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Influence of Glycosidic Linkages and Molecular Weight on the Fermentation of Maltose-Based Oligosaccharides by Human Gut Bacteria

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A structure−function study was carried out to increase knowledge of how glycosidic linkages and molecular weights of carbohydrates contribute toward the selectivity of fermentation by gut bacteria. Oligosaccharides with maltose as the common carbohydrate source were used. Potentially prebiotic alternansucrase and dextransucrase maltose acceptor products were synthesized and separated into different molecular weights using a Bio-gel P2 column. These fractions were characterized by matrix-assisted laser desorption/ionization time-of-flight. Nonprebiotic maltooligosaccharides with degrees of polymerization (DP) from three to seven were commercially obtained for comparison. Growth selectivity of fecal bacteria on these oligosaccharides was studied using an anaerobic in vitro fermentation method. In general, carbohydrates of DP3 showed the highest selectivity towards bifidobacteria; however, oligosaccharides with a higher molecular weight (DP6−DP7) also resulted in a selective fermentation. Oligosaccharides with DPs above seven did not promote the growth of "beneficial" bacteria. The knowledge of how specific structures modify the gut microflora could help to find new prebiotic oligosaccharides.

KEYWORDS: Maltose-based oligosaccharides; molecular weight; structure−function relationship; gut microflora

INTRODUCTION

There is currently much interest in the study of the human gut microbiota to improve health. It is known that several pathogenic species can cause acute gastroenteritis while certain genera such as bifidobacteria and lactobacilli are known to provide some degree of protection against infection (*1*). It is also apparent that certain species may be involved in chronic gut disorders like ulcerative colitis, bowel cancer, and pseudomembranous colitis (*2*). Studies have been recently focused on the use of prebiotics, which are food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of beneficial bacteria (*3*).

Many carbohydrates have been reported to exert a prebiotic effect, such as fructo-oligosaccharides (FOSs), galacto-oligosaccharides, and lactulose (*4*−*6*), and many others are still under investigation for their prebiotic potential (*7*). There is, however, a lack of basic understanding of the mechanisms by which prebiotics operate and the influence of carbohydrate structure on their colonic fermentation, including selectivity.

We have recently studied the effect of glycosidic linkages and monosaccharide composition of a wide range of disaccharides upon selectivity of fermentation (*8*). Glucobioses with α- and β1→2 linkages seemed particularly selective for bifidobacteria, and in general, α-glucosyl-glucose disaccharides showed more positive effects than β-isomers. *trans*-Galacto-oligosaccharides with linkages β1→6 and β1→3 have been shown to be selective for bifidobacteria (*9*), the β-galactosidases of which cleave such isomers faster than β1→4 (*10*).

It is known that polysaccharides are frequently fermented by colonic bacteria and can be considered as dietary fibers, but they are not necessarily selective for desirable bacteria in the gut. On the contrary, all known prebiotics are oligosaccharides with the exception of inulin. FOS is a more rapidly metabolized prebiotic than its parent polysaccharide inulin (*11*). A good example of the importance of molecular weight is afforded by xylan. Xylan is not fermented well by lactobacilli or bifidobacteria, whereas xylo-oligosaccharides, derived from xylan by hydrolysis, are commercial prebiotics on the Japanese market (*12*). Isomaltooligosaccharides (IMO) of degree of polymerization (DP) three have a higher prebiotic activity than IMO of DP2 (*13*). Also, a range of *Lactobacillus* and *Bifidobacterium* strains were not able to utilize the FOS pentasaccharide, although they could utilize the related tri- and tetrasaccharides

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Table 1. Structure of ASR and DSR Maltose Acceptor Oligosaccharide

sample	compound	structure
ASR	ASR3	α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc (panose)
	ASR4a	α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc
	ASR4b	α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc
	ASR5	α-D-Glcp-(1→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc
	ASR6a	α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc
	ASR6b	α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→3)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc
	ASR7	α-D-Glcp-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glcp-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc
DSR	DSR3	α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→4)-D-Glc (panose)
	DSR4	α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc
	DSR5	α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc
	DSR6	α-D-Glcp-(1→6)-α-D-Glcp-(1→6) -α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc
	DSR7	α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→6)-α-D-Glcp-(1→4)-D-Glc

(*14*). These previous studies suggested that the optimal DP may be in the range of 3−4; however, more studies using other oligosaccharides with different structures should confirm this hypothesis.

