtered by 0.2 µm and then
138 lyophilized and kept at -20ºC until analysis. Purification process was carried out three
139 times for each sample (n=3) and monitored by GC-FID as explained below. GOS-1
140 mixture after yeast treatment was mainly constituted by 10% monosaccharides, 34.2%
141 lactose, 22.4% disaccharides and 32.4% trisaccharides (w:w). GOS-2 composition was
142 0.6% monosaccharides, 1.8% lactose, 4.1% disaccharides, 77.1% trisaccharides and
143 16.4% tetrasaccharides (w:w). GOS-3 composition was 1.8% monosaccharides, 25.1%
144 lactose, 26.8% disaccharides and 46.3% trisaccharides (w:w). OsLu was constituted by
145 7.8% monosaccharides, 49.3% lactulose 28.8% disaccharides and 14.1% trisaccharides
146 (w:w).

147 *2.4 Small Intestinal BBMV characterization*

148 Pig small intestinal BBMV (10 mg/mL) was homogenized in ice-cold 0.05 M sodium
149 phosphate buffer solution and then centrifuged at 6,000 x*g* for 15 min. Supernatant was
150 used as enzyme solution for determining protein content and enzymatic activity.

151 *2.4.1 Protein content determination*

152 Total protein content of the pig small intestinal BBMV was quantified according
153 to the Bradford method[28], using the Bio-Rad Protein Assay kit and bovine serum albumin
154 as a standard. The absorbance was monitored at 595 nm.

155 *2.4.2 Hydrolytic activities*

156 *2.4.2.1 β-galactosidase and maltase activities*

157 The determination of the pig intestinal *β*-galactosidase activity was adapted from
158 Warmerdam et al. (2014).[29] Briefly, a solution of *o*-NPG (0.5 mg/mL) in phosphate buffer

159 0.05 M, pH 7.0 was prepared. The enzymatic activity was determined by incubating 1,900
160 µL of the *o*-NPG solution and 100 µL of enzyme solution from BBMV for 2 h at 37 °C.
161 The method is based on the measurement of the continuous release of *o*-NP from *o*-NPG.
162 The absorbance of released *o*-NP was measured at 420 nm every 30 s using a
163 spectrophotometer (Specord Plus, Analytik Jena) together with a temperature controller
164 (Jumo dTRON 308, Jumo Instrument Co.). The specific enzymatic activity (U) was
165 expressed in µmol min^{-1} g^{-1}, where one unit was defined as the amount of enzyme that
166 produced 1 µmol of *o*-NP in one min of reaction (n = 3). Similar procedure was used to
167 determine the maltase activity by using a solution of *p*-NPG in phosphate buffer 0.05 M,
168 pH 6.8 (0.05% w/w) and monitoring the release of *p*-NP at 420 nm every 20 s (n = 3).

169 *2.4.2.2 Sucrose and trehalase activities*

170 Sucrase and trehalase activities were determined following a method described in
171 a previous work.[23] A solution of sucrose or trehalose (0.5% w/v) in sodium phosphate
172 buffer 0.05 M, pH 6.5 was used. An eppendorf tube with 500 µL of sucrose or trehalose
173 solution was preheated at the reaction temperature, 37 °C. Subsequently, 200 µL of
174 enzyme solution was added and the mixture was incubated for 2 h and different aliquots
175 were taken at different times (5, 10, 15, 30, 60, 90 and 120 min). Hydrolysis was stopped
176 by adding 700 µL of a 3,5- dinitrosalicylic acid (DNS) solution. Sucrase and trehalase
177 activity were determined measuring the reducing sugars released from the corresponding
178 disaccharide hydrolysis at 540 nm, according to the DNS method.[30] The specific
179 enzymatic activity (U) was expressed in µmol min^{-1} g^{-1}, where one unit was defined as
180 the amount of enzyme that produced 1 µmol of reducing sugars in one min of reaction (n
181 = 3).

182

183 *2.5 In vitro digestion of prebiotic compounds with BBMV*

184 The digestibility of three different types of GOS, OsLu and lactose and lactulose was
185 evaluated using BBMV.

186 First, a solution of BBMV (10 mg/mL) in PBS solution, 6.8 pH, was prepared. Then,
187 prebiotic or disaccharides samples were added at a concentration of 0.2 mg/mL and
188 digestion was carried out at 37 ºC during 5 h using 750 rpm in an orbital Thermomixer
189 comfort (Eppendorf®). Aliquots were taken at 0, 1, 2, 3, 4 and 5 h of digestion and
190 immediately heated in boiling water for 5 min to stop the reaction.

