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Galacto-oligosaccharides Derived from Lactulose Exert a Selective Stimulation on the Growth of *Bifidobacterium animalis* in the Large Intestine of Growing Rats

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ABSTRACT: There is increasing interest in identifying novel dietary nondigestible carbohydrates capable of modulating the composition and/or metabolic activities of the gut microbiota. This work assessed the differential modulatory influence of novel galacto-oligosaccharides derived from lactulose (GOS-Lu) in comparison with commercial galacto-oligosaccharides derived from lactose (GOS-La) in gut microbiota of growing rats (5 weeks old). Rats were fed either a control diet or diets containing 1% (w/w) of GOS-Lu or GOS-La, and cecal and colonic contents were collected after 14 days of treatment. Compared to controls, GOS-Lu had significantly more bifidobacteria within the large intestine, showing a significant and selective increase of *Bifidobacterium animalis* in the cecum and colon; however, no significant differences in the number of bifidobacteria among GOS-Lu and GOS-La groups were observed. Both types of GOS significantly increased the number of the *Eubacterium rectale/Clostridium coccoides* group. These findings support a prebiotic role of galactosyl-fructoses in functional foods.

KEYWORDS: *bifidobacteria, Bifidobacterium animalis, galacto-oligosaccharides, gut microbiota, lactulose, prebiotic*

INTRODUCTION

The human gastrointestinal tract contains an extraordinary number (10 trillion−100 trillion and more than 200 phylotypes) of resident commensal bacteria existing in homeostasis with the host.[1] This endogenous microbiota establishes a synbiotic mutualistic relationship and has a major impact upon the nutrition and health of the host, via the supply of nutrients, conversion of metabolites, control of epithelial cell proliferation/differentiation, pathogen exclusion, and stimulation of the immune system.[2] Given the emergent evidence of the key role played by the gut microbiota in health and disease, there is growing interest in identifying non-digestible functional food ingredients that are selectively fermented and allow specific changes in the composition and/or activity of the gut microbiota, which confers a beneficial effect on the host.[3] These dietary compounds, named prebiotics, favor the growth of beneficial bacteria and inhibit the growth of undesirable, potentially pathogenic bacteria, including *Salmonella* sp., *Campylobacter jejuni*, *Helicobacter pylori*, and *Escherichia coli*, among others. A growing number of studies support the conclusion that prebiotics could exert beneficial effects not only in the large intestine but also within the entire human body and/or contribute to the prevention/remission of intestinal or systemic pathologies.[4,5] Ingestion of prebiotics typically increases the population of *Bifidobacterium* and *Lactobacillus* species, a widely accepted measure of prebiotic effect to date; in addition, a shift in the populations of other microorganisms, such as *Eubacterium* and *Roseburia*, has been reported to play a key role in butyrate synthesis, which is essential for the maintenance and protection of the normal colonic epithelium. The modulatory effect of prebiotics on gut microbiota has been associated with improvement in overall health, enhancement of defense mechanisms of the host to gut infections, accelerated recovery of gut disturbances, and better absorption of minerals.[6] Currently, the major prebiotic oligosaccharides on the market are inulin, fructo-oligosaccharides (FOS), and galacto-oligosaccharides (GOS).[7] The latter are nondigestible, at least partially, carbohydrates, which are usually composed by 2−10 molecules of galactose and 1 molecule of glucose, being primarily synthesized from lactose by the action of β-galactosidases of fungal, bacterial, or yeast origin, to result in lactose-derived GOS (GOS-La). The prebiotic properties of GOS-La in vitro have been reported consistently.[8−10] GOS-La have been demonstrated to increase the bifidobacterial population of fecal microbiota in healthy human volunteers;[11,12] however, such a positive effect was not observed in other related studies.[13,14] These dissimilarities can be attributed to a number of factors including the type, purity, and composition of the GOS used, as well as differences in the experimental design and methodological aspects.[15] Recently, the synthesis of lactulose-derived GOS (GOS-Lu) has attracted the attention of the scientific community due to their prospective prebiotic applications, being recognized mainly for their ability to promote the growth of bifidobacteria in human fecal slurries.[16,17] A detailed characterization of

