Study on the digestion of milk with prebiotic carbohydrates in a
simulated gastrointestinal model

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Abstract

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

Keywords: lactulose oligosaccharides, prebiotics, digestion, milk, galacto-oligosaccharides
1. Introduction

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

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

One of the requirements for oligosaccharides to be considered as prebiotics is their resistance to digestion in the upper gastrointestinal tract. The susceptibility of prebiotic oligosaccharides to hydrolysis during their passage through the gastrointestinal tract is largely affected by the chemical structure and can impact their final state when they reach the colon to be fermented by the microbiota. Ohtsuka et al. (1990) found that the trisaccharide 4’-galactosyl-lactose was hardly digested in vitro with a homogenate of intestinal mucosa of rats. According to Torres et al. (2010), more than 90% of GOS are stable to digestive enzymes and can reach the colon to exert their positive effect.
Carbohydrate analysis before and after exposure to certain protocols of *in vitro* digestion have shown that xylo-oligosaccharides, palatinose condensates, commercial GOS and lactulose were very resistant to hydrolysis. In contrast, lactosucrose, gentio-oligosaccharides, soybean oligosaccharides, fructo-oligosaccharide and inulin were slightly hydrolysed under such conditions (Playne and Crittenden, 2009).

To our knowledge, limited studies have been carried out on the digestibility of OsLu. Hernandez-Hernandez et al. (2012) pointed out in *in vivo* assays a higher resistance of OsLu compared to GOS during gastrointestinal digestion. This was ascribed to the presence of fructose in β(1→4) linkage with galactose at the reducing end of the OsLu molecules. However, there is a lack of studies on the susceptibility of OsLu to the gastrointestinal digestion when they are added in a food matrix and the impact of these compounds on the digestion of other food components. These considerations are important since standards would be more prone to changes as they are not protected in a food medium. Establishing the digestibility of prebiotic carbohydrates is of great practical application, since this influence the final dose of substrate that reaches the distal portions of gut to exert its prebiotic effect. Thus, the aim of this work has been to study the effect of the OsLu inclusion in milk on the digestion of proteins and the changes in the carbohydrate fraction using standardised *in vitro* digestive conditions with a more physiological relevant gastric digestion approach. A subsequent treatment with a rat small intestine extract has been included to study the effect of intestinal enzymes from mammals. The commercial prebiotics GOS and Duphalac® were also employed for comparison purposes.

2. **Materials and methods**

2.1. **Chemicals and reagents**
Galactose, D-glucose, fructose, lactose, lactulose, raffinose, stachyose, phenyl-β-glucoside and Intestinal acetone powders from rat (rat intestine extract) from Sigma-Aldrich chemical Company (St Louis, MO).

2.2. Obtainment of prebiotic ingredients

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

2.3. Milk samples

Skim Milk Powder (low-heat organic, protein 42.34%, fat 0.89%, lactose 49.8% (w/w) (SMP) was kindly provided by Fonterra NZ. The SMP was reconstituted at 10% with distilled water and, subsequently, lactulose (Duphalac®), GOS or OsLu were added at 5% (w/w), taking into account previous recommendations for prebiotic doses (3.3 g of prebiotic carbohydrates/100 mL) (Walton et al., 2012; Whisner et al., 2013; Lopez-
Sanz et al., 2015). The samples were labeled as SMP+Duphalac®, SMP+GOS and SMP+OsLu and were kept refrigerated until subsequent assays.

2.4. In vitro gastrointestinal digestion

The solutions (see Figure 1) used for the simulation of the oral and gastric phases were based on the standardized static digestion protocol Infogest (Minekus et al., 2014). 5 mL of sample was placed into a 70 mL glass v-form vessel thermostated at 37 °C. To simulate the oral phase, 4 mL of Simulated Salivary Fluid (SSF, Table 1S, Verhoeckx et al., 2015), 25 µL 0.3 M CaCl₂(H₂O) and 0.975 mL Milli-Q water were added and mixed for approximately 2 min using a 3D action shaker (Mini-gyro rocker-SSM3-Stuart, Barloworld Scientific limited, UK) at 35 rpm. The simulation of the gastric phase was conducted using a semi-dynamic model described by Mulet-Cabero et al., (2017). The gastric fluids and enzyme solution were added gradually. Two solutions were added at a constant rate for 2 h: (1) 9 mL of a mixture consisted of 88.9% Simulated Gastric Fluid (SGF), 0.06% 0.3 M CaCl₂(H₂O), 4.4% Milli-Q water and 6.7% 2 M HCl was added using the dosing device of an autotitrator (836 Titrando-Metrohm, Switzerland) and (2) 1 mL of pepsin (3,214 U/mg solid, using haemoglobin as substrate) solution (in water) was added to reach the protease activity of 2,000 U/mL in the final digestion mixture. This enzyme solution was added using a syringe pump (Harvard apparatus, PHD ultra, USA). The system was agitated using the 3D action shaker at 35 rpm during the digestion time.

