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In Vitro Fermentation of Lactulose-Derived Oligosaccharides by Mixed Fecal Microbiota

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ABSTRACT: Fermentation properties of oligosaccharides derived from lactulose (OsLu) and lactose (GOS) have been assessed in pH-controlled anaerobic batch cultures using lactulose and Vivinal-GOS as reference carbohydrates. Changes in gut bacterial populations and their metabolic activities were monitored over 24 h by fluorescent in situ hybridization (FISH) and by measurement of short-chain fatty acid (SCFA) production. Lactulose-derived oligosaccharides were selectively fermented by *Bifidobacterium* and lactic acid bacterial populations producing higher SCFA concentrations compared to GOS. The highest total SCFA production was from Vivinal-GOS > lactulose > OsLu > GOS. Longer incubation periods produced a selective fermentation of OsLu when they were used as a carbon source reaching the highest selective index scores. The new oligosaccharides may constitute a good alternative to lactulose, and they could belong to a new generation of prebiotics to be used as a functional ingredient for improving the composition of gut microflora.

KEYWORDS: *lactulose, oligosaccharides, fermentation, gut microbiota, short-chain fatty acids*

INTRODUCTION

It is well recognized that the human intestinal microbiota plays an important role in human health and disease, and prebiotics can pass unaltered through the small intestine and enter the colon, where they are selectively fermented by the resident beneficial microflora with the associated health benefits. Thus, in the past few years, there has been an increased interest in these functional ingredients, and a number of oligosaccharides have been tested to find new prebiotics with improved or complementary properties such as arabinoxylans,[1] xylooligosaccharides,[2−4] and pectic oligosaccharides.[5] Currently, there is a range of prebiotic carbohydrates on the market, most of them isolated from plant polysaccharides such as inulin and fructooligosaccharides (FOS) or enzymatically synthesized such as galacto-oligosaccharides (GOS) obtained from lactose by glycosyl transfer catalyzed by β-galactosidases from different microorganisms.[6−8]

Lactulose (4-*O*-β-D-galactopyranosyl-D-fructose) is a synthetic sugar that does not occur naturally and is obtained by chemical synthesis from lactose in basic media using different types of catalyst. This disaccharide can also be produced enzymatically using β-galactosidases from different sources using lactose as a glycosyl donor and fructose as an acceptor.[9] Industry has developed many uses for lactulose, and the potential world production is estimated at about 50000 tons per year.[10]

Lactulose is one of the most studied prebiotics; its beneficial effects in the human large intestine have been known for more than 50 years.[11] It is resistant to hydrolysis by human small intestinal disaccharidases, so it reaches the proximal colon unaltered, where it is selectively metabolized by bifidobacteria and lactobacilli, giving rise to the formation of carbon dioxide, hydrogen gas, and short-chain fatty acids (SCFA), causing an increase in fecal biomass and a decrease in pH.[9] However, the distal region of the colon is known to be the site of origin of a number of chronic diseases including colon cancer and ulcerative colitis;[12] therefore, there is great interest in the development of prebiotics able to act in this part of the gut. Several studies investigating the prebiotic properties of carbohydrates have shown the influence of the chemical structure of oligosaccharides (degree of polymerization, sugar monomeric composition, glycosidic linkage, and degree of branching) on fermentation rate.[13]

Recently, the enzymatic synthesis of oligosaccharides derived from lactulose (OsLu) has been described, aiming at the production of a group of more slowly fermenting prebiotics.[14−16] In fact, Cardelle-Cobas et al.[17,18] have demonstrated the bifidogenic properties of mixtures of oligosaccharides derived from lactulose (DP $\geq$ 3) and the fermentative properties of *Lactobacillus* and *Bifidobacterium* toward pure trisaccharides derived from lactulose; however, the selective growth of the most important human gut microbiota has not yet been studied. Therefore, in this study an assessment of the prebiotic properties of OsLu, using human fecal bacteria, was carried out. A comparison of the fermentation profiles of lactulose, oligosaccharides derived from lactose (GOS), and commercial galacto-oligosaccharides (Vivinal-GOS) has also been performed.

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Table 1. Oligonucleotide Probes Used for Fluorescent in Situ Hybridization (FISH) Enumeration of Bacteria.[21]

target organism	probe	sequence from 5′ to 3′ end	hybridization temperature (°C)	ref
Bifidobacterium spp.	*Bif164*	CATCCGGCATTACCACCC	50	22
Bacteroides/Prevotella group	*Bac303*	CCAATGTGGGGGACCTT	45	23
Lactobacillus/Enterococcus	*Lab158*	GGTATTAGCA(T/C)CTGTTTCCA	45	24
Clostridium histolyticum group (*Clostridium* clusters I and II)	*His150*	TTATGCGGTATTAATCA(C/T)CCTTT	50	25
Atopobium cluster	*ATO291*	GGTCGGTCTCTCAAACC	50	26
Eubacterium rectale/Clostridium coccoides group	*Erec482*	GCTTCTTAGTCARGTACCG	37	25

MATERIALS AND METHODS

Reagents. Lactose monohydrate and lactulose (>98% purity) were supplied by Scharlau Chemie S.A. (Barcelona, Spain) and Fluka (Steinheim, Germany), respectively. Soluble enzymatic preparations of β-galactosidase from *Aspergillus aculeatus* (Pectinex Ultra SP-L) and *Kluyveromyces lactis* (Lactozym 3000 L HP G) were kindly provided by Novozymes A/S (Dittingen, Switzerland, and Bagsvaerd, Denmark). A commercial mixture of GOS, Vivinal-GOS, was a gift from Friesland Food Domo (Hanzeplein, The Netherlands). Bio-Gel P-2 fine grade with bead size <45 μm and molecular weight (MW) fractionation range 100−1800 Da was purchased from Bio-Rad Laboratories (Hemel Hempstead, Hertfordshire, U.K.).

