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# Monomer and Linkage Type of Galacto-Oligosaccharides Affect Their Resistance to Ileal Digestion and Prebiotic Properties in Rats[1–3]

Oswaldo Hernández-Hernández,[4] M. Carmen Marín-Manzano,[5] Luis A. Rubio,[5] F. Javier Moreno,[6] M. Luz Sanz,[4] and Alfonso Clemente[5]*

[4]Instituto de Química Orgánica General, Consejo Superior de Investigaciones Científicas, Madrid, Spain; [5]Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas, Granada, Spain; and [6]Instituto de Investigación en Ciencias de la Alimentación, Campus de Cantoblanco, Universidad Autónoma de Madrid-Consejo Superior de Investigaciones Científicas, Madrid, Spain

## Abstract

A detailed study was performed to compare the in vivo ileal digestibility and modulatory effects in fecal microbiota of novel galacto-oligosaccharides (GOS) derived from lactulose [GOS-Lu; degree of polymerization (DP) ≥2, 14.0% trisaccharides] and commercial GOS derived from lactose (GOS-La; DP ≥3, 35.1% trisaccharides) in growing rats (5 wk old). Rats were fed either a control diet or diets containing 1% (wt:wt) of GOS-Lu or GOS-La for 14 d. Quantitative analysis of carbohydrates from dietary and ileal samples demonstrated that the trisaccharide fraction of GOS-Lu was significantly more resistant to gut digestion than that from GOS-La, as indicated by their ileal digestibility rates of 12.5 ± 2.6% and 52.9 ± 2.7%, respectively, whereas the disaccharide fraction of GOS-Lu was fully resistant to the extreme environment of the upper digestive tract. The low ileal digestibility of GOS-Lu was due to the great resistance of galactosyl-fructoses to mammalian digestive enzymes, highlighting the key role played by the monomer type and linkage involved in the oligosaccharide chain. The partial digestion of GOS-La trisaccharides showed that glycosidic linkages (1→6) and (1→2) between galactose and glucose monomers were significantly more resistant to in vivo gastrointestinal digestion than the linkage (1→4) between galactose units. The absence of GOS-La and GOS-Lu digestion-resistant oligosaccharides in fecal samples indicated that they were readily fermented within the large intestine, enabling both types of GOS to have a potential prebiotic function. Indeed, compared with controls, the GOS-Lu group had significantly more bifidobacteria in fecal samples after 14 d of treatment. The number of *Eubacterium rectale* also was greater in the GOS-Lu and GOS-La groups than in controls. These novel data support a direct relationship between patterns of resistance to digestion and prebiotic properties of GOS. J. Nutr. 142: 1232–1239, 2012.

## Introduction

The mammalian intestine harbors a complex microbial ecosystem consisting of an extraordinary number of resident commensal bacteria existing in homeostasis with the host (1). These endogenous microbiota establish a symbiotic, mutualistic relationship and affect numerous physiological functions, including nutrition exchange, control of epithelial cell proliferation/differentiation, pathogen exclusion, and stimulation of the immune system (2,3).

Currently, there is a growing interest in identifying functional dietary compounds capable of modulating the composition and metabolic activities of the intestinal microbiota. These compounds, named prebiotics, were recently redefined as "non-digestible functional ingredients which are selectively fermented and allow specific changes, both in the composition and/or activity of the gastrointestinal microflora that confers benefits upon host well-being and health" (4). Bifidobacteria and lactobacilli species are among the most relevant ones that are thought to play an important role in maintaining and promoting a healthy gut environment (5,6). The major prebiotic oligosaccharides on the market are fructan inulin, fructo-oligosaccharides, and galacto-oligosaccharides (GOS)[7] (7). GOS are nondigestible carbohydrates usually comprised of 2–10 molecules of galactose

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[3] Supplemental Table 1 and Figures 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at htpp://jn.nutrition.org.
* To whom correspondence should be addressed. E-mail: alfonso.clemente@eez.csic.es.

[7] Abbreviations used: DP, degree of polymerization; GOS, galacto-oligosaccharide; GOS-La, lactose-derived galacto-oligosaccharide; GOS-Lu, lactulose-derived galacto-oligosaccharide; $I^T$, retention indices; SI, selectivity index; TMSO, trimethylsilyloxime.



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and a terminal glucose unit, which are derived primarily from transgalactosylation reactions of lactose catalyzed by β-galactosidases of fungal, bacterial, or yeast origin, to result in oligosaccharides with different glycosidic linkages and degrees of polymerization (DP) (8). Recently, the synthesis and detailed chemical characterization of novel lactulose-derived GOS (GOS-Lu) was accomplished (9–11). GOS-Lu are attracting increasing attention due to their prospective prebiotic applications, in particular their ability to promote the in vitro growth of several probiotic strains of *Lactobacillus* and *Bifidobacterium* (12), as also shown for commercial lactose-derived GOS (GOS-La) (9,13).

To exert their potential prebiotic properties, oligosaccharides have to resist and survive, at least to some extent, the acidic environment and enzymatic digestion in the upper digestive tract. Thus, dietary oligosaccharides that escape digestion by endogenous enzymes and absorption in small intestine become available to microbial fermentation in the large intestine (14). Although a number of in vitro and in vivo studies have demonstrated the potential health benefits of GOS-La, there have been few attempts to identify and quantify the intestinal survival of GOS-La and their derivatives (4). Furthermore, the reported results are scarce and seem to be controversial mainly due to methodological difficulties (including complex analytical techniques, sample availability, and experimental models used). As a result, several authors have claimed that GOS-La could not fully meet the criterion of resistance to small intestinal digestion that is necessary to exert prebiotic properties within the large intestine (4,15,16). By using digestive enzymes from several sources, Chonan et al. (17) reported differences in digestibility behavior between disaccharides and trisaccharides of GOS-La, with the former being more susceptible to digestion. Ohtsuka et al. (18) showed that only a small amount of 4′-galactosyl-lactose, a major trisaccharide present in GOS-La, was digested by a homogenate of intestinal mucosa of rats. Additional studies to distinguish the differential digestibility of components within complex mixtures of oligosaccharides are clearly necessary.