The pH was recorded throughout the procedure. Samples (0.5 mL) were taken after 0, 1 and 2 h of digestion and the pepsin activity was stopped with 100 µL of 1 M NaHCO₃ for a subsequent analysis of the protein fraction and the rest of the sample with 150 µL of 5 M NaOH for the following intestinal digestion. This last sample was
labelled as GPhase sample. After gastric digestion two different procedures for small intestinal digestion were carried out:

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

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

2.5. Protein determination

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

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

2.7. Statistical analysis

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

3. Results and discussion

3.1. Effect on protein digestion

Figure 1S (complementary material) shows the pH profile of the different samples of SMP with the addition of prebiotic ingredients (Table 2S, carbohydrate composition analysed by GC-FID) during their digestion in the semi-dynamic gastric model. The initial pH values were close to 7 in all cases and gradually decreased to 1.8 at the end of
the gastric digestion. In general, the profiles of the milk samples with prebiotic ingredients were similar to that of the SMP (no prebiotic ingredient added). The gradual lowering of pH enables the restructuring of the proteins due to acid induced coagulation to be simulated and is based on typical pH profiles measured *in vivo* (Malagelada et al. 1979).

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

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

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

Limited modifications were observed in the carbohydrate fraction following digestion using the Infogest protocol. In spite of the fact that there was a slight decrease of OS and trisaccharides in SMP+GOS after 2 h of digestion, these differences were not statistically significant. None of the carbohydrates derived from the prebiotic ingredients provided any significant change, indicating their stability during this enzymatic digestion by pancreatic fluids and bile salts. Moreover, it seems to be clear
that the presence of other milk components did not impact the passage of GOS, Duphalac® and OsLu throughout the gastrointestinal digestion evaluated by the Infogest protocol.

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

In general, lactose was more hydrolysed than lactulose due to the presence of fructose instead of glucose in the β linkage of the latter (Olaño and Corzo, 2009), being SMP+Duphalac® the sample with the highest degree of hydrolysis of lactose. In general, no significant differences ($p > 0.05$) were found for SMP samples with OsLu and GOS. Lactulose was significantly less susceptible to hydrolysis in SMP+Duphalac® than in SMP+OsLu. Furthermore, lactulose present in OsLu and Duphalac® was more prone to degradation than OS, probably ascribed to its lower Mw, although the difference was only significant after 1 h of digestion. Finally, OS were significantly more hydrolysed in SMP+GOS than in SMP+OsLu reaching values of 35% and 15%, respectively after 2 h;
this was probably due to the more stable β(1-6) linkages in the OsLu mixture as compared to β(1-4) in GOS and the presence of fructose at the terminal end of molecule (Hernandez-Hernandez et al. 2012). These results indicate that OS (DP≥3) present in OsLu were scarcely affected by the gastrointestinal digestion under the conditions used in the present work, being digested in a very low proportion in the small intestine which would favour the presence of a OS in the distal portions of colon to be fermented by beneficial bacteria.

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

Conclusions

According to the results obtained is possible to conclude that the presence of prebiotic carbohydrates in milk, at prebiotic doses, did not affect the gastric digestion of milk proteins, following the Infogest protocol. Similarly, under the same gastrointestinal digestion method, hardly any change was detected in the carbohydrate fraction of milk with GOS, Duphalac® and OsLu after 2 h of digestion. This might indicate the resistance of the three prebiotic mixtures, including OsLu, to gastric and pancreatic
fluids and bile salts. However, when the digested samples of milk with prebiotics were subjected to intestinal digestion by a small gut intestinal extract of rat a dissimilar behaviour in the three cases was observed, OsLu samples being the most resistant to the action of enzymes present in the rat intestine extract, mainly in the case of OS fraction. These results highlight the possibility of OsLu to reach the large intestine, target organ, to exert their potential prebiotic effects.

Acknowledgements

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1292-1303.
Figure caption

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

**Figure 2.** Electrophoretic profiles of milk protein fractions (caseins, β-Lg, α-La, BSA) before and after 2 h of digestion (Bis-Tris-Gel, Novex, NuPage). M: Marker, 1: SMP 0 h, 2: SMP 2 h, 3: SMP+OsLu 0 h, 4: SMP+OsLu 2 h, 5: SMP+ Duphalac 0 h, 6: SMP+Duphalac® 2 h, 7: SMP+GOS 0 h, 8: SMP + GOS 2 h, 9: blank