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Table 1. Group-Specific Primers Based on 16S rRNA Sequences Used for qPCR

target bacterial group	primer	sequence (5′−3′)	PCR product size (bp)	annealing T (°C)	ref
all bacteria	F-Eub 338	ACTCCTACGGGAGGCAGCAG	200	60	35
	R-Eub 518	ATTACCGCGGCTGCTGG			
Bacteroides	F-AllBac 296	GAGAGGAAGGTCCCCCAC	106	60	36
	R-AllBac 412	CGCTACTTGGCTGGTTCAG			
bifidobacteria	F-Bifido	CGCGTCYGGTGTGAAAG	244	60	37
	R-Bifido	CCCCACATCCAGCATCCA			
Clostridium coccoides/Eubacterium rectale group	F-g-Ccoc	AAATGACGGTACCTGACTAA	440	50	38
	R-g-Ccoc	CTTTGAGTTTCATTCTTGCGAA			
Clostridium leptum group	F-sg-Clept	GCACAAGCAGTGGAGT	239	50	38
	R3-sg-Clept	CTTCCTCCGTTTTGTCAA			
lactobacilli	F-Lacto	GAGGCAGCAGTAGGGAATCTTC	126	60	37
	R-Lacto	GGCCAGTTACTACCTCTATCCTTCTTC			

oligosaccharides up to a degree of polymerization (DP) of 7, obtained by transgalactosylation reactions of lactulose by using β-galactosidase from *Aspergillus oryzae*, has been previously reported.[18] Recent studies have demonstrated the extraordinary resistance of GOS-Lu to gastric acid and hydrolysis by digestive enzymes, being significantly higher than that observed for the traditional GOS-La.[19] The low ileal digestibility of GOS-Lu was due to the greater resistance of galactosyl-fructoses to mammalian digestive enzymes as compared to galactosyl-glucoses, which were mainly present in GOS-La, highlighting the key role played by the monomer composition and linkage type involved in the oligosaccharide chain. These data warrant further investigation on the differential ability of GOS-Lu and GOS-La to modulate the gut microbiota to explore a plausible relationship between patterns of resistance to digestion and their potential prebiotic properties.

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. In this study, we have evaluated the modulatory effects of GOS-Lu on microbial composition in the cecum and colon of growing rats in comparison with GOS-La and control groups, with particular emphasis on their bifidogenic effect.

MATERIALS AND METHODS

Synthesis and Preparation of Galacto-oligosaccharides. An industrially available galacto-oligosaccharides mixture derived from lactose (GOS-La) was used in this study. Due to the presence of high levels of digestible lactose, removal of mono- and disaccharides was performed by using size exclusion chromatography, and the DP of collected fractions was determined by electrospray ionization mass spectrometry (ESI-MS).[20] The trisaccharide fraction of GOS-La (35.1% of total carbohydrates) contained mainly 4′-galactosyl-lactose and 6′-galactosyl-lactose, as well as other galactobioses linked to the reducing glucose unit by 1→2 and 1→6 glycosidic linkages.[19]

With regard to the galacto-oligosaccharides derived from lactulose (GOS-Lu), their enzymatic synthesis was carried out via the hydrolysis and transgalactosylation of lactulose (Duphalac, Solvay Pharmaceuticals, Weesp, The Netherlands) by using a β-galactosidase from *Aspergillus oryzae*.[19] The GOS-Lu mixture was treated with activated charcoal to remove the monosaccharide fraction. According to ESI-MS analysis, GOS-Lu were predominantly dominated by the presence of di- and trisaccharides (31 and 42% of total carbohydrates, respectively), followed by tetra- and pentasaccharides. A detailed characterization and quantification of the major di- and trisaccharide fractions of GOS-Lu was accomplished by gas chromatography−mass spectrometry (GC-MS).[18,19] Thus, the disaccharide fraction was mainly composed of galactosyl-fructoses with 1→1, 1→4 (i.e., lactulose), 1→5, and 1→6 glycosidic linkages, in addition to galactobioses linked by 1→1, 1→2, 1→3, 1→4, and 1→6 glycosidic linkages, whereas the trisaccharide fraction was mainly composed by the trisaccharide 6′-galactosyl-lactulose.

Rats and Diets. Male weaned Wistar rats (Charles River Laboratories, Barcelona, Spain), matched by weight (40 ± 5 g; 4 weeks 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 purified diet (AIN-93G; Testdiet, UK), formulated for use during animal growth, based on corn starch (40%), casein (20%), maltodextrin (13.2%), sucrose (10%), and soybean oil (7%) as the main dietary ingredients. A 6 day pre-experimental adaptation period was followed by a 14 day experimental period. At the end of the adaptation period, rats had an average weight of 75 ± 5 g and subsequently entered the experimental period. Thirty rats were randomly assigned to three dietary groups of 10 animals each and were allowed to consume food and water ad libitum. Diets were AIN-93G (control group), AIN-93G plus 1% (w/w) GOS-Lu (GOS-Lu group), and AIN-93G plus 1% (w/w) GOS-La (GOS-La group).