A study of the influence of molecular weight of compounds with common monosaccharide compositions but different glycosidic linkages can be useful to understand how different structures are fermented by the gut microbiota. The synthesis of a series of oligosaccharides from the reaction between the glucosyl group of sucrose and the low molecular weight carbohydrates, as catalyzed by extracellular glucansucrases (dextransucrases, EC 2.4.1.5, DSR) from *Leuconostoc mesenteroides*, has been widely described (*15*, *16*). A variety of DSR with different selectivities have been characterized, and a wide range of oligo- and polysaccharides have been obtained (*17*). In these reactions, there is competition between the formation of acceptor products (oligosaccharides containing one, two, three, or more D-glucopyranosyl groups more than the acceptor) and the normal high-molecular weight glucan product. Côté and Robyt (*18*) used an extracellular glucansucrase (alternansucrase, EC 2.4.1.140, ASR) isolated from *L. mesenteroides* NRRL B-1355, which produces a polysaccharide (alternan) consisting of alternating α-(1→6)- and α-(1→3)-linked D-glucosyl residues. This enzyme was capable of forming both α-(1→6)- and α-(1→3)-linked acceptor products in the presence of a number of low-molecular weight acceptor sugars. However, α-(1→3)-linked structures were only formed when the nonreducing glucose acceptor group was linked through an α-(1→6) bond to another glucose residue (*19*), and the enzyme did not form sequences of more than two consecutive α-(1→6) linkages (*20*). Maltose was found to be the best acceptor, as judged by the high extent of formation of oligosaccharide relative to polysaccharide.

Studies on the fermentation selectivity of ASR maltose acceptor products from DP3 to DP7.4 were recently carried out in our laboratory (*21*). The prebiotic effect decreased from DP3 to DP5 and increased again until DP6.7; however, DP7.4 showed a considerably low population level of bifidobacteria attained during the fermentation. Therefore, more studies with higher DPs are necessary to understand this behavior.

In this work, a comparison of the effect upon the gut microbiota of a range of oligosaccharides produced from a common carbohydrate source was carried out to obtain information on the influence of molecular weight and structure. The oligosaccharides compared were all derived from maltose and included DSR maltose and ASR maltose acceptor products and nonprebiotic maltooligosaccharides (MOSs) for comparison. The structures contained different glycosidic linkages [all α-(1→4); α-(1→4) and α-(1→6); or α-(1→4), α-(1→6) and α-(1→3), respectively]. Molecular sizes ranged from tri- to decasaccharides.

MATERIALS AND METHODS

Glucansucrases. ASR was isolated from sucrose-grown cultures of *L. mesenteroides* NRRL B-21297. The cell-free culture fluid was concentrated by ultrafiltration using a 100000 molecular weight cutoff membrane and dialyzed against 20 mM, pH 5.4, sodium acetate buffer. The only glycansucrase activity detected in this concentrate was ASR (*22*). DSR was prepared according to the method described by Tsuchiya et al. (*23*) and stored as a lyophilized powder.

Carbohydrates. Maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, and MOSs were purchased from Sigma Co. (Poole, United Kingdom). FOSs (Raftilose P-95, DP2−8) were acquired from Orafti (Tienen, Belgium). DSR and ASR maltose acceptor oligosaccharides were synthesized as follows.

Acceptor Reaction Conditions. Acceptor reactions were carried out at room temperature in 20 mM, pH 5.4, sodium acetate buffer containing 0.01% (w/v) sodium azide as described previously (*22*). Reactions were terminated when all of the sucrose had been consumed, typically after 24−48 h. **Table 1** shows the structures of the product oligosaccharides.