Data are not available for the recently characterized GOS-Lu (11) with regard to resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption. The exploitation of these promising oligosaccharides as prebiotic compounds will depend largely upon their ileal survival rates after digestion and their further selective fermentation by microbiota in the large intestine. Therefore, the aims of this work were to carry out a comparative study in vivo regarding the ileal digestibility of GOS-La and GOS-Lu and to evaluate if their major components are fermented by the microbiota to promote the selective growth of beneficial bacteria in the large intestine of rats.

## Materials and Methods

*Materials.* Analytical standards of lactulose [β-Gal-(1→4)-Fru], lactose [β-Gal-(1→4)-Glc], sucrose [α-Glc-(1→2)-β-Fru], maltose [α-Glc-(1→4)-D-Glc], maltotriose [α-Glc-(1→4)-α-Glc-(1→4)-D-Glc], raffinose [α-Gal-(1→6)-α-Glc-(1→2)-β-Fru], α,α-threhalose [α-Glc-(1→1)-α-Glc], and β-galactosidase from *Aspergillus oryzae*, phenyl-β-D-glucoside, hexamethyldisilazane, and hydroxylamine hydrochloride were obtained from Sigma. 1,6-Galactobiose [β-Gal-(1→6)-Gal], 1,4-galactobiose [β-Gal-(1→4)-Gal], and 1,3-galactobiose [Gal-(1→3)-Gal] were supplied by Dextra Laboratories. 6′-Galactosyl-lactose [β-Gal-(1→6)-Gal-β-(1→4)-Glc] was a gift from Professor Nieves Corzo from CIAL-CSIC (Madrid, Spain).

*Synthesis and preparation of GOS.* An industrially available GOS mixture derived from lactose (GOS-La) was used in this study. To remove the mono- and disaccharides, the mixture was fractionated using size-exclusion chromatography (19). Briefly, 80 mL of GOS-La (25% wt:v) was injected in a Bio-Gel P2 (Bio-Rad) column (90 × 5 cm) using water as the mobile phase at a flow of 1.5 mL/min. The DP of collected fractions was determined by electrospray ionization-MS at positive mode. Fractions with DP ≥3 were pooled and freeze-dried to be used in the in vivo experiments. The trisaccharide content of GOS-La was determined by hydrophilic interaction chromatography coupled to MS (20). Then 20 μL of sample was injected onto an ethylene-bridged hybrid amide column (150 mm × 4.6 mm; 3.5 μm; XBridge). The elution was performed using a linear gradient of acetonitrile:MilliQ water, both having 0.1% $NH_4OH$, from 80:20 (v:v) to 50:50 (v:v) for 31 min. The separation and detection were carried out using an Agilent 1200 series HPLC system (Hewlett-Packard) coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard) provided with electrospray ionization and used in positive mode. The trisaccharide fraction was 35.1% of the total purified GOS-La.

Enzymatic synthesis of GOS derived from lactulose (GOS-Lu) was carried out via the hydrolysis and transgalactosylation of the prebiotic carbohydrate lactulose (Duphalac, Solvay Pharmaceuticals) by using a β-galactosidase from *Aspergillus oryzae* and following the procedure described by Clemente et al. (21). The GOS mixture was treated with activated charcoal to remove the monosaccharides fraction (22). A detailed characterization of GOS-Lu, with di- and trisaccharides as 78 and 14% of total carbohydrates, respectively, was recently reported (11).

*Rats and diets.* Male weaned Wistar rats (Charles River Laboratories), matched by weight (40 ± 5 g; 4 wk old), were individually housed in metabolism cages throughout the experiment under controlled conditions of temperature (25°C), moisture (50%), and lighting (12-h cycles). Rats were fed a diet (AIN-93G, Testdiet) formulated for use during growth based on corn starch (40%), casein (20%), maltodextrin (13.2%), sucrose (10%), and soybean oil (7%) as the main dietary ingredients (23). A 6-d preexperimental adaptation was followed by a 14-d experimental period. At the end of the adaptation period, rats had a mean weight of 75 ± 5 g. The 36 rats were then randomly assigned to 3 dietary groups of 12 and consumed food and water ad libitum. Diets were AIN-93G (control group), AIN-93G plus 1% (wt:wt) GOS-Lu (GOS-Lu group), and AIN-93G plus 1% (w:w) GOS-La (GOS-La group). $Cr_2O_3$ was included (2 $g \cdot kg^{-1}$) in all diets as an indigestible marker (24). Fresh fecal samples from rats subjected to the same dietary treatment were collected weekly (d 0, 7, and 14), pooled by collected day (equal weights from 4 rats/pool) in sterile flasks, and frozen prior to storage at −80°C for carbohydrate and microbiological analysis (see below).

All the experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Spanish Council for Scientific Research, and the rats were cared for in accordance with the Spanish Ministry of Agriculture guidelines (RD 1201/2005).

*Ileal sample collection.* At the end of the dietary intervention period (14 d), rats were deprived of food overnight, fed 4 g at timed intervals so that time elapsed between feeding and sacrifice was the same for all rats (2 h). Rats were killed under sodium pentobarbital (40 mg/kg body weight) anesthesia. The ileum (last 20 cm of small intestine) was immediately dissected out and rinsed with sterile distilled water to collect the ileal content. Intestinal contents of individual rats were immediately frozen, freeze-dried, and stored at −80°C. Stomach, cecum, and colon were also washed with sterile distilled water and weighed.