Enzymatic Synthesis and Purification of Oligosaccharides Derived from Lactose and Lactulose. Oligosaccharides derived from lactose (GOS-1) and lactulose (OsLu-1) using Lactozym 3000 L HP G and oligosaccharides derived from lactose (GOS-2) and lactulose (OsLu-2) using Pectinex Ultra SP-L were prepared and purified following the methods described by Cardelle-Cobas et al.[14,15] and Martínez -Villaluenga et al.[16,19]

Analysis of Synthesized Oligosaccharides. Reaction mixtures of oligosaccharides obtained from lactose and lactulose hydrolysis and commercial Vivinal-GOS, as well as isolated fractions of each mixture and purified Vivinal-GOS, were analyzed by HPAEC-PAD on an ICS2500 Dionex system consisting of a GP50 gradient pump and an ED50 electrochemical detector with a gold working electrode and Ag/AgCl reference electrode. Data acquisition and processing were performed with a Chromeleon version 6.7 software (Dionex Corp., Sunnyvale, CA). For eluent preparation, Milli-Q water (Millipore Ibérica, S.A.U., Madrid, Spain), 50% (w/v) NaOH (Fluka, Germany), and NaOAc (Fluka, Germany) were used. All eluents were degassed by flushing with He for 25 min. Separations were performed following the method described by Splechtna et al.[20] Elution was at room temperature on a CarboPac PA-1 column (250 × 4 mm) connected to a CarboPac PA-1 (50 × 4 mm) guard column. Eluent A (100 mM NaOH), eluent B (100 mM NaOH and 50 mM NaOAc), and eluent C (100 mM NaOH and 1 M NaOAc) were mixed to form the following gradient: 100% A from 0 to 20 min and from 0 to 100% B from 20 to 70 min. After each run, the column was washed for 10 min with 100% of eluent C and re-equilibrated for 15 min with the starting conditions of the gradient used. Detection time and voltage parameters were set as follows: E_1 = 0.1 V (t_1 = 400 ms), E_2= 2.0 V (t_2 = 10 ms), E_3 = 0.6 V, E_4= −0.1 V (t_4 = 60 ms); t_t = 500 ms.

Samples and standard solutions were filtered through a nylon Millipore FH (0.45 μm) (Bedford, MA) before injection. A total of 25 μL was injected using an autosampler, and separations were performed at a rate of 1 mL/min. Quantification of oligosaccharides (DP ≥ 3) was performed by external calibration using lactose and lactulose calibration curves.

Batch Culture Fermentation. Fermentation vessels (80 mL) were maintained anaerobically, at 37 °C, by constant sparging with O_2-free N_2. The pH was controlled at pH 6.7 by means of a pH controller (Fermac 260; Electrolab, Tewkesbury, U.K.), which added 1 mol/L NaOH or HCl as appropriate. Carbohydrates (200 mg) were dissolved in autoclaved nutrient basal medium to give a final concentration of 1% (w/v). This medium contained, per liter, 2 g of peptone water (Oxoid Ltd., Basingstoke, U.K.), 2 g of yeast extract (Oxoid), 0.01 g of NaCl (BDH), 0.04 g of K_2HPO_4 (BDH), 0.04 g, of KH_2PO_4 (BDH), 0.01 g of $MgSO_4{\cdot}7H_2O$ (BDH), 0.01 g of $CaCl_2{\cdot}2H_2O$ (BDH), 2 g of $NaHCO_3$ (BDH), 2 mL of Tween 80 (BDH), 0.05 g of Haemin (Sigma, Dorset, U.K.), 10 μL of vitamin K_1 (Sigma), 0.5 g of cysteine-HCl, 0.5 g of bile salts (Oxoid), and 1 mg of Resazurin (Sigma). Samples were inoculated with 2 mL of fecal slurry, which was prepared by homogenizing fresh human feces from healthy donors (10%, w/v) in phosphate-buffered saline (PBS; 0.1 M, pH 7.0) using a stomacher (model 6041; Seward Scientific, U.K.) for 120 s. Three donors, one male and two females aged 25−30 years, who did not have any history of gastrointestinal disorders and had avoided probiotics, prebiotics, and antibiotics for at least 3 months prior to the study, were chosen. Samples (1 mL) were removed after 0, 5, 10, and 24 h of incubation for enumeration of bacteria (FISH) and short-chain fatty acids (SCFA) and lactic acid analysis.