Characterization of Oligosaccharides. Oligosaccharides were separated using a Bio-Gel P2 (fine mesh) column (5 cm × 150 cm), eluted with water under gravity flow. Fractions of 5 mL were collected, and the carbohydrate composition was detected by thin-layer chromatography (TLC) as previously described by Côté and Robyt (*19*) and by HPAEC-PAD using a Dionex DX-300 equipment containing a gradient pump and an eluent degas module. Separation of carbohydrates was carried out on a CarboPac PA-100 anion-exchange column (4 mm × 250 mm) combined with a CarboPac PA 100 guard column (4 mm × 50 mm). Twenty microliters of sample was injected, and elution of carbohydrates (0.7 mL min^{-1}) was performed using a gradient prepared from 1 M sodium acetate (eluent A), deionized water (eluent B), and 1 M sodium hydroxide (eluent C). Eluent C was constant (10%) during the whole process whereas eluent A changed from 3 to 10% at 30 min and increased to 20% at 70 min. This proportion was kept constant till 85 min, where it recovered the initial conditions. The effluent was monitored using a PAD detector (Concorde, Waters) containing a gold working electrode and a hydrogen reference electrode and using triple pulsed amperometry with the following potentials and durations: $E_1 = +0.15$ V ($t_1 = 400$ ms), $E_2 = +0.75$ V ($t_2 = 200$ ms), and $E_3 = -0.8$ V ($t_3 = 200$ ms). The sampling time (t_s) was 20 ms. All analyses were carried out in duplicate. Samples were also characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using a Bruker Daltonics Omniflex spectrometer. Aqueous solutions (1 μL) of oligosaccharides were mixed with an equal volume of saturated 2,5-dihydroxybenzoic acid solution in acetonitrile, allowed to dry on the probe, and subjected to MALDI-TOF mass spectrometry.

In Vitro Fermentations. In vitro fermentations were carried out as previously described (*8*). Seven milligrams of carbohydrates was dissolved in autoclaved nutrient basal medium (630 μL). Samples were

Table 2. Composition (%) of ASR and DSR Maltose Acceptor Oligosaccharide Fractions Obtained by TLC and HPAEC-PAD Analysis[a]

sample	compound	composition (%)
ASR A	ASR 4a	63
	ASR 4b	16
	ASR 5	12
	ASR 3	5
	unknown	4
ASR B	ASR 5	82
	ASR 4a	6
	ASR 6a	6
	ASR 6b	3
	unknown	3
ASR C	ASR 6b + ASR7	49
	ASR 6a	39
	ASR 5	12
ASR D	unknown DP8	20
	unknown DP8	12
	unknown DP9	12
	ASR 7	7
ASR E	unknown DP8–DP10	(*)
ASR F	unknown DP8–DP11	(*)
ASRG	unknown DP9–DP11	(*)
DSR A	DSR 3	84
	unknown (higher DP)	10
	unknown (DP2)	6
DSR B	DSR 4b	86
	DSR 5	7
	unknown (lower DP)	7
DSR C	DSR 5	57
	unknown (lower DP)	26
	DSR 6	9
	DSR 4	8
DSR D	DSR 6	60
	unknown (lower DP)	20
	DSR 7	9
	DSR 5	6
	unknown (higher DP)	5
DSR E	DSR 7	51
	unknown (lower DP)	17
	unknown (higher DP)	13
	DSR 6	11
	DSR 8	8

[a] (*) Unknown composition except from MALDI-TOF (see **Figure 1**).

then inoculated with 70 μL of fecal slurry, which was prepared by homogenizing fresh human feces from healthy donors (10%, w/v) in phosphate-buffered saline (PBS; 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, 1.15 g L^{-1} Na_2HPO_4, and 0.2 g L^{-1} KH_2PO_4), pH 7.3, (Oxoid) with a manual homogenizer (Fisher, Loughborough, United Kingdom) inside an anaerobic cabinet (10% H_2, 10% CO_2, and 80% N_2). Three donors were used 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, and samples were treated separately. One sample was prepared without any carbohydrate addition as a control. All additions, inoculations, and incubations were conducted inside the anaerobic cabinet. Samples (200 μL) were removed after 0 and 12 h of fermentation for enumeration of bacteria and short chain fatty acid (SCFA) analysis.