*Quantitative determination of GOS (di- and trisaccharides) by GC-MS.* Di- and trisaccharides, derived from GOS-La and GOS-Lu, were identified and quantified in dietary, ileum, and fecal samples by GC-MS, following sequential derivatization, using oximation and trimethylsilylation steps. A solution was prepared by dissolving 50 mg of sample in 2 mL of 70% (v:v, ethanol:water) at 4°C and filtered (0.20 μm) and 1 mL mixed with 0.25 mL of phenyl-β-D-glucoside (1 g/L), used as an internal standard, and evaporated under vacuum. Carbohydrates were derivatized to their corresponding trimethylsilyloximes (TMSO) as previously reported (25). GC-MS analyses were carried out using an Agilent Technologies 7890A gas chromatograph (Hewlett-Packard) coupled to a 5975C quadrupole mass detector operating in electronic impact mode at

70 eV and using helium as the carrier gas (1 mL/min) (25). A 30-m × 0.25-mm i.d × 0.25-$\mu$m film thickness fused silica column with cross-linked methyl silicone (Teknokroma) was used. All analyses were carried out in triplicate.

Identification of TMSO derivatives of carbohydrates was carried out by comparison of mass spectra and retention indices ($I^T$) with standard carbohydrates previously derivatized. Earlier data (26,27) were used to identify carbohydrates not commercially available; such identifications were considered to be tentative. Carbohydrate quantitative data were obtained from GC-MS peak areas using the internal standard method; standard solutions from 0.003 to 1 mg of lactulose, maltose, sacarose, maltotriose, and raffinose were prepared to calculate the corresponding response factors relative to internal standard and used to quantify di- and trisaccharides.

*Ileal and fecal digestibility.* The ileal apparent digestibility (%) of GOS-Lu and GOS-La was calculated according to the expression: $[(P_f/Cr_2O_{3f}) - (P_i/Cr_2O_{3i})]/(P_f/Cr_2O_{3f}) \times 100$, where $P_f$ and $P_i$ represent the amount of carbohydrates (mg/100 mg of sample) in feed and ileal samples, respectively, determined by GC-MS analyses, and $Cr_2O_{3f}$ and $Cr_2O_{3i}$ are chromium oxide concentrations (mg/100 mg) in feed and ileal contents (24). In a similar way, fecal digestibility of the different compounds at the end of the experimental period (14 d) was also evaluated. Chromium oxide content was determined in experimental diets, and ileal and fecal samples following the procedure described by Fenton and Fenton (28).

*DNA extraction from fecal samples.* Total DNA was isolated from freeze-dried fecal samples (40 mg) using the QIAamp DNA stool kit (Qiagen) and following the manufacturer's instructions. Eluted DNA was treated with RNase (Invitrogen) and DNA concentration spectrophotometrically assessed by using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies). Purified DNA samples were stored at −80°C.

*qPCR analysis.* qPCR was used to monitor the modulatory effect of GOS-Lu and GOS-La on the fecal microbiota of rats during 14 d of treatment. Different microbial groups, including total bacteria, *Bacteroides*, *Lactobacilli*, *Bifidobacteria*, *Eubacterium rectale/Clostridium coccoides*, and *Clostridium leptum*, were distinguished and quantified using qPCR. The 16S rDNA-targeted group-specific primers used in this study are listed in **Supplemental Table 1**. qPCR assays were performed using an iQ5 Cycler Multicolor PCR detection system (BioRad Laboratories). The reaction mixture (25 $\mu$L) comprised 12.5 $\mu$L of iQ SYBR Green Supermix (BioRad), 0.75 $\mu$L of each of the specific primers (10 $\mu$mol/L; Roche Diagnostics), 9 $\mu$L of sterile distilled water, and 2 $\mu$L of DNA template. For total bacteria, *Bacteroides*, *Bifidobacteria*, and *Lactobacilli* group, PCR conditions included a first step at 50°C for 2 min, followed by 95°C for 10 min for initial denaturation and 40 cycles at 95°C for 15 s and 60°C for 1 min for primer annealing and product elongation. In the case of *Eubacterium rectale/Clostridium coccoides* and *Clostridium leptum* groups, PCR conditions were an initial denaturation step at 94°C for 5 min followed by 40 cycles at 94°C for 20 s, 50°C for 20 s, and 72°C for 1 min. A plasmid standard containing the target region was generated for each specific primer set using DNA extracted from pooled fecal samples of rats fed an AIN-93G diet. The amplified products were cloned using the TOPO TA cloning kit for Sequencing (Invitrogen) and transformed into *Escherichia coli* One Shot Top 10 cells (Invitrogen). Sequences were submitted to the ribosomal RNA database to confirm the specificity of the primers (29). For quantification of target DNA copy number, standard curves were generated using serial 10-fold dilutions of the extracted products by using at least 6 non-zero standard concentrations per assay (30). The bacterial concentration in each sample was measured as $\log_{10}$ copy number by the interpolation of the $C_t$ values obtained by the fecal samples and the standard calibration curves. Each plate included triplicate reactions per DNA sample and the appropriate set of standards.

*Selectivity index.* To obtain a general quantitative measure of the prebiotic effect, selectivity index (SI) values were calculated for the different treatments. The SI represents a comparative relationship between the growth of "beneficial" bacteria, including bifidobacteria, lactobacilli, and *Eubacterium rectale*, and that of the "less desirable" ones, such as bacteroides and clostridia, in relation to the change in the total number of bacteria. The equation to estimate the SI values was adapted from Palframan et al. (31) as follows: $[(Bif_t/Total_t)/(Bif_0/Total_0)] + [(Lact_t/Total_t)/(Lact_0/Total_0)] + [(Erec_t/Total_t)/(Erec_0/Total_0)] - [(Bact_t/Total_t)/(Bact_0/Total_0)] - [(Clost_t/Total_t)/(Clost_0/Total_0)]$, where Bif, Lact, Erec, Bact, Clost, and Total are the $\log_{10}$ copy number/g of freeze-dried fecal sample of bifidobacteria, lactobacilli, *Eubacterium rectale/Clostridium coccoides* group, bacteroides, *Clostridium leptum* subgroup, and total bacteria, respectively, at the time of sampling (7,14 d), all related to their starting levels (0 d).