Enumeration of Bacteria. Bacterial populations were counted using fluorescent in situ hybridization (FISH). Samples (375 μL) were fixed overnight at 4 °C with 4% (w/v) filtered paraformaldehyde (pH 7.2) at a ratio of 1:3 (v/v). Samples were then centrifuged at 8973*g* for 5 min, and cells were washed twice with filtered PBS (0.2 M, pH 7), resuspended in 300 μL of a mixture of PBS/99% ethanol (1:1 v/v), and stored at −20 °C until further analysis. Hybridization of the samples was carried out as described previously[21] using appropriate genus-specific 16S rRNA-targeted oligonucleotide probes labeled with the fluorescent dye Cy3 (MWG Biotech, Germany) for the different bacteria or with the nucleic acid stain 4′,6-diamidino-2-phenylindole (DAPI; Sigma) for total cell counts. Probes used for each of the bacteria, previously validated by different authors,[22−26] are shown in Table 1.

Analysis of SCFA. Analysis of SCFA (acetic, propionic, and butyric acids) and lactic acid was carried out by high-performance liquid chromatography with refractive index detection (HPLC-RI). Separation was performed in a Phenomenex PCOA organic acid column (Phenomenex, Chester, U.K.) maintained at 85 °C at a flow rate of 0.6 mL/min using 0.005 mol/L H_2SO_4 in HPLC grade water as eluent. Before analysis, samples were centrifuged at 13400*g* for 5 min to remove bacteria and particulate matter, and 20 μL was injected into the HPLC system (Hewlett-Packard HP 1050 series). Quantification of the samples was carried out using calibration curves for acetic, propionic, butyric, and lactic acids at concentrations ranging from 5 to 100 mM.

Statistical Analysis. Statistical analysis was performed using SPSS for Windows version 15.0 (IBM SPSS, Chicago, IL). Univariate analysis of variance (ANOVA) and the DMS test were also used to determine significant differences among bacterial populations using the various oligosaccharides. Differences were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

The hydrolysis and transglycosylation reactions of lactose and lactulose using β-galactosidases from commercial preparations Lactozym 3000 L HP G and Pectinex Ultra SP-L gave rise to complex mixtures of monosaccharide, disaccharides, trisaccharides with linkages $\beta(1\rightarrow6)$, $\beta(1\rightarrow4)$, $\beta(1\rightarrow1)$, and $\beta(1\rightarrow3)$, and other unidentified oligosaccharides.[17] After hydrolysis, reaction mixtures were submitted to a purification process to obtain enriched oligosaccharide fractions. The carbohydrate composition of reaction mixtures of lactose and lactulose and commercial Vivinal-GOS before and after purification is shown

Table 2. Oligosaccharide Composition of Commercial Vivinal-GOS and Reaction Mixtures of Lactose and Lactulose Hydrolysis before and after Purification Process

	oligosaccharide composition		
sample[a]	in reaction mixtures, before purification (% total carbohydrates)	in isolated fractions (% total carbohydrates)[b]	main glycosidic linkages in the mixture
Vivinal-GOS	16% monosaccharides	39% (trisaccharides:	
	10% lactose	25% 4′-galactosyl-lactose +	β (1→4)
	10% 4′-galactosyl-lactose	14% 6′-galactosyl-lactose)	
	6% 6′-galactosyl-lactose	61% OGOS	β (1→6)
	58% OGOS[c]		
GOS-1	49% monosaccharides		
	21% lactose	81% 6′-galactosyl-lactose	β (1→6)
	17% 6′-galactosyl-lactose	19% OGOS	
	13% OGOS		
GOS-2	25% monosaccharides	42% 6′-galactosyl-lactose	β (1→6)
	52% lactose	8% 4′-galactosyl-lactose	
	17% 6′-galactosyl-lactose	50% OGOS	
	6% OGOS		
OSLu-1	31% monosaccharides		
	42% lactulose[d]	36% 6′-galactosyl-lactulose	β (1→6)
	10% 6′-galactosyl-lactulose	47% 1-galactosyl-lactulose	
	11% 1-galactosyl-lactulose	17% OOSLu	β (1→1)
	6% OOSLu[e]		
OSLu-2	35% monosaccharides		
	36% lactulose	47% 6′-galactosyl-lactulose	β (1→6)
	15% 6′-galactosyl-lactulose	6% 1-galactosyl-lactulose	
	2% 1-galactosyl-lactulose	47% OOSLu	
	12% OOSLu		

[a]GOS-1, lactose-derived oligosaccharides obtained using Lactozym 3000 L HP G; GOS-2, lactose-derived oligosaccharides obtained using Pectinex Ultra SP-L; OsLu-1, lactulose-derived oligosaccharides obtained using Lactozym 3000 L HP G; OsLu-2, lactulose-derived oligosaccharides obtained using Pectinex Ultra SP-L. [b]Data from Cardelle-Cobas et al.[17] [c]OGOS, unidentified GOS + disaccharides different from lactose. [d]Lactulose, gal β (1→4) fru. [e]OOSLu, unidentified OSLu + disaccharides different from lactulose.