Enumeration of Bacteria. Bacteria were counted using fluorescent in situ hybridization. Samples (100 μL) were fixed overnight at 4 °C with 4% (w/v) filtered paraformaldehyde (pH 7.2) in a ratio of 1:3 (v/v). Samples were then washed twice with filtered PBS, resuspended in 200 μL of a mixture of PBS/ethanol (1:1, v/v), and stored at −20 °C until further analysis. Hybridization of the samples was carried out as described previously (*24*) using appropriate 16S rRNA-targeted oligonucleotide probes labeled with the fluorescent dye Cy3 (MWG Biotech, Germany) for the different bacteria or the nucleic acid stain DAPI (4′, 6-diamidino-2-phenylindole) for total cell counts. Probes used for each of the bacteria, previously validated by different authors, were Bif164, specific for *Bifidobacterium* (*25*); Bac303, specific for *Bacteroides* (*26*); His150, for *Clostridium* (*histolyticum* subgroup) (*27*); EREC482 for *Eubacterium* (*Clostridium coccoides-Eubacterium rectale* group) (*27*); Lab158, for *Lactobacillus/Enterococcus* (*28*); and ATO291 for *Atopobium* (*Coriobacterium* group) (*29*). The samples were then filtered onto 0.2 μm pore size filters (Millipore Corp., Watford, United Kingdom), and cells were counted using a Nikon Eclipse E400 fluorescent microscope. A minimum of 15 random fields were counted in each slide.

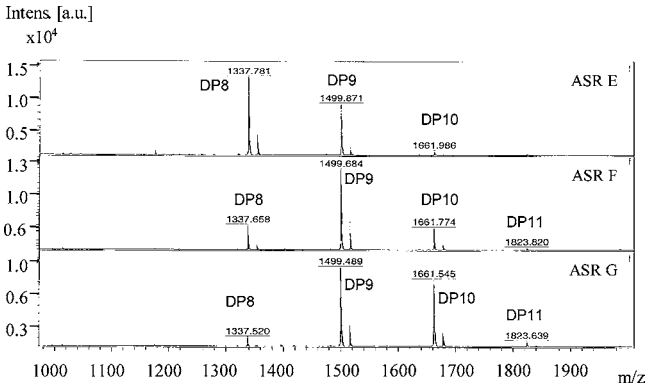

Figure 1. MALDI-TOF analysis of ASR maltose acceptor oligosaccharide fractions (ASR E–ASR G) obtained after separation by Bio-gel P2.

Prebiotic Index (PI). To obtain a general quantitative comparative measure of changes in bacterial populations among the studied samples, a PI was calculated. The PI has been previously reported in the literature as a relationship between changes in the "beneficial" and "undesirable" bacteria within the microflora, all of them related to their starting levels (*8*). The equation used was as follows: $PI = \alpha + \beta + \chi - \delta - \epsilon$, where α = (Bif12/Bif0)/total; β = (Lac12/Lac0)/total; χ = (EREC12/EREC0)/total; δ = (Bac12/Bac0)/total; ϵ = (His12/His0)/total; total = total count (12 h)/total count (0 h); Bif12 = bifidobacterial count at 12 h; and Bif0 = bifidobacterial count at 0 h, etc.