*Statistics.* Individual rats were considered the experimental unit. The effect of dietary treatment on fecal microbiota composition was analyzed by 2-way repeated-measures ANOVA using the GLM procedure (SPSS Statistics version 18.0), according to the following model: $Y_{ijk} = \mu + \alpha_i + \omega_k + \alpha\omega_{ik} + \varepsilon_{ijk}$, where $\mu$ is the mean, $\alpha_i$ is the effect of diet (control, GOS-Lu, GOS-La), $\omega_k$ is the time (0, 7, 14 d), $\alpha\omega_{ik}$ is the interaction between diet and time, and the term $\varepsilon_{ijk}$ represents the random error. Similarly, SI data were analyzed for dietary treatment and time effects. Given that the time × diet interaction was significant, groups at a time and time points within group were compared using 1-factor ANOVA. Means that significantly differed were identified using the Minimum Significant Difference test. Differences were considered significant at $P < 0.05$.Values in the text are means ± SD.

## Results

*Animal performance.* There were no differences among the groups in food intake (14.2 ± 0.1 g/d) or body weight gain (4.7 ± 0.1 g/d) during the 14-d experiment. In addition, dietary treatments did not significantly affect the relative weight of different organs, including stomach, small intestine, cecum, and colon, except that rats fed GOS-La had a lower relative colon weight compared with those fed control diets ($P < 0.05$) (data not shown).

*Ileal and fecal digestibility of GOS-Lu and GOS-La.* Quantitative evaluation of carbohydrates from dietary and ileal samples demonstrated that the disaccharide fraction of GOS-Lu, mostly composed of $\beta$-galactobioses and galactosyl-fructoses (11), was fully resistant to digestion in the small intestine (**Table 1**). GC-MS profiles of GOS-Lu disaccharides from dietary and ileal samples of rats were almost identical, with slight differences in minor chromatographic peaks, which did not match carbohydrates according to their mass spectra and retention times (**Supplemental Fig. 1***A*). The chromatographic profile of fecal samples of rats fed GOS-Lu demonstrated the complete fermentation of the disaccharide fraction in the large intestine; none of the detected chromatographic peaks of fecal samples showed the typical mass fragmentation patterns of TMSO derivatives of carbohydrates. Such peaks were also present in fecal samples of rats fed the control diet (not shown). As a result of the high levels of digestible lactose present in the disaccharide fraction, mono- and disaccharides were removed from the starting GOS-La. Therefore, no data regarding the resistance of disaccharides from GOS-La to digestion are reported in this study.

The GOS-Lu trisaccharide fraction exhibited a limited digestion, as evaluated in ileal samples of treated rats, showing a digestibility rate of 12.5 ± 2.6% (Table 1); in contrast, GOS-La trisaccharides were clearly susceptible to small intestinal hydrolysis, having a much higher digestibility rate (52.9 ± 2.7%) (**Table 2**). An exhaustive analysis of their single chromatographic peaks, obtained by GC-MS, was carried out as it is described below. The chromatographic profile of dietary

**TABLE 1** Ileal digestibility, $I^T$, and tentative structural identification of GOS (di- and trisaccharides) of GOS-Lu[1]

| Peak number[2] | Disaccharides | $I^T$ | Ileal digestibility[2] (%) |
|---|---|---|---|
| Total | | | 0.0 |
| 1+2 | Gal-(1→4)-Fru (lactulose) | 2878–2887 | |
| 3 | Gal-(1→1)-Gal + Gal-(1→4)-Gal *E* | 2903 | |
| 4 | Gal-(1→5)-Fru 1 | 2915 | |
| 5a | Gal-(1→3)-Gal *E* + Gal-(1→2)-Gal *E* | 2932 | |
| 5b | Gal-(1→5)-Fru 2 | 2937 | |
| 6 | Gal-(1→4)-Gal *Z* + unknown | 2959 | |
| 7 | Gal-(1→3)-Gal *Z* – Gal-(1→2)-Gal Z | 2979 | |
| 8 | Gal-(1→6)-Fru 1 | 3003 | |
| 9 | Gal-(1→6)-Fru 2 | 3012 | |
| 10 | Gal-(1→1)-Fru 1 | 3029 | |
| 11a | Gal-(1→6)-Gal *E* | 3046 | |
| 11b | Gal-(1→1)-Fru 2 | 3049 | |
| 12 | Gal-(1→6)-Gal *Z* | 3094 | |

| Peak number[2] | Trisaccharides | $I^T$ | Ileal digestibility[2] (%) |
|---|---|---|---|
| Total | | | 12.5 ± 2.6 |
| 13 | Unknown | 3785 | |
| 14 | Unknown | 3794 | |
| 15 | Gal-(1→6)-Gal-(1→4)-Fru 1 | 3809 | |
| 16 | Gal-(1→6)-Gal-(1→4)-Fru 2 | 3826 | |
| 17 | Unknown | 3835 | |
| 18 | Gal-(1→4)-Gal-(1→1)-Fru | 3841 | |

[1] Data are mean ± SD, $n$ = 3 (pools of samples from 4 rats), calculated as ileal digestibility of the whole fraction of di- or trisaccharides. Fru, fructose; Gal, galactose; GOS, galacto-oligosaccharide; GOS-Lu, lactulose-derived galacto-oligosaccharide; $I^T$, retention indices.
[2] Labeled peaks are described in Supplemental Figure 1.