All of the experimental protocols were reviewed and approved (December 18, 2008) by the Ethics Committee for Animal Research at the Animal Nutrition Unit (EEZ, CSIC, Spain), and the rats were cared for in accordance with the Spanish Ministry of Agriculture guidelines (RD 1201/2005).

Sample Collection. At the end of the dietary intervention period (14 days), rats were deprived of food overnight and then fed 4 g at timed intervals so that time elapsed between feeding and sacrifice was the same for all rats (2 h). Rats were euthanized under sodium pentobarbital (40 mg/kg body weight) anesthesia. Stomach, small intestine, cecum, and colon were dissected out immediately, washed with sterile distilled water, and weighed. Total cecum and colon contents were collected, homogenized, immediately frozen, freeze-dried, weighed, and stored at −80 °C until further analysis.

DNA Extraction from Luminal Samples. Total DNA was isolated from freeze-dried luminal samples (40 mg) of the cecum and colon, using the QIAamp DNA stool kit (Quiagen, West Sussex, UK) and following the manufacturer's instructions. DNA concentration was assessed spectrophotometrically by using a NanoDrop ND-100

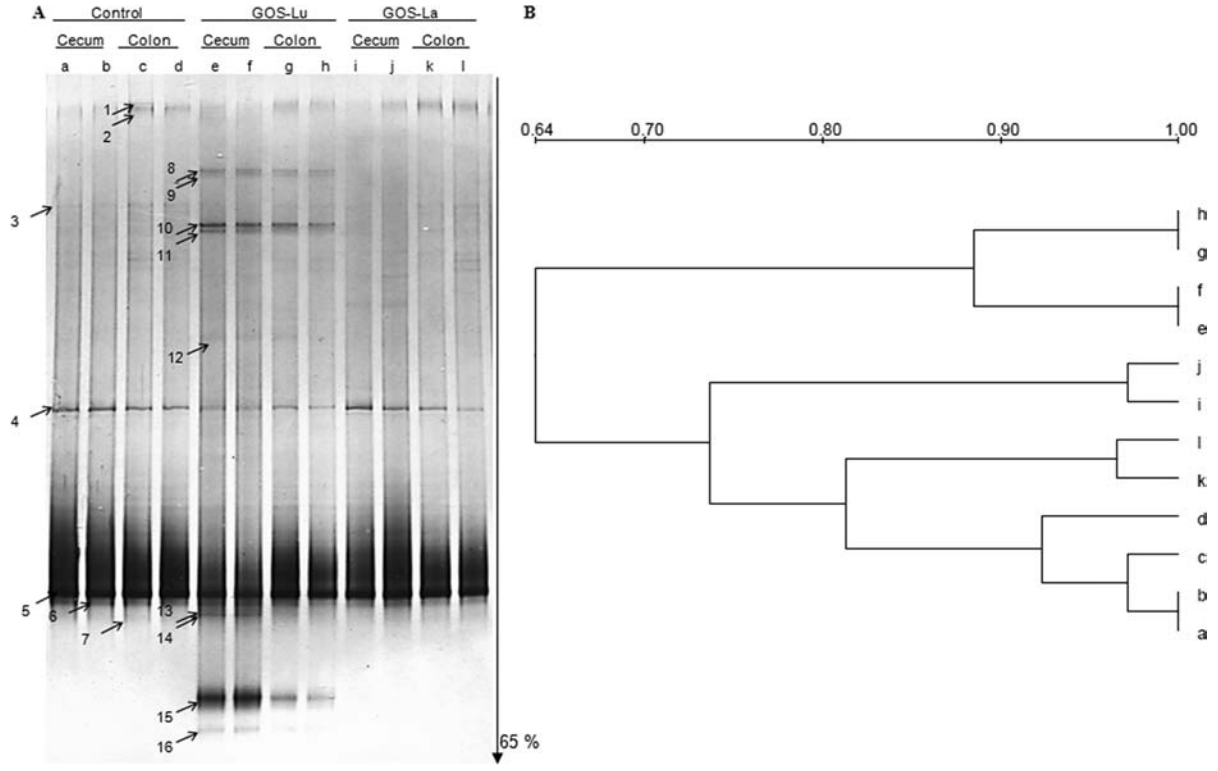

Figure 1. (A) DGGE profiles and (B) dendrogram of the *Bifidobacterium* population from cecal and colonic content of two rats fed control (lanes a−d) and experimental diets (GOS-Lu, lanes e−h; and GOS-La, lanes i−l). Vertical arrow shows the direction and concentration of the denaturing gradient. The numbers indicate the bands extracted from DGGE gel and cloned into p-TOPO. Cluster analysis of DGGE pattern profiles was performed by using the Dice similarity coefficient and the unweighted-pair group method by means of arithmetic average clustering algorithm (UPGMA).

spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Purified DNA samples were stored at −80 °C.