in Table 2. The main oligosaccharide formed by trans-galactosylation of lactose was 6′-galactosyl-lactose for both enzymatic preparations. For lactulose, two trisaccharides, 6′-galactosyl-lactulose and 1-galactosyl-lactulose, were obtained, the former being the most abundant when the reaction was carried out with Pectinex Ultra SP-L. Similar amounts of both oligosaccharides were found in the case of transgalactosylation of lactulose with Lactozym 3000 L HP G. Commercial Vivinal-GOS presented 4′-galactosyl-lactose as the main oligosaccharide in addition to 6′-galactosyl-lactose (Table 2). In commercial Vivinal-GOS the carbohydrate content was 16% monosaccharides (glucose and galactose), 10% lactose, and 74% GOS (disaccharides different from lactose and oligosaccharides with DP $\geq$ 3).

Changes in Bacterial Concentrations. Although it is very difficult to fully characterize all changes occurring in the colonic microflora, monitoring of populations of selected species could be an indicator of the state of colon health.[27] The growth of fecal bacteria on the synthesized and purified oligosaccharides derived from lactose and lactulose was tested and compared with those produced by lactulose and enriched fraction of Vivinal-GOS used as reference. Figures 1 and 2 show the changes in bacterial counts at 0, 5, 10, and 24 h of incubation for each substrate and bacterial group tested. High levels of total bacteria were maintained in all incubated substrates. From 0 to 5 h of incubation, no significant changes ($P > 0.05$) in the bacterial growth of enumerated groups were observed except for the *Lab158* group, which grew in purified fractions of Vivinal GOS, GOS-1, lactulose, OsLu-1, and OsLu-2, and for the *Ato291* bacterial group, which also grew in purified fractions of oligosaccharides GOS-1 and OsLu-1.

At 10 h of fermentation, however, the differences in bacterial populations compared to 0 h of fermentation were significantly higher; thus, an increase of *Lab158* counts was observed in all substrates studied. Also, an increase of *Bif164* was observed in Vivinal-GOS ($P < 0.05$) and lactulose-derived oligosaccharides (OsLu-1 and OsLu-2) ($P < 0.05$). An increase of these lactic bacteria and a significant decrease of *Bac303* were found in mixed continuous fecal cultures using lactulose as a carbon source.[7] Taking into account only the synthesized oligosaccharides, OsLu-1 (Figure 1B), OsLu-2 (Figure 1), and GOS-2 (Figure 2C) produced the highest values for bifidobacteria population. In these oligosaccharides a significant decrease ($P < 0.05$) on growth of *His150*, *Ato291* and *Erec482* populations was also found. *Bac303* concentrations remained stable throughout the entire fermentation time for all substrates studied and did not show significant changes ($P > 0.05$). *Bacteroides* is one of the predominant colonical bacteria genera comprising about 30% of the total culturable microflora.[28]