GOS-Lu trisaccharides and those found in ileal samples of rats fed GOS-Lu are shown (Supplemental Fig. 1*B*). The similarity of both chromatographic profiles confirms the high resistance to digestibility of their major components. As previously reported, the major trisaccharides of GOS-Lu were identified as Gal-(1→6)-Gal-(1→4)-Fru (6′-galactosyl-lactulose) (peaks 15 and 16) and Gal-(1→4)-Gal-(1→1)-Fru (peak 18) (Supplemental Fig. 1*B*) (9,11). In contrast, some individual GOS-La trisaccharides were completely digested (**Supplemental Fig. 2***B*); thus, the chromatographic peaks 13, 14, 19, and 22 were not found in ileal samples of rats fed GOS-La (Table 2; Supplemental Fig. 2*B*). Indeed, such susceptibility to hydrolysis was clear from the presence of disaccharides in ileal samples of rats fed GOS-La (Supplemental Fig. 2*A*), demonstrating that some oligosaccharides of DP ≥3 were hydrolyzed. Di- and trisaccharides of GOS-La reaching the large intestine were fully fermented, as indicated by the absence of such oligosaccharides in fecal samples (Supplemental Fig. 2*A*,*B*).

*Tentative identification of GOS-La trisaccharides and disaccharides derived from hydrolysis of oligosaccharides of DP ≥3.* To determine the chemical structure of GOS-La oligosaccharides digested at the ileum, a tentative identification of the TMSO trisaccharides and disaccharides formed as a result of digestion was carried out by GC-MS analyses. The $I^T$ of carbohydrates present in dietary and ileal samples of rats fed GOS-La are shown (Table 2). The mass spectrum of peak 1 ($I^T$ = 2686) showed a ratio of *m/z* 191:204:217 ions of 1.4:1:1.1, characteristic of nonreducing sugars with 1→1 glycosidic linkages and consistent with Gal-(1→1)-Gal (1,1-galactobiose) or Gal-(1→1)-Glc (1,1-galactosyl-glucose). Given that 1,1-galactobiose shows an $I^T$ value of 2903 (11), the chromatographic peak was assigned to 1,1-galactosyl-glucose. Peak 2 was composed of a mixture of 2 disaccharides, Gal-(1→4)-Glc (1,4-galactosyl-glucose) and Gal-(1→3)-Glc (1,3-galactosyl-glucose). The former was assigned by comparison with a GOS previously obtained by using β-galactosidase from *Aspergillus aculeatus* (9), whereas the latter was assigned based on the presence of relatively highly abundant *m/z* 205, 244, and 307 ions. Such a fragmentation pattern is characteristic of the 1→3 glycosidic linkage and similar to that of the Gal-(1→3)-Gal (1,3-galactosyl-galactose) standard but with different $I^T$ (2699 and 2932, respectively), hampering its tentative identification. Peak 3 showed the typical fragmentation pattern of TMSO carbohydrates; however, it could not be identified by its mass spectrum and retention index. Peak 4 was composed of a mixture of Gal-(1→4)-Gal (1,4-galactosyl-galactose), identified by comparison with its corresponding commercial standard, and Gal-(1→4)-Glc (1,4-galactosyl-glucose, isomer *Z*), identified in a similar way to its corresponding *E* isomer. Similarly, peak 5 was composed of a mixture of the second peak of Gal-(1→3)-Glc (1,3-galactosyl-glucose, isomer *Z*) and an unknown disaccharide. Peaks 6 and 7 were identified as Gal-(1→2)-Glc (1,2-galactosyl-glucose), isomers *E* and *Z*, respectively, by the presence of the *m/z* 319 ion with a high abundance, which is characteristic of 1→2 glycosidic linkages and that corresponds to the loss of a TMSOH group from the C3-C6 chain of an hexose group. Peaks 8 and 9 were characterized by a high intensity ion of *m/z* 422 corresponding to C1-C4 of the oxime chain, typical of 1→6 glycosidic linkages, and identified as Gal-(1→6)-Glc (1,6-galactosyl-glucose) isomers due to a difference in the $I^T$ between these peaks and the corresponding Gal-(1→6)-Gal (1,6-galactosyl-galactose) standards.

Up to 17 chromatographic peaks corresponding to GOS-La trisaccharides were detected. Peak 10 had a ratio of ions 191:204:217, typical of 1→1 glycosidic linkages. Peak 16 was identified as Gal-(1→4)-Gal-(1→4)-Glc (4′-galactosyl-lactose) by comparison with the most abundant trisaccharide of Vivinal-GOS (26) and supported by previous data reported by Cardelle-Cobas et al. (13). In peak 17, coelution of 2 compounds occurred, with the first one being identified as Gal-(1→6)-Gal-(1→4)-Glc (6′-galactosyl-lactose) by comparison with the compound previously isolated and identified by NMR (10), and the second one identified as Gal-(1→4)-Gal-(1→2)-Glc 1 by its characteristic *m/z* 319 ion and by comparison with that previously identified in Vivinal-GOS by NMR (26). Peak 20 was composed of 2 compounds, Gal-(1→4)-Gal-(1→2)-Glc 2 and Gal-(1→6)-Gal-(1→2)-Glc 1, identified in a similar way as the previous peak. Peak 18 corresponded to the isomers of 4′-galactosyl-lactose and coeluted with another unknown carbohydrate. Peak 23 was identified as Gal-(1→6)-Gal-(1→6)-Glc by the presence of the ion at *m/z* 422 and by comparison with 6′-galactosyl-galactose (13); although the mass spectrum was similar, the retention index was different due to the presence of a terminal galactose unit instead of a glucose molecule. Unfortunately, it was not possible to determine the chemical structure of some GOS-La trisaccharides having low ileal digestibility (peaks 11, 12, 15, 24, 25, and 26) (Table 2) due to the absence of diagnostic ions in the mass spectra, which, however, were typical of TMSO carbohydrates.