Analysis of SCFAs and Lactic Acid. Analysis of SCFA and lactic acid was carried out as previously indicated by Sanz et al. (*8*). Samples were centrifuged at 13000*g* for 5 min, and 20 μL was injected onto the high-performance liquid chromatography (HPLC) system (Hewlett-Packard HP1050 series) equipped with a UV detector and an automatic injector. The column was an ion-exclusion Aminex HPX-87H (7.8 mm × 300 mm, BioRad) maintained at 50 °C. The eluent was 0.005 mM sulfuric acid in HPLC-grade water, and the flow was 0.6 mL min^{-1}. Detection was performed at 210 nm, and data were acquired using Chem Station for LC3D software (Agilent Technologies). Quantification of the samples was carried out using calibration curves for acetic, propionic, butyric, and lactic acids at concentrations between 0.5 and 100 mM.

Statistical Analysis. Statistical analysis was performed using SPSS for Windows version 11.5. Univariate analysis of variance and Tukey's posthoc test was also used to determine significant differences among bacteria populations using the different oligosaccharides. Differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

In this work, new ASR maltose acceptor products were obtained and separated by molecular size using gel filtration columns. **Table 2** shows the results obtained from TLC and HPAEC-PAD analysis of ASR and DSR fractions. These samples were mainly composed of DP4 and DP5 (ASR A), DP5 and DP6 (ASR B), DP6 and DP7 (ASR C), and DP6−8 (ASR D). Moreover, three ASR fractions were constituted of oligosaccharides higher than DP7 [fractions composed mainly of DP8 (ASR E), DP9 (ASR F), and DP10 (ASR G)] were studied to determine the effect of higher oligosaccharides in the growth of gut microflora. The chemical composition of these fractions could be quantified neither by TLC nor by HPAEC, and the results obtained from MALDI-TOF spectra are shown in **Figure 1**.

Table 3. Changes in Bacterial Population (Log Cell mL^{-1}) after 12 h of in Vitro Fermentation with FOSs, MOSs (G3, Maltotriose; G4, Maltotetraose; G5, Maltopentaose; G6, Maltohexaose; and G7, Maltoheptaose), and ASR Maltose Acceptor and DSR Maltose Acceptor Products ($n = 3$)[a]