191 Furthermore, incubation of BBMV without any carbohydrate source was also analyzed.
192 Results showed quantifiable amounts of glucose as the digestion proceeded. These values
193 were conveniently withdrawn to avoid any overestimation of the monosaccharide
194 fraction.

195 *2.6 Carbohydrates quantification by GC-FID*

196 Carbohydrates present in the samples and digested mixtures were analysed as
197 trimethylsilylated oximes (TMSO) by gas chromatography coupled to ionization flame
198 detector (GC-FID) following the method of Brobst & Lott Jr, (1966).[31] First, 500 μL of
199 samples (0.1 mg carbohydrates) was added to 500 μL of phenyl-*β*-glucoside (Internal
200 Standard, IS) and the mixture was dried in a rotary evaporator (Büchi Labortechnik AG,
201 Flawil, Switzerland). TMSO derivatives were formed by adding 250 μL of
202 hydroxylamine chloride in pyridine (2.5% w/v) and heating the mixture at 70 ºC for 30
203 min, followed by the addition of hexamethyldisilazane (250 μL) and trifluoroacetic acid
204 (25 μL) and incubated at 50 ºC for 30 min. Mixtures were centrifuged at 6,700 *g* for 2
205 min and supernatants were injected in the GC-FID.

206 TMSO derivatives were separated using a fused silica capillary column DB-5HT (5%-
207 phenyl-methylpolysiloxane; 30m x 0.25mm x 0.10μm, Agilent). Nitrogen at 1 mL/min
208 was used as carrier gas. Injector and detector temperatures were set at 280 and 385 ºC,
209 respectively. The oven temperature was set from 150 ºC to 380 ºC at a ratio of 3 ºC/min.
210 Data acquisition and integration were done using Agilent ChemStation software
211 (Wilmington, DE, USA). Response factors were calculated after duplicate analysis of
212 standard solutions (fructose, glucose, galactose, lactose, lactulose and raffinose) over the
213 expected concentration range in samples, (0.005–1 mg) and IS (0.25 mg).

214

215 *2.7 Statistical Analysis*

216 Statistical analysis was carried out using SPSS for Windows, version 23.0. One-way
217 analysis of variance (ANOVA) and Tukey's *post hoc* test was used to determine
218 significant differences ($p < 0.05$) between concentrations of carbohydrates in each
219 prebiotic sample (n=3).

3. Results and Discussion

Monosaccharides are the major impurities in GOS obtainment, therefore, removal of these compounds is recommended due to, mainly, their undesirable caloric value.[16] Furthermore, inhibition of *β*-galactosidase by glucose and galactose in transgalactosylation and hydrolysis reaction of carbohydrates was reported.[32] Among the different purification strategies to remove these compounds, selective fermentation with *K. marxianus* has been proposed as a sound technology being amenable for scale-up at industrial level.[33,34]

3.1 BBMV enzymatic characterization

The brush border of the mammalian intestinal mucosa contains several key enzymes present as multienzyme complexes, i.e. sucrase-isomaltase, lactase-phlorizin hydrolase, maltase-glucoamylase and trehalase.[35] Accordingly, it is well reported the presence of those carbohydrases in the brush border of the intestinal mucosa of pig.[36,22,37] **Table 1** shows the protein content and main enzymatic activities (*β*-galactosidase, maltase, sucrase and trehalase) of BBMV measured under the assayed digestion conditions. Maltase activity (753.1 U/g) was the highest with ten-fold higher values than the other measured activities. Likewise, *β*-galactosidase (70.1 ± 1.4 U/g) showed the second highest activity in the substrate whereas trehalase (21.4 ± 7.6 U/g) and sucrase (19.9 ± 2.2 U/g) presented statistically similar values. To date, some studies have characterized the carbohydrase activities of small intestinal enzymes in pigs,[38,37,39,22] showing a clear predominance of maltase activity as compared to other activities, which agrees with the data obtained in this work.