*Effect of GOS on fecal microbiota composition.* In all treatments, including the control group, bifidobacteria populations

**TABLE 2** Ileal digestibility, $I^T$, and tentative structural identification of GOS (di- and trisaccharides) of GOS-La[1]

| Peak number[2] | Disaccharides[3] | $I^T$ |
|---|---|---|
| 1 | Gal-(1→1)-Gal or Gal-(1→1)-Glc | 2685 |
| 2 | Gal-(1→4)-Glc *E* + Gal-(1→3)-Glc *E* | 2699 |
| 3 | Unknown | 2701 |
| 4 | Gal-(1→4)-Glc *Z*+ Gal-(1→4)-Gal *E* | 2709 |
| 5 | Gal-(1→3)-Glc *Z* + unk | 2727 |
| 6 | Gal-(1→2)-Glc *E* | 2736 |
| 7 | Gal-(1→2)-Glc *Z* | 2765 |
| 8 | Gal-(1→6)-Glc *E* | 2824 |
| 9 | Gal-(1→6)-Glc *Z* | 2868 |

| Peak number[2] | Trisaccharides | $I^T$ | Individual ileal digestibility (%) | Ileal digestibility[4] (%) |
|---|---|---|---|---|
| Total | | | | 52.9 ± 2.7 |
| 10 | (1→1)[5] | 3661 | 6.0 ± 1.2 | |
| 11 | Unknown | 3675 | 47.7 ± 2.6 | |
| 12 | Unknown | 3711 | 22.6 ± 1.8 | |
| 13 | Unknown | 3723 | 99.5 ± 2.9 | |
| 14 | Unknown | 3755 | 100.0 ± 0.0 | |
| 15 | Unknown | 3766 | 30.2 ± 3.3 | |
| 16 | Gal-(1→4)-Gal-(1→4)-Glc *E* | 3775 | 29.5 ± 4.8 | |
| 17 | Gal-(1→6)-Gal-(1→4)-Glc *E* + Gal-(1→4)-Gal-(1→2)-Glc | 3794 | 77.0 ± 6.6 | |
| 18 | Gal-(1→4)-Gal-(1→4)-Glc *Z* + unk | 3801 | 77.9 ± 4.6 | |
| 19 | Unknown | 3811 | 100.0 ± 0.0 | |
| 20 | Gal-(1→6)-Gal-(1→2)-Glc 1 + Gal-(1→4)-Gal-(1→2)-Glc 2 | 3826 | 0.0 ± 0.0 | |
| 21 | Unknown | 3862 | 54.1 ± 1.5 | |
| 22 | Unknown | 3880 | 100.0 ± 0.0 | |
| 23 | Gal-(1→6)-Gal-(1→6)-Glc 1 | 3889 | 81.3 ± 3.6 | |
| 24 | Unknown | 3963 | 25.3 ± 8.6 | |
| 25 | Unknown | 3997 | 30.4 ± 3.5 | |
| 26 | Unknown | 4012 | 29.7 ± 1.9 | |

[1] Data are mean ± SD, $n = 3$ (pools of samples from 4 rats). Gal, galactose; Glc, glucose; GOS, galacto-oligosaccharide; GOS-La, lactose-derived galacto-oligosaccharide; $I^T$, retention indices.
[2] Labeled peaks are described in Supplemental Fig. 2.
[3] Disaccharides were not present in GOS-La diet.
[4] Calculated as ileal digestibility of the whole fraction of trisaccharides.
[5] Nonidentified monomers.

from fecal samples increased throughout the experimental period (0–14 d). By d 14, the bifidogenic effect was significantly greater in rats fed GOS-Lu than in those fed GOS-La (**Table 3**). The GOS-La group had significantly more lactobacilli in fecal samples than control or GOS-Lu groups. The number of *Eubacterium rectale/Clostridium coccoides* was greater in the GOS-Lu and GOS-La groups than in controls. At d 7, the SI suggested that there was no prebiotic effect of GOS-Lu or GOS-La, because that in the GOS-La group was significantly less than in the control group and that of the GOS-Lu group did not differ (**Fig. 1**). The SI increased from

**TABLE 3** Microbiota composition in fecal samples obtained from growing rats fed a control, GOS-Lu, or GOS-La diet on d 0, 7, and 14[1]

| | Diets | | | Time | | | | *P* value[2] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Bacterial groups | Control | GOS- Lu | GOS- La | 0 | 7 | 14 | Pooled SEM | Diets | Time | Interaction |
| | *log$_{10}$ copy number/g of freeze-dried fecal sample* | | | | | | | | | |
| All bacteria | 10.5$^a$ | 10.7$^b$ | 10.8$^b$ | 10.6$^a$ | 10.6$^a$ | 10.9$^b$ | 0.007 | <0.001 | <0.001 | NS[3] |
| Bacteroides | 10.6$^a$ | 10.7$^b$ | 10.9$^c$ | 10.7$^b$ | 10.6$^a$ | 10.8$^c$ | 0.007 | <0.001 | <0.001 | NS |
| *Bifidobacteria* | 9.82$^a$ | 10.5$^c$ | 10.1$^b$ | 9.68$^a$ | 10.1$^b$ | 10.7$^c$ | 0.024 | <0.001 | <0.001 | NS |
| *Lactobacilli* | 9.64$^a$ | 9.78$^b$ | 10.1$^c$ | 9.52$^a$ | 9.77$^b$ | 10.2$^c$ | 0.006 | <0.001 | <0.001 | NS |
| *Eubacterium rectale/Clostridium coccoides* group | 9.47$^a$ | 10.1$^b$ | 10.2$^b$ | 9.59$^a$ | 9.83$^b$ | 10.2$^c$ | 0.019 | <0.001 | <0.001 | NS |
| *Clostrodium leptum* subgroup | 9.33$^a$ | 9.48$^b$ | 9.62$^c$ | 9.57$^c$ | 9.38$^a$ | 9.47$^b$ | 0.007 | <0.001 | <0.001 | NS |