	DAPI	*Bifidobacteria*	*Bacteroides*	*Clostridia*	EREC	*Lactobacillus*	*Atopobium*
time = 0	9.20 (0.05) a[b]	7.93 (0.23) a,b,c	8.05 (0.12) a,b,c	6.93 (0.13) a	8.20 (0.01) a,b	6.92 (0.17) a	7.48 (0.19) a
control	9.21 (0.02) a	7.89 (0.05) a,b	7.99 (0.05) a,b	6.95 (0.03) a	8.02 (0.07) a	6.80 (0.15) a	7.56 (0.06) a,b
FOS	9.21 (0.12) a	8.49 (0.12) a,b,c,d,e,f,g	8.09 (0.03) a,b,c	6.94 (0.17) a	8.23 (0.06) a,b	7.31 (0.16) a	7.73 (0.22) a,b
MOS	9.2 (0.11) a	8.76 (0.12) d,e,f	7.97 (0.03) a	7.06 (0.14) a	8.09 (0.19) a	6.78 (0.17) a	7.64 (0.31) a,b
G3	9.24 (0.07) a	8.86 (0.16) e,f	8.24 (0.06) a,b,c,d,e	7.30 (0.27) a	8.82 (0.16) b	7.11 (0.20) a	8.02 (0.19) a,b
G4	9.51 (0.09) a	9.05 (0.09) f	8.30 (0.08) a,b,c,d,e,f	7.87 (0.22) a	8.62 (0.09) a,b	7.73 (0.03) a	8.03 (0.23) a,b
G5	9.35 (0.09) a	8.80 (0.13) d,e,f	8.18 (0.05) a,b,c,d	7.21 (0.20) a	8.59 (0.14) a,b	7.55 (0.08) a	7.87 (0.15) a,b
G6	9.36 (0.09) a	8.93 (0.14) e,f	8.20 (0.09) a,b,c,d,e	7.43 (0.23) a	8.41 (0.10) a,b	7.77 (0.11) a	7.88 (0.26) a,b
G7	9.34 (0.09) a	8.74 (0.05) d,e,f	8.25 (0.04) a,b,c,d,e,	7.66 (0.49) a	8.40 (0.17) a,b	7.83 (0.05) a	7.88 (0.14) a,b
DSR A	9.44 (0.10) a	9.02 (0.07) f	8.24 (0.11) a,b,c,d,e	7.19 (0.05) a	8.28 (0.12) a,b	7.08 (0.01) a	7.84 (0.16) a,b
DSR B	9.34 (0.06) a	8.78 (0.11) d,e,f	8.13 (0.02) a,b,c,d	7.15 (0.04) a	8.07 (0.21) a	7.10 (0.31) a	7.53 (0.21) a
DSR C	9.46 (0.09) a	9.01 (0.07) f	8.14 (0.07) a,b,c,d	7.36 (0.15) a	8.39 (0.09) a,b	7.57 (0.44) a	8.54 (0.29) b
DSR D	9.36 (0.04) a	8.82 (0.18) d,e,f	8.57 (0.03) d,e,f,g	7.97 (0.16) a	8.60 (0.13) a,b	8.01 (0.30) a	7.87 (0.15) a,b
DSR E	9.23 (0.05) a	8.63 (0.09) b,c,d,e,f	8.47 (0.11) c,d,e,f,g	7.11 (0.04) a	8.66 (0.12) a,b	7.42 (0.23) a	7.94 (0.15) a,b
ASR A	9.31 (0.15) a	8.89 (0.10) e,f	8.40 (0.07) a,b,c,d,e,f,g	7.40 (0.16) a	8.29 (0.18) a,b	7.12 (0.36) a	8.03 (0.18) a,b
ASR B	9.32 (0.03) a	8.65 (0.13) c,d,e,f	8.44 (0.04) b,c,d,e,f,g	7.21 (0.01) a	8.15 (0.15) a,b	7.58 (0.28) a	7.70 (0.04) a,b
ASR C	9.21 (0.03) a	8.85 (0.00) e,f	8.38 (0.06) a,b,c,d,e,f,g	7.82 (0.15) a	8.43 (0.07) a,b	7.46 (0.22) a	7.82 (0.16) a,b
ASR D	9.17 (0.03) a	8.09 (0.08) a,b,c,d	8.64 (0.08) e,f,g	7.57 (0.08) a	8.50 (0.07) a,b	7.91 (0.00) a	7.74 (0.05) a,b
ASR E	9.31 (0.05) a	8.20 (0.20) a,b,c,d,e	8.73 (0.16) f,g	6.78 (0.32) a	8.52 (0.10) a,b	6.91 (0.23) a	7.78 (0.14) a,b
ASR F	9.13 (0.04) a	7.79 (0.20) a	8.78 (0.07) g	7.54 (0.23) a	8.38 (0.09) a,b	7.11 (0.21) a	7.69 (0.21) a,b
ASR G	9.15 (0.06) a	7.90 (0.17) a,b,c	8.75 (0.07) f,g	7.64 (0.23) a	8.44 (0.11) a,b	7.40 (0.16) a	7.55 (0.17) a,b

[a] A control sample without carbohydrate source is also included. Different letters indicate significant differences ($P < 0.05$) for each bacterial genus. [b] Standard error in brackets.

Table 4. PI Scores after 12 h of in Vitro Fermentation with MOSs, DSR, and ASR Maltose Acceptor Products ($n = 3$) in Relation with Their DP

	PI		
DP	MOS	DSR	ASR
3	8.83 (G3)[a]	5.32 (DSR A)	5.32 (DSR A)
4	3.49 (G4)	4.06 (DSR B)	5.31 (ASR A)
5	6.68 (G5)	6.03 (DSR C)	5.22 (ASR B)
6	8.05 (G6)	4.27 (DSR D)	1.77 (ASR C)
7	4.03 (G7)	6.26 (DSR E)	4.26 (ASR D)
8			–0.81 (ASR E)
9			–7.10 (ASR F)
10			–5.81 (ASR G)

[a] Sample identity in brackets.