3.2 Digestion of prebiotic carbohydrates by BBMV

Figure 1 shows GC-FID profiles of oligosaccharides before and after 5h of digestion with BBMV. Differences were observed between the three GOS mixtures profiles, 1,4-galactobiose (*β*-Gal-(1→4)-Gal) and 1,6-galactobiose (*β*-Gal-(1→6)-Gal) were identified as peaks 2 and 5, respectively in all samples. (*β*-Gal-(1→3)-Glc) and allolactose (*β*-Gal-(1→6)-Glc) isomers of lactose were also detected in all samples as peak 3 and peak 4, respectively. Further structural differences were found in the trisaccharides fraction. *β*-1,4-galactosyl-lactose (*β*-Gal-(1→4)-*β*-Gal-(1→4)-Glc, peak 6) was detected in all samples, *β*-1,6-galactosyl-lactose (*β*-Gal-(1→6)-*β*-Gal-(1→4)-Glc, peak 8) was detected in GOS-2 and GOS-3 samples and *β*-1,3-galactosyl-lactose (*β*-Gal-(1→3)-*β*-Gal-(1→4)-Glc, peak 7) was only detected in GOS-1 mixture. Tetrasaccharides were also detected in GOS-2 mixture (data not shown) and this fraction was mainly constituted by *β*-Gal-(1→4)-*β*-Gal-(1→4)-*β*-Gal-(1→4)-Glc and other tetrasaccharides not identified in this work.[40,11,41]

OsLu mixture was constituted by *β*(1→6) as the main glycosidic linkage and mostly by galactosyl galactoses (Gal-Gal) and galactosyl fructoses (Gal-Fru). *β*-(1→6)-galactosyl-lactulose (*β*-Gal-(1→6)-*β*-Gal-(1→4)-Fru) was identified as the main trisaccharide in the sample. In general, all assessed GOS and OsLu showed a diminution after the BBMV digestion, although considerable differences among all studied samples were observed. .

Tables 2 and 3 show the quantitative determination of individual carbohydrates in GOS and OsLu during digestion, respectively. A progressive increase in the level of monosaccharides was found in all samples as digestion proceeded, which was concomitant with the decrease in di- and trisaccharide fractions. Digestion of standard

267 solutions of lactose or lactulose with BBMV is also shown for comparative purposes. As
268 expected, lactose was much more prone to degradation than lactulose due to the presence
269 of fructose instead of glucose in the *β*-linkage of the latter.[42] Lactose degradation in GOS
270 samples was remarkably lower (50 - 68 %) when compared to the standard solution (97
271 %) (**Table 1S, Supplementary Information)**, probably due to the fact that the
272 degradation of particular GOS trisaccharides or tetrasaccharides could revert released
273 lactose, as well as to the presence of other carbohydrates in the GOS mixtures which
274 might mitigate the straightforward digestion of lactose when is present alone. Regarding
275 lactulose digestion, the standard solution showed a slight lower hydrolysis than that
276 observed for lactulose present in OsLu (29.5 and 32.8 %, respectively, after 5 h of
277 digestion). Similar behaviour was obtained in a previous work comparing the digestibility
278 of prebiotics added to milk in an *in vitro* study with a rat extract.[20]

279 Concerning disaccharides degradation, *β*-Gal-(1→3)-Glc and *β*-Gal-(1→6)-Glc
280 (allolactose) exhibited a slight decrease in their content after the BBMV digestion.
281 Allolactose (*β*(1→6)) was the most resistant to hydrolysis when compared to lactose
282 (*β*(1→4)) and *β*(1→3) structures. In this regard, it has been previously reported the high
283 resistance of allolactose to intestinal mucosa with less than 5% of hydrolysis compared
284 with lactose in an *in vitro* human assay[43] and in an *in vivo* study with rats.[14] Concerning
285 galactosyl galactoses, none of these carbohydrates provided any noticeable change,
286 indicating their stability during the digestion with BBMV. Indeed, an increase of these
287 compounds was found in some samples. Concretely, GOS-2 mixture showed an increase
288 of 4' and 6'-galactosyl galactose, respectively, suggesting the possible breakdown of the
289 *β*(1→4) linkage of the terminal glucose in their trisaccharide fraction. Regarding OsLu
290 disaccharides, high resistance of galactosyl galactoses was also observed. Scarce

hydrolysis of galactosyl-fructoses was found, with β(1→6)-galactosyl-fructose linkages as the lowest decrease among all determined disaccharides (**Table 3**). According to Hernandez-Hernandez et al.[14] it is plausible that, in a similar way to lactulose, other galactosyl-fructoses can be highly resistant to digestion within the mammalian small intestinal system. In line with our results, Julio-Gonzalez et al. (2019)[44] have recently reported the potential higher resistance to mammalian digestion of galactosyl-galactoses than galactosyl-glucoses.