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

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

<table>
<thead>
<tr>
<th>Carbohydrate content (%)</th>
<th>Galactose</th>
<th>Glucose</th>
<th>Lactulose</th>
<th>Lactose</th>
<th>Disaccharides</th>
<th>Trisaccharides</th>
<th>Tetrasaccharides</th>
<th>Oligosaccharides*</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP</td>
<td>0h</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>N.D.</td>
<td>99.4 ± 0.2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>1h</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>N.D.</td>
<td>99.2 ± 0.1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>2h</td>
<td>0.3 ± 0.0</td>
<td>0.4 ± 0.2</td>
<td>N.D.</td>
<td>99.4 ± 0.2</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>SMP + GOS</td>
<td>0h</td>
<td>0.5 ± 0.1</td>
<td>7.6 ± 1.0</td>
<td>N.D.</td>
<td>65.6 ± 3.7</td>
<td>11.0 ± 0.8</td>
<td>12.9 ± 1.8</td>
<td>2.4 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>1h</td>
<td>0.5 ± 0.0</td>
<td>7.7 ± 1.5</td>
<td>N.D.</td>
<td>66.3 ± 3.3</td>
<td>12.0 ± 2.2</td>
<td>12.3 ± 1.4</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>2h</td>
<td>0.5 ± 0.0</td>
<td>6.9 ± 0.2</td>
<td>N.D.</td>
<td>68.4 ± 1.4</td>
<td>10.8 ± 1.3</td>
<td>10.9 ± 0.7</td>
<td>2.4 ± 1.7</td>
</tr>
<tr>
<td>SMP + Duphalac®</td>
<td>0h</td>
<td>3.6 ± 0.4</td>
<td>0.4 ± 0.4</td>
<td>22.0 ± 5.1</td>
<td>73.6 ± 4.9</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>1h</td>
<td>3.4 ± 0.8</td>
<td>0.2 ± 0.2</td>
<td>20.6 ± 1.1</td>
<td>76.5 ± 1.1</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>2h</td>
<td>3.1 ± 0.2</td>
<td>0.4 ± 0.2</td>
<td>21.6 ± 1.9</td>
<td>75.6 ± 1.7</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>SMP + OsLu</td>
<td>0h</td>
<td>5.0 ± 0.3</td>
<td>0.3 ± 0.1</td>
<td>6.3 ± 2.1</td>
<td>68.4 ± 1.7</td>
<td>9.8 ± 0.3</td>
<td>9.3 ± 0.2</td>
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<tr>
<td></td>
<td>1h</td>
<td>5.0 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>7.1 ± 1.4</td>
<td>67.4 ± 1.3</td>
<td>9.8 ± 0.4</td>
<td>9.5 ± 0.4</td>
<td>0.8 ± 0.3</td>
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<tr>
<td></td>
<td>2h</td>
<td>5.3 ± 0.3</td>
<td>0.3 ± 0.0</td>
<td>6.0 ± 0.4</td>
<td>69.0 ± 1.1</td>
<td>10.2 ± 0.5</td>
<td>8.6 ± 1.0</td>
<td>0.8 ± 0.6</td>
</tr>
</tbody>
</table>

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.**

**Gastric digestion**

Samples
- SMP (Skim Milk Powder)
- SMP + GOS
- SMP + OsLo

**Semidynamic gastric model conditions** (Minaeke 2014)
- 5 ml of samples
- 4 ml Simulated Salivary Fluid (SSF)
- 30 μl 0.3 M CaCl₂, 0.75M H₂O₂
- 4 ml Simulated Gastric Fluid (SGF)
- 1.37 ml milli-Q water
- 4.6 ml 2 M HCl (in SGF)
- 1 ml pepsin
- Incubated at 37 °C for 2 h

**Intestinal digestion**

Intestinal digestion (Minaeke 2014)
- 30 ml 1-GPase
- 11 ml Simulated Intestinal Fluid (SIF)
- 5 ml pancreatin solution 800 U/ml
- 2.5 ml fresh bile solution
- 40 ml 0.3 M CaCl₂
- 0.15 ml 1 M NaOH (to pH 7.0)
- 1.31 ml water, incubated at 37 °C for 2 h

**Unintended digestion (RSIE)**
- 5 mg 1-GPase
- 100 mg Rat Small Intestine Extract (RSIE)
- 1 ml distilled water
- Incubated at 37 °C for 2 h

**Steps**
- Sampling (0.5 mL)
  - 0-GPase (0 h)
  - 1-GPase (1 h)
  - 2-GPase (2 h)
- Stopping reaction with:
  - 100 μl NaHCO₃, 1 M for protein analysis
  - 150 μl NaOH 5 M for intestinal digestion

**Freeze-drying for analysis of carbohydrates**

**Steps**
- Sampling (5 mL)
  - 0-GPase (0 h)
  - 1-GPase (1 h)
  - 2-GPase (2 h)

**Steps**
- Stopping reaction in a boiling bath
- Centrifuging for carbohydrate analysis
Figure 2.
Figure 3.
Figure 4.
- 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)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>SSF (pH 7) /mmol/L</th>
</tr>
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<tbody>
<tr>
<td>K⁺</td>
<td>18.8</td>
</tr>
<tr>
<td>Na⁺</td>
<td>13.6</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>19.5</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>3.7</td>
</tr>
<tr>
<td>HCO₃⁻, CO₃²⁻</td>
<td>13.7</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.15</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>0.12</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.2</td>
</tr>
</tbody>
</table>

α-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®.

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

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.