DSR acceptor products were also analyzed by TLC, HPAEC-PAD, and MALDI-TOF (**Table 2**). Fraction DSR A was mainly DP3 and DP4. DSR B and C were composed of DP4 and DP5 and DP5 and DP6, respectively. DP6−8 were the main constituents for DSR D, whereas DP7−9 were for DSR E. MOSs [from DP3 (G3) to DP7 (G7)] were commercially acquired and were practically pure.

Table 3 shows changes in bacterial populations after 12 h of incubation with the different fractions of oligosaccharides. A mixed MOS and mixed FOS were also included in this study. In general, a significant increase in the bifidobacterial population was observed in most of the samples, with G4, G6, DSR A, and DSR C showing the highest values. Bacteroides did not undergo significant changes relative to the control with the exception of ASR D, ASR E, ASR F, and ASR G, which showed a significant increase during the incubation. In general, clostridia, EREC, *Lactobacillus*, and *Atopobium* populations did not show significant differences with the control. Highest levels of lactobacilli were observed for G7, DSR D, and ASR D, whereas G3 showed the greatest population of EREC.

PI values were calculated to achieve clearer information on the selective fermentation of these oligosaccharides by the gut microbiota and to facilitate comparisons among them. **Table 4** shows PI values obtained for each carbohydrate, organized according to their DP. Fractions of ASR and DSR oligosaccharides were not completely pure (**Table 2**); however, for the purposes of presentation, they have been assigned to the highest DP constituent of each one. With this criteria, sample DSR A, which is composed mainly of DP3 and also by DP4, has been assigned to DP3, etc. In general, the PI trend for ASR oligosaccharides was similar to that found in previous work (*21*). ASR D oligosaccharides resulted in relatively high PI values (4.26); however, the PI of ASR E was actually negative (−0.81) mainly due to the low population of lactobacilli. This behavior was confirmed by the results obtained for the oligosaccharide fractions with the highest DPs (ASR F and ASR G), which also showed negative PI values. In general, although selective growth of bacteria decreased with molecular weight, PI values of DP6 for MOS and DP7 for DSR and ASR products increased. However, larger DP oligosaccharides gave rise to considerably lower PI values, which was characterized by the low growth of lactobacilli or high growth of bacteroides (ASR products). The FOS mixture showed a PI value of 4.1 lower than some of the studied samples.

A comparison among linkages showed similar or higher PI values for α-(1→4)-linked glucosides (MOS) than for α-(1→6)-linked glucosides (DSR) except for DP7. These values are lower for alternating α-(1→3), α-(1→6)-linkages (ASR). In this context, however, it must be remembered that MOSs are digestible and could not be classified as prebiotics. The aim in this study was to generate data on the structural basis of fermentation selectivity.

Table 5 shows lactic acid and SCFAs produced during fermentation of the oligosaccharide fraction. The generation of these products depends upon the fermented carbohydrate source and has beneficial effects for human health. These acids are produced by almost all intestinal bacteria; however, acetic and lactic acids are considered typical fermentation products of the bifidus pathway, whereas butyrate comes from clostridia and EREC (among others). High concentrations of lactic and acetic acids, significantly different from the control, were produced during fermentation of the different fractions of MOS. However, lower concentrations were observed for ASR products, with the

Table 5. Concentrations (mM) of Lactic, Acetic, Propionic, and Butyrc Acids Produced after 12 h of in Vitro Fermentation with FOS, MOS, and ASR Maltose Acceptor and DSR Maltose Acceptor Products ($n = 3$)[a]