Regarding trisaccharides fraction, **Table 2** data shows that β(1→3)-galactosyl-lactose in GOS-1 exhibited a higher hydrolysis than β(1→4)-galactosyl-lactose in GOS-2 and β(1→6)-galactosyl-lactose in GOS-3. However, to get more insight in the effect on linkage on trisaccharides fraction, **Table 4** shows the hydrolysis degree of each different linkage trisaccharide present in all samples. In addition, the slope of the representation of hydrolysis degree (%) vs time (h), which could be considered as the hydrolysis rate, can also be seen. By considering a standard intestinal digestion time of 2 h, the hydrolysis degree of trisaccharides showed β(1→3)-galactosyl-lactose (hydrolysis rate of 21.9% as determined in GOS-1) to be more prone to degradation by intestinal enzymes followed by β(1→4)-galactosyl-lactose (7.8-17.4%), whereas β(1→6)-galactosyl-lactose (5.0-7.1%) and β(1→6)-galactosyl-lactulose (4.9%) showed the highest resistance to hydrolysis.

Concerning oligosaccharides as a whole (that is, the sum of di, tri and tetrasaccharides), in general GOS-3 and OsLu demonstrated to be the most resistant to intestinal degradation (**Figure 2, Table 1S, Supplementary Information)**, where the presence of fructose at the reducing end of molecules provides OsLu a slight better resistance to digestion with 22.8 % against 27.1 % of hydrolysis for GOS-3 after 5 h

(**Figure 2C**). Furthermore, hydrolysis rate for GOS-3 and OsLu (**Table 5**) showed a lower degradation for OsLu as compared to GOS-3 after 2 and 5 h of digestion. GOS-2 oligosaccharides mixture was slightly more prone to degradation with a higher hydrolysis rate after the BBMV digestion whereas GOS-1 oligosaccharides mixture exhibited the highest degree of hydrolysis with 50.1 % degradation and the highest hydrolysis rate after 2 h (12.3) and 5 h (9.6) of treatment with BBMV from pig small intestine as compared to the other samples.

In this sense, a recent work highlighted the utility of a similar BBMV from pig small intestine to produce prebiotic GOS, and have revealed that BBMV preferably synthesizes GOS linked by $\beta(1\rightarrow3)$ bonds, finding β-Gal-(1→3)-β-Gal-(1→4)-Glc as the main trisaccharide after comprehensive NMR analysis.[44] This study also pointed out no presence of β-Gal-(1→6)-β-Gal-(1→4)-Glc, whereas the β-Gal-(1→4)-β-Gal-(1→4)-Glc trisaccharide was present but only at trace amounts. These findings support the data obtained in the current work since the most abundant glycosidic linkages, formed when mammalian intestinal β-galactosidase act as transgalactosidase, are expected to be preferentially broken under hydrolytic conditions.

In the other hand, regarding monosaccharides release, galactose amounts were higher compared to glucose release, in accordance to the composition of the main oligosaccharides in the samples. **Table 2** showed that the highest hydrolysis of GOS-1 oligosaccharides produced a higher release of total monosaccharides (62 mg/100 mg of total carbohydrates) after 5 h of digestion as compared to GOS-2, GOS-3 and OsLu (34.6, 38.9 and 33.8 mg/100 mg total carbohydrates, respectively). In this sense, the highest resistance of galactobioses and galactosyl-fructoses could affect positively to regulate the caloric intake and diminish the possible absorption of free monosaccharides in the small

intestine, highlighting the key role of the monomer composition and type of glycosidic linkage in prebiotic oligosaccharide samples.