Quantitative PCR (qPCR) Analysis. Quantitative PCR was used to evaluate the effects of GOS-Lu and GOS-La on microbial composition in luminal samples of cecum and colon of rats after 14 days of treatment in comparison with controls. Different microbial groups including total bacteria, *Bacteroides*, lactobacilli, bifidobacteria, *Eubacterium rectale*/*Clostridium coccoides*, and *Clostridium leptum* were distinguished and quantified using qPCR. The 16S rRNA gene-targeted group-specific primers used in this study are listed in Table 1. qPCR assays were performed using an iQ5 Cycler Multicolor PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). The reaction mixture (25 μL) comprised 12.5 μL of iQ SYBR Green Supermix (Bio-Rad), 0.75 μL of each of the specific primers (10 μM; Roche Diagnostics, Barcelona, Spain), 9 μL of sterile distilled water, and 2 μL of DNA template. For total bacteria, *Bacteroides*, bifidobacteria, and lactobacilli, PCR conditions included a first step at 50 °C for 2 min, followed by 95 °C for 10 min for initial denaturation, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min for primer annealing and product elongation. The same PCR conditions were also used for quantitative analysis of the species *Bifidobacterium pseudolongum* and *B. animalis*. The species-specific primer pair for *B. pseudolongum* was 5′-CCCTTTTTCCGGGTCCTGT-3′ and 5′-ATCCGAACTGAGACCGGTT-3′; in the case of *B. animalis*, the primer pair was 5′-GCATGTTGCCAGCGGGTGA-3′ and 5′-ATCCGAACTGAGACCGGTT-3′.[21] In the case of *E. rectale*/*C. coccoides* and *C. 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 for primer annealing and product elongation. A plasmid standard containing the target region was generated for each specific primer set using DNA extracted from pooled fecal samples of rats fed AIN-93G diet. The amplified products were cloned using the TOPO TA cloning kit for sequencing (Invitrogen) and transformed into *E. coli* One Shot Top 10 cells (Invitrogen). Sequences were submitted to the rRNA database to confirm the specificity of the primers. For quantification of target DNA copy number, standard curves were generated using serial 10-fold dilutions of the extracted products by using at least six nonzero standard concentrations per assay. The bacterial concentration in each sample was measured as $\log_{10}$ copy number by the interpolation of the C_t values obtained by the luminal samples and the standard calibration curves. Each plate included triplicate reactions per DNA sample and the appropriate set of standards.

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) Analysis of Bifidobacteria. The 16S rRNA genes were amplified by PCR from extracted DNA of cecum and colon luminal content, using *Bifidobacterium* genus-specific primers Bif164-F (5′-GGGTGG-TAATGCCGGATG-3′) and Bif662-GC-R (5′-CGCCCGCCGCGC-GCGGCGGGCGGGGCGGGGCACGGGGGGCCACCGTTACAC-CGGGAA-3′).[22] PCR amplification conditions used were an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 62 °C for 20 s, and 72 °C for 1 min and a final elongation step at 72 °C for 7 min. The resulting amplicons were visualized on agarose gels to confirm the presence of a PCR product of expected size (520 bp). PCR fragments were separated by DGGE by using a denaturing gradient of 40−65%. The gels were visualized by silver-staining, dried at 37 °C, and scanned. Within an experimental group, the cecal and colonic samples revealed similar DGGE profiles (data not shown). The total number and dendogram of similarity cluster analysis of DGGE bands from cecal and colonic content of two animals per treatment were determined by Quantity One analysis software (Bio-Rad) (Figure 1). Cluster analysis of DGGE profiles was performed using the Dice similarity coefficient and the unweighted-pair group method by means of arithmetic average clustering algorithm (UPGMA). The richness (S) of the bacterial community was determined from the number of bands in each sample. Shannon index (H), an index of diversity, was calculated following the methodology proposed by Buckland et al.[23] as $H = -\sum (p_i \cdot \ln p_i)$,