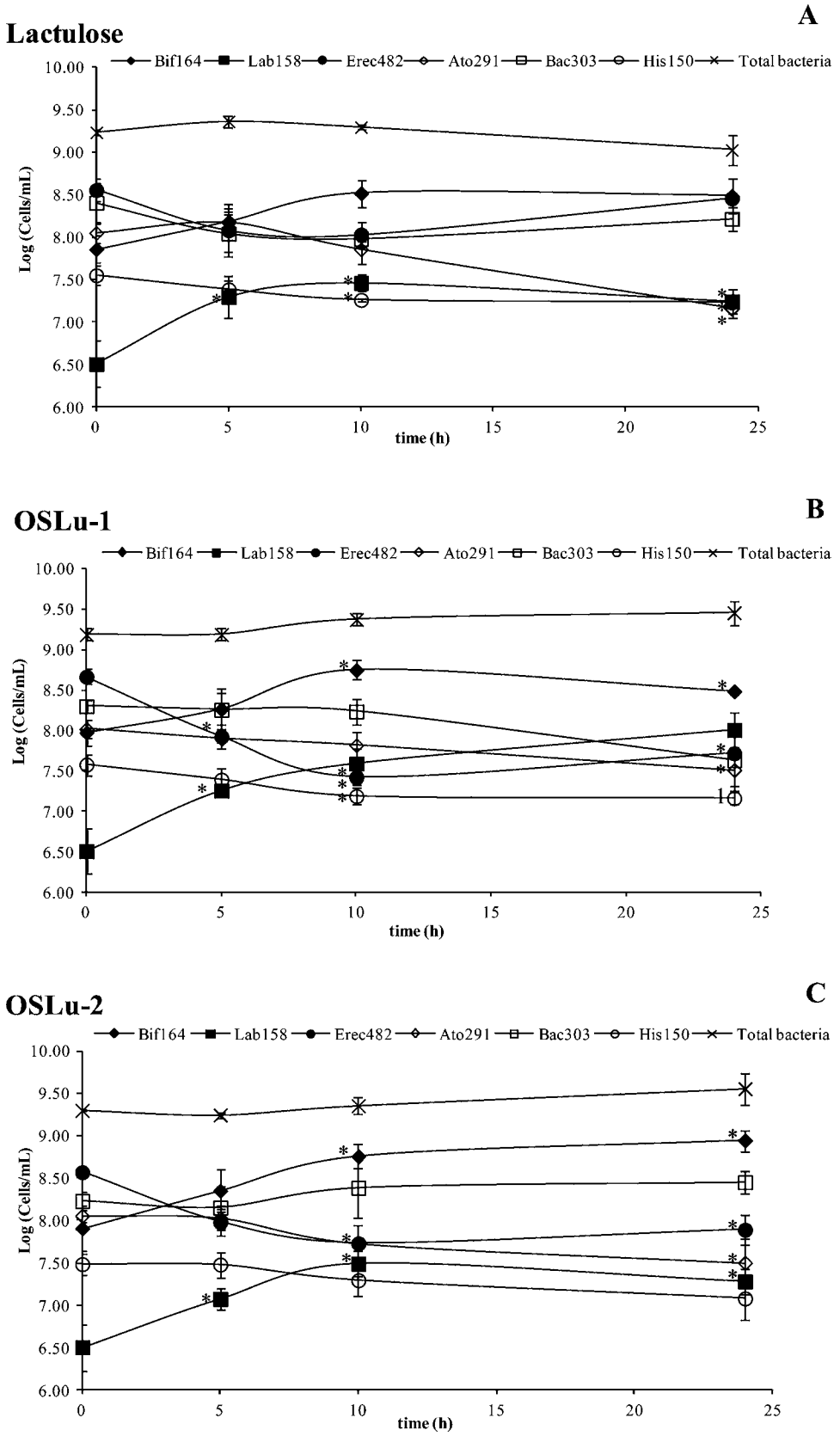

Figure 1. Changes in bacterial counts in batch culture fermentations (24 h) in the presence of (A) lactulose, (B) OSLu-1 (enriched fraction of oligosaccharides derived from hydrolysis and transglycosylation of lactulose using Lactozym 3000 L HP G), and (C) OsLu-2 (enriched fraction of oligosaccharides derived from hydrolysis and transglycosylation of lactulose using Pectinex Ultra SP-L). Bif, *Bifidobacterium* spp.; Lab, *Lactobacillus/Enterococcus*; Erec, *Eubacterium*; Bac, *Bacteroides*; His, *Clostridium*; Ato, *Atopobium*. (∗) Significant differences ($P < 0.05$).

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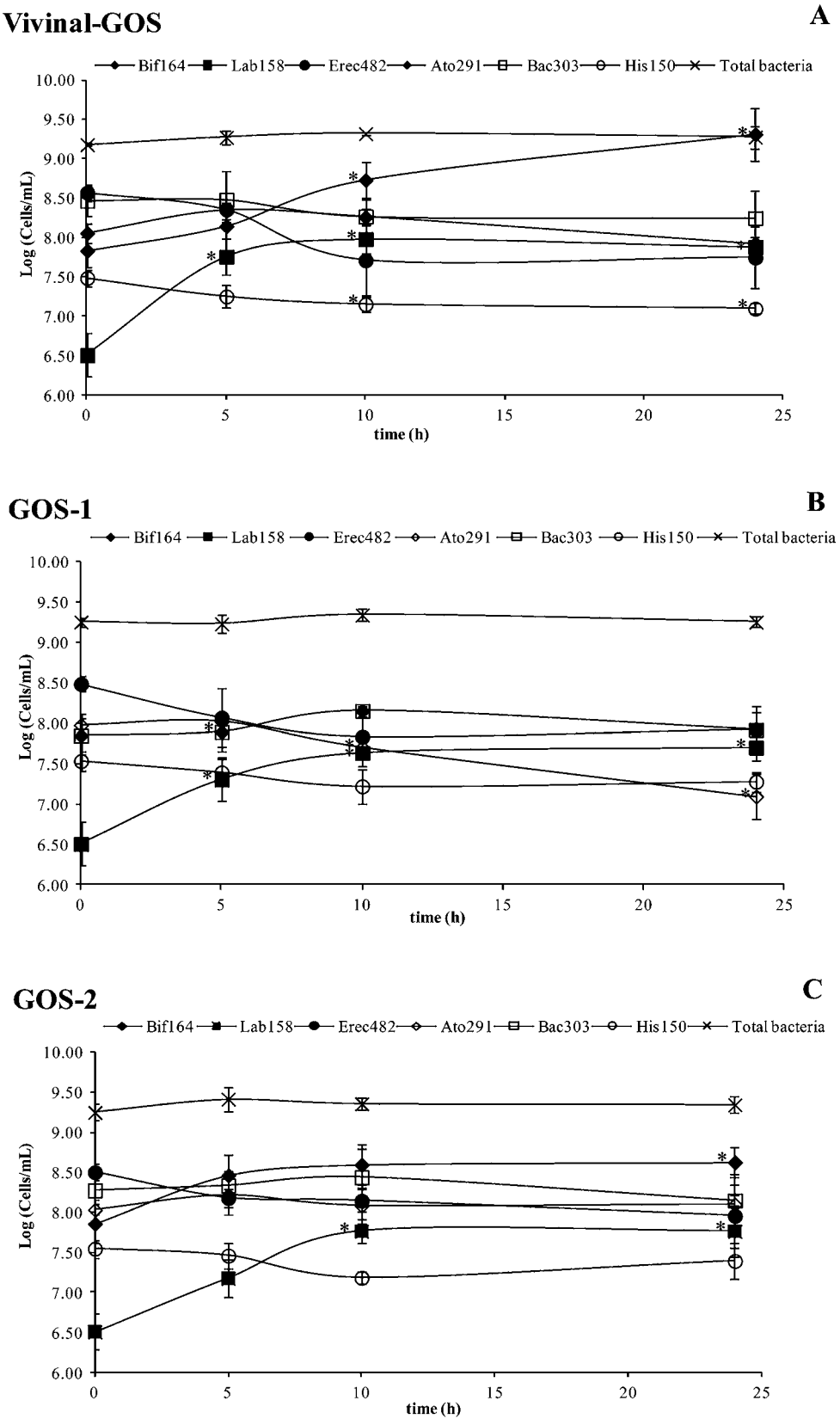