In this sense, results obtained in this work have demonstrated that the use of small intestinal BBMV from pig is a reliable and useful strategy to evaluate prebiotic carbohydrate digestibility. Intestinal *in vitro* digestion with BBMV revealed the degradation of recognized prebiotics such as lactulose, different mixtures of GOS and an emerging prebiotic OsLu at considerably dissimilar levels. Our findings have revealed a stronger resistance of $\beta(1\rightarrow6)$ linkages oligosaccharides to *in vitro* digestion when compared to $\beta(1\rightarrow4)$ and $\beta(1\rightarrow3)$ linkages GOS. In general, $\beta(1\rightarrow3)$ followed by $\beta(1\rightarrow4)$ linkages were more prone to small intestinal degradation using BBMV. This less resistance to intestinal digestion was also found for galactosyl-glucose disaccharides as compared to galactosyl-galactoses (galactobioses). The key role of monomer composition was also underlined by the presence of fructose in OsLu mixture, providing, thus, a higher resistance to digestion of galactosyl-fructoses. Findings described in this work could be extrapolated to humans providing evidence on the structure-function relationship, as well as an increase on the knowledge of the different resistance of β-linkages for the sake of a future potential development of new tailored prebiotics. Moreover, the observed hydrolysis with mammalian small intestinal enzymes of recognized prebiotics could challenge the general belief that these compounds reach the colon without any alterations in their structure. More investigation should be done in order to gain more insight in the concept of prebiotics' digestibility.

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Abbreviations used

GOS, Galactooligosaccharides

OsLu, Oligosaccharides derived from lactulose

BBMV, Brush Border Membrane Vesicles

DP, Degree of Polymerization

References

1. Heintz-Buschart, A., & Wilmes, P. (2018). Human Gut Microbiome: Function Matters. *Trends in Microbiology*, *26*(7), 563–574.
2. Carabotti, M., Scirocco, A., Antonietta, M., & Severi, C. (2015). The gut-brain axis : interactions between enteric microbiota, central and enteric nervous systems. *Annals of Gastroenterology*, *28*(2), 203–209.
3. Forsythe, P., Bienenstock, J., & Kunze, W. A. (2014). Vagal Pathways for Microbiome-Brain-Gut Axis Communication. In M. Lyte and J. F. Cryan (eds.) *Microbial Endocrinology: The Microbiota-Gut-Brain Axis in Health and Disease, 817* (pp. 115-136) Springer New York 2014.
4. Sherwin, E., Sandhu, K. V., Dinan, T. G., & Cryan, J. F. (2016). May the Force Be With You: The Light and Dark Sides of the Microbiota–Gut–Brain Axis in Neuropsychiatry. *CNS Drugs*, *30*(11), 1019–1041.
5. Ibáñez, L., Rouleau, M., Wakkach, A., & Blin-Wakkach, C. (2018). Gut microbiome and bone. *Joint Bone Spine*, *In press.* https://doi.org/10.1016/j.jbspin.2018.02.008
6. Sozańska, B. (2018). Microbiome in the primary prevention of allergic diseases and bronchial asthma. *Allergologia et Immunopathologia*, In press. https://doi.org/10.1016/j.aller.2018.03.005
7. Moreno, F. J., Corzo, N., Montilla, A., Villamiel, M., & Olano, A. (2017). Current state and latest advances in the concept, production and functionality of prebiotic oligosaccharides. *Current Opinion in Food Science, 13*, 50–55.
8. Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K.

396 and Reid, G. (2017). Expert consensus document: The International Scientific
397 Association for Probiotics and Prebiotics (ISAPP) consensus statement on the
398 definition and scope of prebiotics. *Nature Reviews Gastroenterology and*
399 *Hepatology*, *14*(8), 491–502.
400 9. Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R.,
401 Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F.,
402 Respondek, F., Whelan, K., Coxam, V., Davicco, MJ., Léotoing, L., Wittrant, Y.,
403 Delzenne, NM., Cani, PD., Neyrinck, AM., and Meheust, A. (2010). Prebiotic
404 effects: metabolic and health benefits. *British Journal of Nutrition*, *104*, S1-63.
405 10. Sangwan, V., Tomar, S. K., Singh, R. R. B., Singh, A. K., & Ali, B. (2011).
406 Galactooligosaccharides: Novel Components of Designer Foods. *Journal of Food*
407 *Science*, *76*(4). R103-11.
408 11. Moreno, F. J., Montilla, A., Villamiel, M., Corzo, N., & Olano, A. (2014).
409 Analysis, structural characterization, and bioactivity of oligosaccharides derived
410 from lactose. *Electrophoresis*, *35*(11), 1519–1534.
411 12. Gänzle, M. G. (2012). Enzymatic synthesis of galacto-oligosaccharides and other
412 lactose derivatives (hetero-oligosaccharides) from lactose. *International Dairy*
413 *Journal*, *22*(2), 116–122.
414 13. Torres, D. P. M., Gonçalves, M. do P. F., Teixeira, J. A., & Rodrigues, L. R.
415 (2010). Galacto□oligosaccharides: production, properties, applications, and
416 significance as prebiotics. *Comprehensive Reviews in Food Science and Food*
417 *Safety*, *9*(5), 438–454.
418 14. Hernández-Hernández, O., Marin-Manzano, M. C., Rubio, L. A., Moreno, F. J.,
419 Sanz, M. L., & Clemente, A. (2012). Monomer and linkage type of galacto-

oligosaccharides affect their resistance to ileal digestion and prebiotic properties in rats. *The Journal of Nutrition*, *142*(7), 1232–1239.