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Table 2. Effect of Control and Experimental Diets, GOS-Lu and GOS-La, on Cecal and Colonic Microbiota Composition in Growing Rats Fed for 14 Days[a]

bacterial group	cecum			colon		
	control	GOS-Lu	GOS-La	control	GOS-Lu	GOS-La
all bacteria	10.78 (0.26)	10.91 (0.17)	10.90 (0.21)	11.09A (0.27)	11.27AB (0.20)	11.34B (0.15)
Bacteroides	9.79 (0.91)	10.05 (0.62)	10.06 (0.67)	10.86A (0.39)	11.10AB (0.28)	11.20B (0.26)
bifidobacteria	8.52a (1.26)	9.86b (1.02)	8.90ab (0.96)	9.20A (0.74)	10.07B (0.98)	9.29AB (0.97)
lactobacilli	8.98 (1.17)	9.00 (0.91)	9.54 (0.49)	9.29AB (0.64)	9.08A (0.79)	9.70B (0.52)
Eubacterium rectale/*Clostridium coccoides* group	9.63a (0.29)	9.92b (0.21)	9.90b (0.16)	9.67A (0.27)	10.05B (0.25)	10.09B (0.19)
Clostridium leptum group	9.49b (0.22)	9.21a (0.26)	9.19a (0.37)	9.34 (0.35)	9.43 (0.31)	9.41 (0.20)

[a]Data are means (n = 10), expressed as $\log_{10}$ copy number/g of freeze-dried luminal sample. Standard deviation is in parentheses. Within cecum (a, b) or colon sections (A, B), means without a common letter differ (LSD test, $P \leq 0.05$). GOS-Lu, galacto-oligosaccharides derived from lactulose; GOS-La, galacto-oligosaccharides derived from lactose.

Table 3. Diversity Indices of Cecal and Colonic Samples Obtained from Growing Rats Fed Control, GOS-Lu, or GOS-La Diet for 14 Days[a]

	cecum			colon		
	control	GOS-Lu	GOS-La	control	GOS-Lu	GOS-La
richness	9.25a (0.96)	13.00b (0.82)	9.75a (0.50)	6.75A (0.50)	10.50B (1.00)	6.50A (0.58)
Shannon index	2.22a (0.10)	2.56b (0.07)	2.28a (0.05)	1.91A (0.08)	2.35B (0.09)	1.87A (0.09)
evenness index	0.70a (0.03)	0.81b (0.02)	0.71a (0.02)	0.62A (0.03)	0.76B (0.03)	0.61A (0.03)

[a]Data are mean (n = 10). Standard deviation is in parentheses. Within cecum (a, b) or colon sections (A, B), means without a common letter differ (LSD test, $P \leq 0.05$). GOS-Lu, galacto-oligosaccharides derived from lactulose; GOS-La, galacto-oligosaccharides derived from lactose.

where p_i is the proportion of species i in each sample. The evenness (E) of the bacterial community was further estimated as $E = H/\ln S$.

Isolation and Sequencing of the Main DGGE Bands. A selection of the main DGGE bands was excised and eluted by overnight incubation in 20 μL of sterilized distilled water at 4 °C. Two microliters from each tube was used as template to amplify the band of interest by using the primers Bif164-F and Bif662-R: 5′-CCACCG-TTACACCGGGAA-3′ under PCR conditions previously described (see above). PCR products were purified from agarose gels with the NucleoSpin extract II isolation kit (Macherey-Nagel, Germany) and then cloned using the TOPO TA cloning kit for sequencing (Invitrogen). Plasmid DNA was isolated from selected transformants with the GenElute Plasmid Miniprep kit (Sigma-Aldrich), being inserts sequenced by using sequencing primers included in the TOPO TA cloning kit (Invitrogen). Sequence similarity searches were conducted using the BLAST algorithm of the GenBank database (www.ncbi.nlm.nih.gov) to identify the nearest relatives of the partial 16S rRNA gene sequences. A sequence similarity ≥98% of the 16S rRNA gene was used as the criterion for species identification.

Statistical Analysis. Individual rats were considered the experimental unit. The effect of dietary treatment on the microbiota composition of cecal and colonic contents was analyzed by one-way ANOVA. Means that differed significantly ($P \leq 0.05$) were identified using the least significant difference (LSD) test.

RESULTS

Animal Growth Performance. GOS-Lu and GOS-La were incorporated in a single daily dose (1%, w/w) to growing rats (5 weeks old) for a period of 14 days. There were no significant differences in food intake (14.2 ± 0.1 g/day) or body weight gain among groups (4.7 ± 0.1 g/day) at the end of the experimental period. Dietary treatments did not have any significant effect on the relative weight of different organs including the stomach, small intestine, cecum, and colon, except that rats fed GOS-La had a significantly lower ($P < 0.05$) relative colon weight (0.50 ± 0.07) compared with those fed control diets (0.59 ± 0.07).