Figure 2. Changes in bacterial counts in batch culture fermentations (24 h) in the presence of (A) enriched fraction of oligosaccharide from commercial Vivinal-GOS, (B) GOS-1 (enriched fraction of oligosaccharides derived from lactose hydrolysis and transglycosylation using Lactozym 3000 L HP G), and (C) GOS-2 (enriched fraction of oligosaccharides derived from lactose hydrolysis and transglycosylation using Pectinex Ultra SP-L). Bif, *Bifidobacterium* spp.; Lab, *Lactobacillus/Enterococcus*; Erec, *Eubacterium*; Bac, *Bacteroides*; His, *Clostridium*; Ato, *Atopobium*. (∗) Significant differences ($P < 0.05$).

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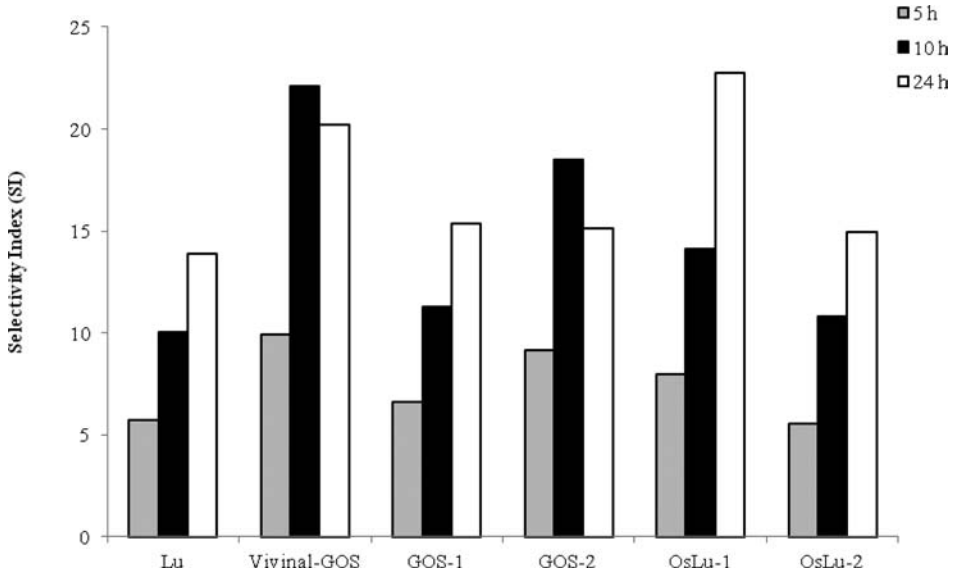

Figure 3. Selective index (SI) scores from pH-controlled batch culture fermentations (5, 10, and 24 h) of lactulose (Lu), enriched fraction of oligosaccharides derived from lactose (GOS-1 and GOS-2), lactulose (OsLu-1 and OsLu-2), and commercial Vivinal-GOS. 1, Oligosaccharides obtained using Lactozym 3000 L HP G; 2, oligosaccharides obtained using Pectinex Ultra SP-L.

At 24 h of fermentation all bacterial counts remained constant or even decreased with respect to 10 h of fermentation and showed the most significant differences for *Bac303* and *Ato291*. Purified oligosaccharides from commercial Vivinal-GOS, GOS-2, OSLu-1, and OsLu-2 favored the growth of *Bif164*. According to these results, to establish an optimal fermentation time, is necessary to consider the type of oligosaccharide used as the carbon source.