15. Díez-Municio, M., Herrero, M., Olano, A., & Moreno, F. J. (2014). Synthesis of novel bioactive lactose-derived oligosaccharides by microbial glycoside hydrolases. *Microbial Biotechnology, 7(4)*, 315–331.

16. Gosling, A., Stevens, G. W., Barber, A. R., Kentish, S. E., & Gras, S. L. (2010). Recent advances refining galactooligosaccharide production from lactose. *Food Chemistry*, *121*(2), 307–318.

17. Drechsler, K. C., & Bornhorst, G. M. (2018). Modeling the softening of carbohydrate-based foods during simulated gastric digestion. *Journal of Food Engineering*, *222*, 38–48.

18. Egger, L., Ménard, O., Delgado-andrade, C., Alvito, P., Assunção, R., Balance, S., Barberá, R., Brodkorb, A., Cattenoz, T., Clemente, A., Comi, I., Dupont, D., Garcia-llatas, G., Lagarda M.J., Feunteun S.L., Janssenduijghuijsen, L., Karakaya, S., Lesmes, U., Mackie, A.R., Martins, C., Meynier, A., Miralles, B., Murray, B.S., Pihlanto, A., Picariello, G., Santos, C.N., Simsek, S., Recio, I., Rigby, N., Rioux, L.E., Stoffers, H., Tavares, A., Tavares, L., Turgeon, S., Ulleberg, E.K., Vegarud, G.E., Vergères, G. and Portmann, R. (2016). The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. *Food Research International*, 88, 217–225.

19. McCleary, Barry V, Jeanne Rader, Martine Champ, K. O. (2010). Determination of total dietary fiber (CODEX definition) by Enzymatic-Gravimetric method and liquid chromatography : Collaborative Study. *Journal of AOAC International*, 93(1), 221-233.

20. Ferreira-Lazarte, A., Montilla, A., Mulet-Cabero, A.-I., Rigby, N., Olano, A., Mackie, A., & Villamiel, M. (2017). Study on the digestion of milk with prebiotic carbohydrates in a simulated gastrointestinal model. *Journal of Functional Foods*, *33*, 149–154.

21. Strube, M. L., Jensen, T. K., Meyer, A. S., & Boye, M. (2015). In situ prebiotics: enzymatic release of galacto-rhamnogalacturonan from potato pulp in vivo in the gastrointestinal tract of the weaning piglet. *AMB Express*, *5*(1), 1–8.

22. Tanabe, K., Nakamura, S., Omagari, K., & Oku, T. (2015). Determination Trial of Nondigestible Oligosaccharide in Processed Foods by Improved AOAC Method 2009.01 Using Porcine Small Intestinal Enzyme. *Journal of Agricultural and Food Chemistry*, *63*(24), 5747–5752.

23. Ferreira-Lazarte, A., Olano, A., Villamiel, M., & Moreno, F. J. (2017). Assessment of *in Vitro* Digestibility of Dietary Carbohydrates Using Rat Small Intestinal Extract. *Journal of Agricultural and Food Chemistry*, *65*(36), 8046–8053.

24. Jantscher-Krenn, E., Marx, C., & Bode, L. (2013). Human milk oligosaccharides are differentially metabolised in neonatal rats. *British Journal of Nutrition*, *110*(4), 640–650.

25. Marín-Manzano, M. C., Abecia, L., Hernández-Hernández, O., Sanz, M. L., Montilla, A., Olano, A., Rubio L. A., Moreno, F. L. & Clemente, A. (2013). Galacto-oligosaccharides derived from lactulose exert a selective stimulation on the growth of *Bifidobacterium animalis* in the large intestine of growing rats. *Journal of Agricultural and Food Chemistry, 61(31)*, 7560–7567.

26. Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M., & Semenza, G. (1978). A Modified procedure for the rapid preparation of efficiently transporting vesicles

from small intestinal brush border membranes. *Biochimica et Biophysica Acta*, *506*, 136–154.