[1] Data are mean and pooled SEM, $n = 3$ (pools of samples from 4 rats), expressed as log10 copy number/g of freeze-dried fecal sample. Within diet or time, means without a common letter differ, $P > 0.05$. GOS, galacto-oligosaccharide; GOS-La, lactose-derived galacto-oligosaccharide; GOS-Lu, lactulose-derived galacto-oligosaccharide.
[2] Significance main effects (diet and treatment time) were determined by General Linear Model by 2-way repeated-measures ANOVA.
[3] NS, nonsignificant, $P > 0.05$.

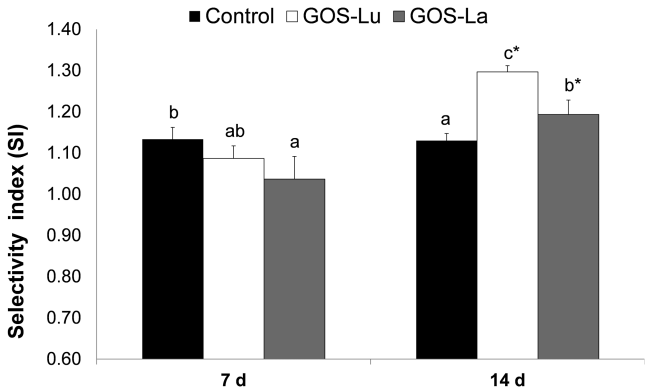


**FIGURE 1** SI scores of fecal samples obtained from growing rats fed a control, GOS-Lu, or GOS-La diet on d 7 and 14. Data are mean ± SD, $n = 3$ (pools of samples from 4 rats). Effects of diet ($P = 0.004$), time ($P = 0.001$), and their interaction ($P = 0.008$) were significant. Means at a time without a common letter differ, $P < 0.05$. *Differences from corresponding d 7, $P < 0.05$. GOS-La, lactose-derived galacto-oligosaccharide; GOS-Lu, lactulose-derived galacto-oligosaccharide; SI, selectivity index.

d 7 to 14 in the GOS-Lu and GOS-La groups but not in the controls. At d 14, the SI was greater in the GOS-Lu group than in the GOS-La group and the values in both were greater than in controls. These data suggest that GOS-Lu exerted a stronger prebiotic effect than GOS-La.

## Discussion

It is generally accepted that the major beneficial effects of prebiotic carbohydrates occur in the large intestine due to the slow transit of the substrates to be fermented and their effects on microbiota diversity, which plays an important role in host health (32). Several studies have demonstrated a modulatory effect of commercially available GOS-La on fecal microbiota of healthy human volunteers (33–35), whereas others have not shown a significant effect (15,36). Interestingly, the administration to human volunteers of a GOS-La mixture containing mainly $\beta$ (1→3) as well as $\beta$ (1→4) and $\beta$ (1→6) linkages proved to have a better bifidogenic effect than a commercially available GOS-La mixture consisting of GOS having $\beta$ (1→4) and $\beta$ (1→6) linkages only (35). These dissimilarities on the selective growth of bifidobacteria could be attributed to several factors, including resistance to hydrolysis and/or fermentation selectivity of dietary oligosaccharides.

Whereas most research interest on prebiotics has focused on their role as modulators of intestinal microbiota, few efforts have been made to study the small intestinal resistance of dietary GOS, 1 of the 3 major criteria that determine prebiotic potential (4,33). Indeed, the elevated complexity of such samples poses a challenge in analytical chemistry that increases when biological samples are evaluated (11). Even if there are suggestions that GOS-La reach the large intestinal sections intact, several studies have revealed their susceptibility to partial hydrolysis (4); in addition, no data regarding the in vivo digestibility of GOS-Lu were previously reported. In the present study, we demonstrated that GOS-La and GOS-Lu are resistant to in vivo digestion to different extents, with the former being much more susceptible to hydrolysis (Tables 1 and 2). Di- and trisaccharides resistant to digestion in the upper gastrointestinal tract were completely fermented by intestinal microbiota, as none were found in fecal samples (Supplemental Figs. 1 and 2).

GOS, either produced from lactose or lactulose, are composed of a very complex mixture of carbohydrates, differing in their linkage type, number, and order of monomers in the oligosaccharide chain. GOS derived from lactose and lactulose usually comprise oligomers with a terminal glucose or fructose, respectively. According to our results, these structural differences seem to have a considerable impact on susceptibility to in vivo gastrointestinal digestion and, as a result, their potential as prebiotic carbohydrates. Thus, GOS-Lu trisaccharides were more resistant to hydrolysis than those derived from GOS-La (Tables 1 and 2). Several intestinal brush border enzymes are able to catalyze the hydrolysis of glycosidic linkages, such as amylases and sucrases; in addition, other glycolytic enzymes such as isomaltases and $\beta$-glycosidases can also contribute to the digestion of dietary carbohydrates (37). Considering the structures present in GOS-La trisaccharides, it is likely that $\beta$-glycosidases present in the brush border of the small intestine hydrolyze glycosidic linkages between galactoses and glucoses. In this study, we demonstrated the susceptibility to hydrolysis of 4′-galactosyl-lactose [Gal-(1→4)-Gal-(1→4)-Glc] (peaks 16 and 18, Table 2), as previously reported in vitro by Ohtsuka et al. (18). The susceptibility to hydrolysis of GOS-La having a DP ≥3 was also confirmed by the presence of their derivative disaccharides in ileal samples (Supplemental Fig. 2*A*); likewise, the abundance of Gal-(1→6)-Glc and Gal-(1→2)-Glc disaccharides in ileal samples pointed to the high resistance of these glycosidic linkages to the extreme conditions of the upper digestive tract (peaks 6–9, Table 1 and Supplemental Fig. 2*A*). In addition, a partial and selective fermentation of GOS-La by the small intestinal microbiota may also occur and could explain the reported growth stimulation of probiotic bacteria in the ileum of weaned piglets after administration of several prebiotic carbohydrates, including GOS-La (38).