Moreover, significant differences were calculated between substrates (synthesized and commercial oligosaccharides) in regard to the growth of each bacterial genus tested and incubated at 10 and 24 h. The *Bif164* population increment was statistically different from 0 h of fermentation for all substrates studied, but no differences between them were observed with the exception of GOS-1 ($P > 0.5$). Growth of *Lab158* using lactulose as a carbon source at 10 h of fermentation was similar to that found in their derivatives (OsLu-2 and OsLu-1) ($P = 0.508$ and $P = 0.682$, respectively); however, significant growth was obtained when OsLu-1 was incubated at 24 h ($P = 0.010$). No significant differences in bacterial growth among purified oligosaccharides of Vivinal-GOS, GOS-1, and GOS-2 were observed. For the *Erec482* population, the major statistical differences were found at 10 h, with substrates GOS-2 and OsLu-1. The first showed a value similar to 0 h of fermentation ($P = 0.357$), whereas the second showed a lower statistically different value ($P = 0.007$). At 24 h, *Ato291* genus grew better in GOS-2, and significant differences were found for Vivinal-GOS, GOS-1, and OsLu-2. For the *His150* group, fermentation assays with the six tested substrates did not produce changes in bacterial counts in comparison to time 0 h, except for OsLu-2, which caused a decrease at 10 h of fermentation ($P = 0.041$). No significant differences in the growth of *Bac303* between the six substrates tested compared to 0 h of fermentation were detected.

To obtain a relative quantitative measurement of all changes in bacterial population, the selectivity index (SI) (or prebiotic index, PI) was calculated using the formula described by Ruiz-Matute et al.[29]

$$\mathrm{SI} = ((\mathrm{Bif}_t/\mathrm{Bif}_0) + (\mathrm{Lac}_t/\mathrm{Lac}_0) + (\mathrm{EREC}_t/\mathrm{EREC}_0) - (\mathrm{Bac}_t/\mathrm{Bac}_0) - (\mathrm{His}_t/\mathrm{His}_0))/(\text{total count}_t/\text{total count}_0)$$

where Bif_t = bifidobacterial count at 5, 10, or 24 h, Bif_0 = bifidobacterial count at 0 h, etc. This measurement gives a comparative relationship between the growth of beneficial fecal bacteria (bifidobacteria, *Lactobacillus/Enterococcus* group, and *Eubacterium* rectale (EREC) group) and less desirable ones (for example, clostridia and bacteroides), related to the changes of the total number of bacteria.[30] Figure 3 shows SI scores for all fermentation times studied and all oligosaccharides assayed. Whereas at 24 h of fermentation, the SI scores of OsLu-1 were the highest, similar values were observed for lactulose, GOS-1, GOS-2, and OsLu-2. The commercial prebiotic Vivinal-GOS and the purified fraction GOS-2 had the maximum SI scores at 10 h of incubation. Considering the synthesized oligosaccharides, OsLu-1 presented values higher than lactulose at 10 and 24 h of fermentation.

SI values of all oligosaccharides assayed were higher than those obtained by Ruiz-Matute et al.[29] for gluco-oligosaccharides derived from cellobiose and similar to those found by Sanz et al.[30] for kojibiose, sophorose, and 4 β-galactobiose, which showed PI values of 21.62, 18.63, and 17.39, respectively. Therefore, PI measurement should be used carefully because the value differs between individuals and it cannot be meaningfully compared between published studies.[31]

SCFA. Fermentation of carbohydrates by colonic bacteria mainly produces SFCAs and gas. In this study, SCFA production has been determined in fecal fermentation supernatants using lactulose and a purified fraction of oligosaccharides from Vivinal-GOS as reference carbohydrates and purified oligosaccharide fractions derived from lactose and lactulose. Similarly to the bacterial analyses, the quantification of SCFA was carried out at 0, 5, 10, and 24 h (Table 3). During

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Table 3. Lactic Acid, Short-Chain Fatty Acid (SCFA), and Total (Lactic + Acetic + Propionic + Butyric) Concentrations (Millimolar) Produced during Fermentation with Lactose-Derived Oligosaccharides (GOS-1 and GOS-2), Lactulose-Derived Oligosaccharides (OsLu-1 and OsLu-2), and the Commercial Prebiotics Lactulose and Vivinal-GOS[a]