Effect of GOS-Lu and GOS-La on Microbiota of Cecal and Colonic Contents. Quantitative differences between bacterial groups in luminal samples of cecum and colon of rats fed control, GOS-Lu, and GOS-La diets were assessed by using qPCR. After a period of 14 days, the GOS-Lu diet significantly stimulated ($P < 0.05$) the growth of bifidobacteria in both cecum and colon compared with control group; however, no significant differences in the number of bifidobacteria between GOS-Lu and GOS-La groups within the large intestine were observed (Table 2). In colonic content, the number of lactobacilli was significantly greater ($P < 0.05$) in rats fed GOS-La than in those fed GOS-Lu, but not when compared to controls. In GOS-Lu and GOS-La dietary treatments, the number of *E. rectale*/*C. coccoides* groups in the cecum and colonic content was higher ($P < 0.05$) than in controls, whereas *C. leptum* significantly decreased ($P < 0.05$) in the cecal content of GOS-Lu and GOS-La groups.

To obtain a broader assessment of the impact of the different dietary treatments on the structure of the bifidobacteria population in the colon and cecum, 16S rRNA gene profiles were generated by genus-specific PCR-DGGE. A representative DGGE of cecal and colonic samples from two animals fed control diet, GOS-Lu, and GOS-La is shown (Figure 1A). Dendogram analysis of PCR-DGGE indicated that the cecal and colonic bifidobacteria community patterns were similar and clustered according to the dietary treatment (Figure 1B). Interestingly, the GOS-Lu group clustered separately from the control and GOS-La groups (64% of similarity) and formed an individual cluster with an 88% similarity coefficient. In the case of control and GOS-La groups, the coefficients of similarity within the group were 92 and 74%, respectively. To provide an ecological interpretation of the DGGE pattern, three diversity indices of the DGGE profiles were calculated (Table 3). The number of bands or richness in cecal and colonic samples of rats fed GOS-Lu was significantly higher ($P < 0.05$) compared to those from rats fed control or GOS-La diets. Shannon and evenness indices followed the same pattern in the cecum and colon for each treatment.

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Table 4. Identification of Bifidobacteria Species from Isolated DGGE Bands[a]

DGGE band[b]	presence	identity of "best hits" deduced from sequences[c]	identity (%)
1	all treatments	uncloned	
2	all treatments	uncloned	
3	all treatments	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	99−100
4	all treatments	*B. pseudolongum* subsp.*pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	98−99
5	all treatments	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	99
6	all treatments	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	98−99
7	all treatments	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	99
8	only in GOS-Lu[d]	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	99
9	only in GOS-Lu	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	99−100
10	only in GOS-Lu	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	99−100
11	only in GOS-Lu	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	99
12	only in GOS-Lu	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	98−99
13	only in GOS-Lu	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	99
14	only in GOS-Lu	*B. pseudolongum* subsp. *pseudolongum* 16S rRNA, partial sequence, strain: JCM 7072	99
15	only in GOS-Lu	*B. animalis* subsp. *animalis* 16S rRNA, partial sequence, strain: JCM 1190	99
16	only in GOS-Lu	*B. animalis* subsp. *animalis* 16S rRNA, partial sequence, strain: JCM 1190	98−99

[a]At least three clones obtained from each individual DGGE band for identification were analyzed. [b]Numbers correspond to DGGE bands of Figure 1. [c]The first species to appear on the BLAST output from NCBI (www.ncbi.nlm.nih.gov/BLAST/) is shown. The accession numbers of *16 rRNA* sequences corresponding to *B. pseudolongum* subsp. *pseudolongum* and *B. animalis* subsp. *animalis* are AB507147.1 and AB507070.1, respectively. [d]GOS-Lu, galacto-oligosaccharides derived from lactulose.

Table 5. Effect of Control and Experimental Diets, GOS-Lu and GOS-La, on Intestinal *Bifidobacterium pseudolongum* and *Bifidobacterium animalis* Composition in Growing Rats Fed for 14 Days[a]

	cecum			colon		
	control	GOS-Lu	GOS-La	control	GOS-Lu	GOS-La
B. pseudolongum	8.72 (1.23)	9.42 (1.14)	8.95 (0.91)	7.43 (0.69)	7.48 (1.07)	7.30 (0.95)
B. animalis	8.14a (0.87)	9.58b (1.14)	8.44a (0.70)	6.84A (0.60)	7.84B (1.15)	6.96A (0.77)

[a]Data are means (n = 10), expressed as $\log_{10}$ copy number/g of freeze-dried fecal sample. Standard deviation is in parentheses. Within cecum (a, b) or colon (A, B), means without a common letter differ (LSD test, $P \leq 0.05$). GOS-Lu, galacto-oligosaccharides derived from lactulose; GOS-La, galacto-oligosaccharides derived from lactose.