GOS-Lu disaccharides were highly resistant to the acidic environment and enzymatic digestion in the upper digestive tract (Table 1), exhibiting a superior resistance to hydrolysis compared with GOS-Lu trisaccharides. A plausible explanation for this differential behavior could be the role that both monomer and linkage type may play in resisting the action of the digestive enzymes (39). Thus, the higher digestibility of GOS-Lu trisaccharides compared with disaccharides could be attributed to the higher susceptibility to the action of hydrolytic enzymes of some specific linkages between galactose residues, as observed for those contained in the 4′-galactosyl-lactose [Gal-(1→4)-Gal-(1→4)-Glc], compared with those involved in the formation of galactosyl-fructoses. In this context, lactulose is not hydrolyzed or absorbed in the small intestine but selectively fermented by the colonic microbiota (33,40). GOS-Lu disaccharides are mostly composed of $\beta$-galactobioses and galactosyl-fructoses, as previously reported by Hernandez-Hernandez et al. (11). Therefore, in a similar way to lactulose, it is very plausible that other galactosyl-fructoses, such as those contained in peaks 4, 5b, 8, 9, 10, 11b, 15, 16, and 18 (Table 1), are quite resistant to digestion within the mammalian gastrointestinal system and have the ability to reach the large intestine intact as fermentable substrates for the resident intestinal microbiota.

In this study, we also evaluated the impact of GOS-La and GOS-Lu, with significant differences in their major components (Tables 1 and 2), on the fecal microbiota of growing rats. Interestingly, the novel GOS-Lu exerted a stronger bifidogenic effect on fecal microbiota than GOS-La, with the latter showing a significant increase in lactobacilli population relative to the control (**Table 3**). It has been suggested that variation in daily dose may contribute to differences in the modulatory effect on intestinal

microbiota. GOS-La, administrated at doses of 5 g or higher, was bifidogenic as observed in fecal samples of human healthy volunteers, whereas a dose of 2.5 g had no significant effect (41). These authors suggested that a minimum or threshold dose may exist below which a bifidogenic effect is not observed. Consequently, it seems likely that GOS having an extended period of digestion in the upper digestive tract will have a stronger dose-dependent effect on their ability to modulate the intestinal microbiota, where higher doses are necessary for such an effect. We suggest that the amounts of GOS that reach the large intestine depend on their structural characteristics that, therefore, may influence their potential prebiotic effects. Given the differences in the digestive physiology between rat and humans, the results obtained cannot automatically predict the prebiotic responses in humans. Our data suggest that a detailed chemical characterization and studies of digestibility using in vivo model systems, in support of human intervention studies, are relevant for dietary recommendations and help to determine criteria for developing novel prebiotics.

In spite of rapid research advances in gut microbial ecology, the systematic understanding of this complex ecosystem and its microbial interactions are still limited. Our microbiological data reflect the effects of GOS-Lu and GOS-La at a group level only. Not all bifidobacteria are likely to be able to utilize or compete for these prebiotics. Regarding this relevant area, further studies are in progress to investigate which types or species of bifidobacteria are selectively affected by these prebiotics in the large intestine. Analyses of microbiota have shown the stimulation on growth of not only bifidobacteria or lactobacilli but also the *E. rectale/C. coccoides* group (Table 3). An increase in the populations of *E. rectale/C. coccoides* has been reported in human intervention studies after treatment with prebiotics (42). These bacteria are known to produce relatively high amounts of butyrate (43), which could exert a protective role in protection against inflammatory bowel disease and colorectal cancer (44). Although its physiological relevance needs to be investigated more deeply, some authors have claimed that the prebiotic concept may be expanded toward other genera, including *Eubacterium* and *Roseburia* (14).

In conclusion, GOS-La and novel GOS-Lu were incorporated in a single dose (1%, wt:wt) to rats for a period of 14 d. Under such conditions, these compounds have met the 3 main criteria that a food ingredient must satisfy to be considered as prebiotic (4,45): *1*) show resistance, at least to some extent, to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption; *2*) act as a substrate for fermentation by intestinal microorganisms; and *3*) show selective stimulation of the growth of intestinal bacteria associated with health and well-being. Our findings revealed that GOS-Lu had a higher resistance to in vivo gastrointestinal digestion and absorption in the small intestine, likely to be due to the presence of a fructose residue at the reducing end of the oligosaccharides. The partial digestion of GOS-La trisaccharides suggested that glycosidic linkages Gal-(1→6)-Glc and Glc-(1→2)-Glc are more resistant to in vivo gastrointestinal digestion than the linkage type Gal-(1→4)-Gal. The absence of resistant GOS in fecal samples indicated that the oligosaccharides served as fermentation substrates in the large intestine. As a result, a stronger bifidogenic effect was observed in fecal samples of rats fed GOS-Lu compared with those fed GOS-La. Interestingly, a significant increase in the population of the *E. rectale/C. coccoides* group was also revealed following treatment with GOS-Lu or GOS-La. To the best of our knowledge, this work provides the first evidence for the in vivo prebiotic effects of GOS-Lu, indicating that these novel oligosaccharides could have the ability to reach the large intestine in physiologically relevant doses due to their low digestibility. Further in vivo studies addressing the effect of GOS-Lu on the selective growth of the intestinal microbiota are underway currently.


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