substrate	lactic acid				acetic acid				propionic acid				butyric acid				total SCFA			
	0 h	5 h	10 h	24 h	0 h	5 h	10 h	24 h	0 h	5 h	10 h	24 h	0 h	5 h	10 h	24 h	0 h	5 h	10 h	24 h
lactulose	2.41	4.62	11.46	5.07	3.06	21.74	23.37	31.98	1.20	2.98	11.00	15.06	nd	nd	1.7	2.72	7.86	36.57a	48.13a	59.97ac
	(0.05)	(2.89)	(4.87)	(4.03)	(0.67)	(12.42)	(5.01)	(8.84)	(0.14)	(0.28)	(2.65)	(4.27)			(0.71)	(2.08)	(0.84)	(15.52)	(2.15)	(3.17)
Vivinal-GOS	2.53	12.99	12.39	8.98	3.36	16.00	35.20	42.15	1.29	1.78	8.76	15.10	nd	nd	2.05	2.74	8.54	24.26a	58.68c	68.98a
	(0.11)	(2.43)	(3.93)	(3.47)	(0.65)	(5.67)	(3.45)	(8.31)	(0.06)	(0.44)	(4.65)	(7.45)			(1.17)	(2.06)	(0.67)	(6.80)	(5.89)	(5.44)
GOS-1	2.52	5.07	5.61	6.21	4.41	3.43	10.25	12.40	1.29	1.40	1.99	4.65	nd	nd	nd	nd	7.29	12.63a	17.86a	23.26b
	(0.56)	(0.29)	(0.25)	(1.18)	(0.34)	(1.95)	(4.43)	(5.39)	(0.07)	(0.02)	(0.51)	(2.93)					(0.66)	(1.66)	(4.84)	(7.32)
GOS-2	2.18	8.18	10.18	13.99	3.13	12.65	26.60	24.31	1.26	3.64	6.36	7.35	nd	nd	nd	2.25	7.14	26.63a	43.13b	48.67ad
	(0.41)	(2.93)	(3.57)	(12.46)	(0.19)	(6.96)	(4.90)	(1.83)	(0.05)	(1.29)	(3.47)	(4.00)				(0.97)	(0.53)	(9.07)	(1.02)	(2.66)
OsLu-1	2.07	7.01	11.98	7.07	5.92	10.07	32.52	32.10	1.23	2.88	12.73	14.43	nd	nd	nd	nd	8.06	34.24a	57.23c	54.80cbd
	(0.61)	(2.25)	(2.49)	(2.02)	(0.43)	(6.14)	(3.59)	(4.78)	(0.03)	(0.92)	(6.64)	(7.34)					(1.06)	(8.63)	(1.07)	(1.63)
OsLu-2	3.43	8.18	12.01	7.74	8.85	12.65	31.61	34.53	1.20	2.83	10.54	14.35	nd	nd	nd	1.10	7.24	28.41a	52.06c	51.94ad
	(0.07)	(2.93)	(2.73)	(2.50)	(0.36)	(6.96)	(3.72)	(5.46)	(0.07)	(0.40)	(4.64)	(5.42)				(0.34)	(0.419	(9.94)	(0.71)	(5.81)

[a]Different letters indicate significant differences ($P < 0.05$) for the same acid. Standard deviation is given in parentheses. nd, not detected.

fermentation, the main products formed were lactic acid and the SCFA acetic and propionic. Concentrations were significantly different from those found in the control sample. OsLu-1 and OsLu-2 produced a higher acetate level and similar lactate and propionate contents to lactulose. The low value of SCFA found in fecal cultures containing GOS-1 was in agreement with the result shown above for the growth of bacterial populations, because only a small increase for the genus *Bifidobacterium* was observed. Butyrate was detected in small concentrations in cultures containing lactulose, purified fraction of Vivinal-GOS, GOS-2, and OsLu-2. This can be due to the fact that the most numerous butyrate-producing bacteria found in human feces belong to the *Clostridium* group,[32] and slight changes (although not significant) in these populations during the fermentation process could have promoted the production of this SCFA. Butyric acid is considered to be a desirable metabolite of gut bacterial function, and some in vitro studies have shown that it can promote apoptosis of cancer cells.[33]

SCFA production increased during the 24 h of fermentation in most of the substrates studied. SCFA production was in the order Vivinal-GOS > lactulose > OsLu-1> OsLu-2 > GOS-2 > GOS-1. Batch cultures containing GOS-1 and GOS-2 produced less SCFA than commercial Vivinal-GOS at 10 and 24 h. At both fermentation times, GOS-1 showed statistical differences with Vivinal-GOS ($P = 0.013$ and $P = 0.001$, respectively) and also showed the lowest SCFA value in comparison with all of the substrates tested. Bacterial fermentation with OsLu-1 and OsLu-2 produced, therefore, a higher SCFA level than lactulose at 10 h of fermentation ($P = 0.068$; $P = 0.500$). These levels were then matched (24 h), and statistical differences were not found.

All of these results are in agreement with those obtained for bacterial counts. Thus, the lower value of SCFA found in fecal cultures containing GOS-1 is due to a small increase of the genus *Bifidobacterium* spp. However, higher values of SCFA values for the rest of the substrates could be explained by an increase of this bacterial population.

Although it is very difficult to attribute a fermentation product to a specific bacterial group in a mixed culture system, it has been described that the major end-products of the bifidobacterial pathway are acetic and lactic acids.[34] *Bacteroides* is a metabolically versatile group that is able to use many types of substrates and produce propionic acid.[35] In this study, although *Bacteroides* was an important group in the inocula used, the low bifidobacteria and lactobacilli populations were able to successfully compete for the tested substrates and proliferate.

In conclusion, this study shows the variations in fermentation properties of lactulose, enriched fractions of oligosaccharides derived from lactose and lactulose hydrolysis, and enriched fraction oligosaccharides from commercial Vivinal-GOS. The four purified fractions, derived from lactose and lactulose, promoted the in vitro growth of *Bifidobacterium* and enhanced production of SCFA. The results presented here indicate that the lactulose-derived oligosaccharides were selectively fermented by *Bif164* and *Lab158* populations, producing a higher concentration of SCFA than other bacteria tested. The new oligosaccharides studied here may constitute a good alternative to lactulose as a prebiotic, and they could complement the lactulose fermentation properties by selectively stimulating the gut microflora in different regions of the colon and present added functionality due to their characteristic structures. This

study, while providing an initial assessment of the prebiotic potential of lactulose-derived oligosaccharides, should be completed by evaluating in vivo their fermentation properties before being finally used as a functional ingredient for improving the composition of gut microflora.

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Notes

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