The bands excised for sequencing are numbered in Table 4. Some of the predominant bands (1−7) were shared in all groups; however, GOS-Lu showed up to nine specific bands (8−16). The electrophoretic bands present in cecal and colonic samples and corresponding to all treatments (3−7) and those present only in the GOS-Lu group (8−14) were identified as *B. pseudolongum* subsp. *pseudolongum* (≥98% identity) (Table 4). Two electrophoretic bands (15 and 16) were exclusively present in cecal and colonic samples of rats fed GOS-Lu, both being identified as *B. animalis* subsp. *animalis* (≥98% identity). In addition, qPCR analysis of *B. pseudolongum* and *B. animalis* from cecal and colonic samples of rats fed different dietary treatments was performed. The number of *B. animalis* was significantly greater ($P < 0.05$) in GOS-Lu than in GOS-La or control groups (Table 5).

DISCUSSION

GOS are dietary nondigestible, at least partially, carbohydrates capable of modulating the growth and/or metabolic activities of the gut microbiota to confer health-promoting effects to the host. Commercially available GOS-La have been demonstrated to be resistant to the extreme conditions within the gastrointestinal tract and to stimulate selectively the growth of bifidobacteria and/or lactobacilli in infants,[24] healthy adults,[11] and elderly people.[25] In addition, it has been suggested that GOS-La could alleviate certain symptoms of irritable bowel syndrome[26] and reduce colitis severity in *Smad3*-deficient mice treated with the pathogen *Helicobacter hepaticus*.[27]

Little is known about how structural differences influence the bifidogenic properties of GOS. The administration of a GOS-La mixture, containing mainly β-(1→3) as well as β-(1→4) and β-(1→6) glycosidic linkages, to healthy human volunteers proved to have a higher bifidogenic effect than a mixture having β-(1→4) and β-(1→6) linkages only.[12] In our study, two GOS types (GOS-Lu and GOS-La) having significant differences in terms of oligosaccharides composition (monomer and linkage type) and, associated with that, different resistance to digestions were evaluated.[18,19] In the case of GOS-Lu, the presence of highly resistant galactobioses and galactosyl-fructoses with the ability to reach the large intestine intact as fermentable substrates for the resident intestinal microbiota has been recently demonstrated.[19] When compared with GOS-Lu, GOS-La showed a significantly lower resistance to in vivo digestion; the glycosidic linkages Gal-(1→6)-Glc and Glc-(1→6)-Glc were shown to be more resistant to gastrointestinal digestion than the linkage type Gal-(1→4)-Gal. Interestingly, both GOS types have demonstrated to be readily fermented within the large intestine, as they were not detected in fecal samples, enabling them to have a potential prebiotic function. In this study, we have assessed the modulatory influence of GOS-La and GOS-Lu in gut microbiota of growing rats after a period of 14 days of treatment. GOS-Lu treatment significantly ($P < 0.05$) increased the bifidobacterial population in luminal content of the cecum and colon compared to controls, whereas no significant differences between both GOS groups within the large intestine were observed (Table 2). Such differential behaviors could be

related to the higher resistance to in vivo gut digestion and absorption in the small intestine of GOS-Lu when compared to GOS-La under the same experimental conditions and also to a dissimilar fermentation selectivity of both GOS types according to their structural differences.[18,19] The bifidogenic effect of GOS-Lu was exerted at moderate and rational doses (1%, w/w). This fact could be very advantageous to avoid possible side effects, such as intestinal discomfort from gas production, abdominal disorders, and diarrhea, after prebiotic intake at higher doses, compromising its potential prebiotic effect. GOS-La did not increase the bifidobacterial population in the large intestine of rats compared to control diet, probably because a higher dose is necessary to achieve a bifidogenic effect for the period of treatment tested (14 days). It has been suggested that variations in the daily dose may contribute to differences in the modulatory effect on gut microbiota. In the case of GOS-La, a dose-dependent bifidogenic effect of GOS-La has been previously reported; when administrated to healthy human volunteers at daily doses of 5 g or higher, GOS-La exerted a bifidogenic effect as observed in fecal samples, whereas a dose of 2.5 g had no significant effect.[28]

It is noteworthy that the feeding of GOS-Lu and GOS-La diets resulted in a significant decrease ($P < 0.05$) of cecal bacteria belonging to the *C. leptum* group (Table 2). A high proportion of these micro-organisms may be pathogenic through their proteolytic capabilities and toxin production. The *E. rectale*/*C. coccoides* group was significantly increased ($P < 0.05$) in the intestinal contents of rats fed GOS-Lu or GOS-La as compared to control. This bacterial group, which is also predominant in the human gut, includes species that are known as butyrate-producing bacteria, thereby contributing to important processes linked to colonic health, including protection against inflammatory bowel disease and colorectal cancer.