	lactic	acetic	propionic	butyric
time = 0	2.23 (0.19) a[b]	0.38 (0.00) a	0.05 (0.00) a	0.06 (0.00) a
control	0.44 (0.00) a	11.52 (0.84) a,b	2.23 (0.26) b	2.19 (0.21) a,b,c,d
FOS	29.14 (2.15) c,d	36.64 (2.60) c,d,e	2.24 (0.24) b	1.70 (0.53) a,b
MOS	39.42 (2.15) d	44.89 (1.18) e,f	2.49 (0.32) b,c	2.05 (0.58) a,b,c
G3	35.81 (4.11) d	48.86 (2.32) e,f	2.61 (0.22) b,c	2.16 (0.53) a,b,c,d
G4	37.43 (4.04) d	46.93 (3.63) e,f	2.51 (0.40) b,c	2.09 (0.61) a,b,c,d
G5	38.45 (3.46) d	48.98 (2.36) e,f	2.55 (0.48) b,c	2.21 (0.65) a,b,c,d
G6	37.65 (2.96) d	47.48 (3.65) e,f	2.61 (0.36) b,c	2.19 (0.64) a,b,c,d
G7	33.42 (3.66) d	48.08 (2.28) e,f	2.71 (0.39) b,c	2.05 (0.66) a,b,c
DSR A	17.93 (0.86) b,c	45.42 (3.47) e,f	2.61 (0.31) b,c	2.67 (0.86) a,b,c,d
DSR B	33.22 (0.36) d	52.97 (1.08) e,f	2.73 (0.34) b,c	2.04 (0.89) a,b,c
DSR C	33.08 (1.46) d	56.22 (4.09) f	2.78 (0.10) b,c	2.05 (0.57) a,b,c,
DSR D	11.79 (1.62) a,b	43.63 (6.41) e,f	5.19 (0.23) d,e	4.90 (0.85) c,d
DSR E	3.70 (0.43) a	37.95 (1.33) d,e	7.14 (0.12) e,f	5.38 (1.38) d
ASR A	9.59 (4.01) a,b	37.86 (5.53) d,e	2.97 (0.26) b,c	3.53 (0.78) b,c,d
ASR B	28.81 (2.68) c,d	55.45 (5.45) f	3.13 (0.14) b,c	2.34 (0.51) a,b,cd
ASR C	2.76 (1.33) a	36.28 (4.70) c,d,e	4.41 (0.67) c,d	3.48 (0.38) b,c,d
ASR D	1.23 (0.79) a	24.23 (1.91) b,c,d	7.90 (0.31) f	3.15 (0.30) a,b,c,d
ASR E	1.18 (0.74) a	23.86 (0.94) b,c,d	8.92 (0.25) f	3.43 (0.50) b,c,d
ASR F	1.07 (0.63) a	20.40 (2.45) b,c	7.48 (0.84) f	2.95 (0.34) a,b,c,d
ASR G	1.04 (0.60) a	21.79 (1.13) b,c,d	8.10 (0.42) f	3.20 (0.36) a,b,c,d

[a] A control sample without a carbohydrate source is also included. Different letters indicate significant differences ($P < 0.05$) for each acid. [b] Standard error in brackets.

exception of ASR B. DSR E also showed low levels of lactic acid and the greatest concentration of butyric acid, possibly produced by the high EREC population. Moreover, samples with low lactic acid contents showed the greatest values of propionic acid.

This study was conceived as a structure−function study rather than an attempt to manufacture novel prebiotics. At the present time, we have no data on the digestibility of these materials in the human gut; consequently, they cannot yet be considered as candidate prebiotics unless delivered in a colon-specific delivery system. The structure−function information obtained in this work can, however, contribute toward a better knowledge of the fermentation of carbohydrates by the gut microbiota depending on glycosydic linkages and molecular weight. However, more studies could be carried out using pure cultures to investigate whether the increase of bacterial groups was due to selective metabolism of these carbohydrates as a substrate or whether their growth was stimulated indirectly through cross-feeding.

Although previous studies with prebiotic oligosaccharides suggested that the optimial DP was in the range of 3−4, in this work, a relatively high selectivity toward beneficial bacteria has also been shown by oligosaccharides with DP5−7. There is much interest in the production of oligosaccharides with higher colonic persistence, which can reach the most distal regions where most of the chronic intestinal disorders originate (*30*) and conceivably higher molecular weight oligosaccharides may be more slowly fermented. This would, however, require further studies on the rate of fermentation of these materials.

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