27. López-Sanz, S., Montilla, A., Moreno, F. J., & Villamiel, M. (2015). Stability of oligosaccharides derived from lactulose during the processing of milk and apple juice. *Food Chemistry*, *183*, 64–71.

28. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1), 248–254.

29. Warmerdam, A., Zisopoulos, F. K., Boom, R. M., & Janssen, A. E. M. (2014). Kinetic characterization of galacto-oligosaccharide (GOS) synthesis by three commercially important β-galactosidases. *Biotechnology Progress*, *30*(1), 38–47.

30. Miller, G. L. (1959). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry*, *31*(3), 426–428.

31. Brobst, K. M., & Lott Jr, C. E. (1966). Determination of some components in corn syrup by gas-liquid chromatography of the trimethylsilyl derivatives. *Cereal Chemistry*, *43*, 35–43.

32. Vera, C., Guerrero, C., & Illanes, A. (2011). Determination of the transgalactosylation activity of *Aspergillus oryzae* β-galactosidase: effect of pH, temperature, and galactose and glucose concentrations. Carbohydrate Research, 346(6), 745–752.

33. Guerrero, C., Vera, C., Novoa, C., Dumont, J., Acevedo, F., & Illanes, A. (2014). Purification of highly concentrated galacto-oligosaccharide preparations by selective fermentation with yeasts. *International Dairy Journal*, *39*(1), 78–88.

34. Cheng, C. C., Yu, M. C., Cheng, T. C., Sheu, D. C., Duan, K. J., & Tai, W. L. (2006). Production of high-content galacto-oligosaccharide by enzyme catalysis

494 and fermentation with Kluyveromyces marxianus. Biotechnology Letters, 28(11),
495 793–797.
496 35. Feher, J. (2012). Digestion and Absorption of the Macronutrients (Ch. 5). In J.
497 Feher (Ed.), Quantitative Human Physiology (pp. 731–743). Boston: Academic
498 Press.
499 36. Kidder, D. E., & Manners, M. J. (1978). Digestion in the pig. Bristol, UK:
500 Scientechnica.
501 37. Rubio, L. A., Ruiz, R., Peinado, M. J., & Echavarri, A. (2014). Morphology and
502 enzymatic activity of the small intestinal mucosa of Iberian pigs as compared with
503 a lean pig strain, *Journal of Animal Science, 88*, 3590–3597.
504 38. Gnoth, M. J., Kunz, C., Kinne-Saffran, E., & Rudloff, S. (2000). Human milk
505 oligosaccharides are minimally digested in vitro. *The Journal of Nutrition*,
506 *130*(12), 3014–20.
507 39. Sørensen, S. H., Norén, O., Sjöström, H., & Danielsen, E. M. (1982). Amphiphilic
508 Pig Intestinal Microvillus Maltase/Glucoamylase Structure and Specificity.
509 *European Jouranl of Biochemistry*, *126*, 559–568.
510 40. Hernández-Hernández, O., Montañés, F., Clemente, A., Moreno, F. J., & Sanz,
511 M. L. (2011). Characterization of galactooligosaccharides derived from lactulose.
512 *Journal of Chromatography A*, *1218*(42), 7691–7696.
513 41. Ruiz-Matute, A. I., Corzo-Martínez, M., Montilla, A., Olano, A., Copovi, P., &
514 Corzo, N. (2012). Presence of mono-, di- and galactooligosaccharides in
515 commercial lactose-free UHT dairy products. *Journal of Food Composition and*
516 *Analysis*, *28*(2), 164–169.
517 42. Olano, A., & Corzo, N. (2009). Lactulose as a food ingredient. *Journal of the*
518 *Science of Food and Agriculture*, *89*(12), 1987–1990.

519 43. Burvall, A., Asp, N.-G., & Dahlqvist, A. (1980). Oligosaccharide formation
520 during hydrolysis of lactose with Saccharo myces lactis lactase (Maxilact®)--Part
521 3: Digestibility by human intestinal enzymes in vitro. *Food Chemistry, 5,* 189-
522 194.
523 44. Julio-González L.C., Hernandez-Hernandez O., Moreno F.J., Olano A., Jimeno
524 M.L., and Corzo N. (2019). Trans-β-galactosidase activity of pig enzymes
525 embedded in the small intestinal brush border membrane vesicles. Scientific
526 Reports, *In press*. DOI: 10.1038/s41598-018-37582-8
527
528