The reported changes in bifidobacteria population might be considered a major shift in the gut microbiota toward a potentially healthier composition.[29,30] Bifidobacteria are thought to exert a protective role against pathogens via production of antimicrobial agents and/or blocking of adhesion of pathogens, to promote gut integrity, and to modulate the host immune response. However, not all bifidobacteria are likely to be able to utilize or compete for these GOS. In this work, we have investigated which bifidobacteria species are selectively affected by dietary GOS in the large intestine of rats. By sequence analysis of PCR-DGGE bands, *B. animalis* subsp. *animalis* was found in cecal and colonic samples of rats fed GOS-Lu only (Figure 1). Although the absence of these bands in cecal and colonic samples of rats fed control or GOS-La diets does not exclude its presence, it does indicate that this particular species was not dominant in those groups. Several electrophoretic bands, which were present in all treatments, were identified as *B. pseudolongum* subsp. *pseudolongum*. Sequence analysis of such electrophoretic bands demonstrated small differences in a few nucleotides being responsible for differential migration behavior in the gel (data not shown). The detection of multiple bands associated with single isolates has been previously reported by Satokari et al.,[22] who described up to three different bands for *B. adolescentis*: two different operons of the 16rRNA gene differing by a few nucleotides and a third band containing heteroduplexes of both sequences. Species of the *Bifidobacterium* genus have shown between one and five operon copies of the 16S rRNA, which differ in their sequences.[31] The presence of a doublet with two adjacent bands due to an abortion of the elongation reaction during PCR caused by the GC clamp has also been reported to affect band migration. By qPCR analysis, no significant differences were observed in the levels of *B. pseudolongum* in intestinal samples of rats fed GOS compared to those fed control diet. In agreement with qualitative analysis derived from PCR-DGGE, the GOS-Lu group had significantly more *B. animalis* in intestinal samples than control or GOS-La groups (Table 5). This species evolved to be a dominant community member in rats fed GOS-Lu for a period of 14 days. This could be attributed to the selective ability of this species to metabolize specific oligosaccharides present in GOS-Lu. Although access to genome sequences has increased our knowledge of the biochemical capabilities within the genus *Bifidobacterium*, little is still known about the carbohydrate-degrading enzymes of bifidobacteria involved in the breakdown of oligosaccharides. Most of the enzymes described are α-galactosidases, β-galactosidases, and enzymes active against gluco-oligosaccharide.[32] Further studies regarding the bifidobacterial enzymes involved in the selective degradation of GOS-Lu could help us in the development of novel prebiotics with enhanced bifidogenic properties.

Our previous findings showed that GOS-Lu are resistant to gastric acidity, to hydrolysis by mammalian digestive enzymes, and to gastrointestinal absorption, being fermented by gut microbiota.[19] In the present work, we have demonstrated that GOS-Lu selectively stimulate the growth and/or activity of bifidobacteria and *E. rectale*/*C. coccoides* group in the intestinal microbiota, which could contribute to the health and well-being of the host. Given the differences in the digestive physiology between rats and humans, the results obtained cannot per se predict the prebiotic responses in humans. Further intervention studies with human volunteers can be expected to shed more light on the prospective applications of novel GOS-Lu as commercial prebiotics. Finally, it should also be kept in mind that special efforts have to be addressed toward the better understanding of the mechanism through which prebiotics affect positively the gut health of the host. Recent applications of health claims based on bifidogenic effects have been rejected by European regulatory bodies. The identification of clear and measurable effects in clinical end points and/or accepted biomarkers for beneficial effects on the host rather than the selective stimulation of nonpathogenic microorganism, including lactobacilli and bifidobacteria, would be necessary.[33] The difficulty still remaining in prebiotic research is to demonstrate that their claimed health benefits are directly associated with observed changes in the gut microbiota composition.[34]

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

DGGE, denaturing gradient gel electrophoresis; DP, degree of polymerization; ESI-MS, electrospray ionization mass spectrometry; GC-MS, gas chromatography−mass spectrometry; GOS, galacto-oligosaccharides; GOS-La, galacto-oligosaccharides derived from lactose; GOS-Lu, galacto-oligosaccharides derived from lactulose

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■ NOTE ADDED AFTER ASAP PUBLICATION

This article published July 25, 2013 with an error in the second paragraph of the Materials and Methods section, noting the percentages of total carbohydrates. The correct version published July 26, 2013.