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Prebiotic Properties of Alternansucrase Maltose-Acceptor Oligosaccharides

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α-(1−6) and α-(1−3)-linked oligosaccharides were obtained from the reaction between sucrose and maltose, catalyzed by an alternansucrase isolated from *Leuconostoc mesenteroides* NRRL B-21297 and separated using a Bio-Gel P2 column in six fractions. Fractions 1, 2, and 3 were mainly composed of DP3, DP4, and DP5, respectively. However, fractions 4, 5, and 6 consisted of mixtures from DP5 to DP9, and they are identified here as DP5.7, DP6.7, and DP7.4, respectively. Potential prebiotic properties of these oligosaccharides were tested using pure and mixed cultures. Generally, in pure studies, most of the tested bacteria failed to grow or grew poorly using the DP6.7 and DP7.4 fractions and showed the greatest growth on DP3. Growth of fecal bacteria on the maltose-acceptor products was tested following an in vitro fermentation method. DP3 showed the highest prebiotic effect, followed by DP4 and DP6.7, whereas DP7.4 did not present any prebiotic activity.

KEYWORDS: Alternansucrase; prebiotic; maltose-acceptor products; molecular weight

INTRODUCTION

Nowadays there is an increasing interest in the use of foods that are able to modulate the composition of human colonic microflora in a way that is beneficial to health (*1*). Beneficial bacteria (probiotics) are added to many dairy products, and some carbohydrates (prebiotics) are used to selectively stimulate the growth of certain intestinal bacteria (currently lactobacilli and bifidobacteria), which beneficially affect host health (*2*, *3*). Many oligosaccharides with prebiotic properties such as fructo-oligosaccharides (FOS; *4*, *5*) and galacto-oligosaccharides (GOS; *6*) are on the market currently, and there is interest in the development of new prebiotic products. Many researchers are investigating the manufacture of prebiotic carbohydrates by enzymatic transfer reactions from cheap resources such as sucrose and lactose (*2*, *7*) or different polysaccharides (*8*, *9*).

The synthesis of a series of oligosaccharides from the reaction between the glucosyl group of sucrose and low molecular weight acceptor sugars catalyzed by extracellular glucansucrases (dextransucrases, DS) from *Leuconostoc mesenteroides* has been described (*10*, *11*). A variety of DS with different selectivities have been characterized, and a wide range of oligo- and polysaccharides have been obtained (*12*). The effect of several acceptors and the kinetics of these reactions have been extensively investigated (*12*−*15*), providing valuable information for optimizing the production of oligosaccharides and determining the consumption of the reactants. 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 (*16*) used an extracellular glucansucrase (alternansucrase, 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) structures were formed only when the nonreducing glucose acceptor group is linked through an α-(1−6) bond to another glucose residue (*17*). Maltose was found to be the best acceptor, as judged by the high rate of formation of oligosaccharide relative to polysaccharide.

Recently, the activity of some alternansucrase-derived oligosaccharides (acceptor products of gentiobiose, maltitol, maltose, melibiose, and raffinose) on various pure colonic bacteria has been tested (*18*, *19*). These products showed some bifidogenic properties, whereas coliform and pathogenic bacteria displayed no or little growth on them. However, there are no data available on their fermentation properties using mixed fecal bacteria, and also there are no data on the effect of the molecular weight of the acceptor product. In this work maltose-acceptor products have been synthesized and separated depending on their degree of polymerization (DP). Their prebiotic properties via in vitro fermentation by human gut bacteria (in pure and mixed cultures)

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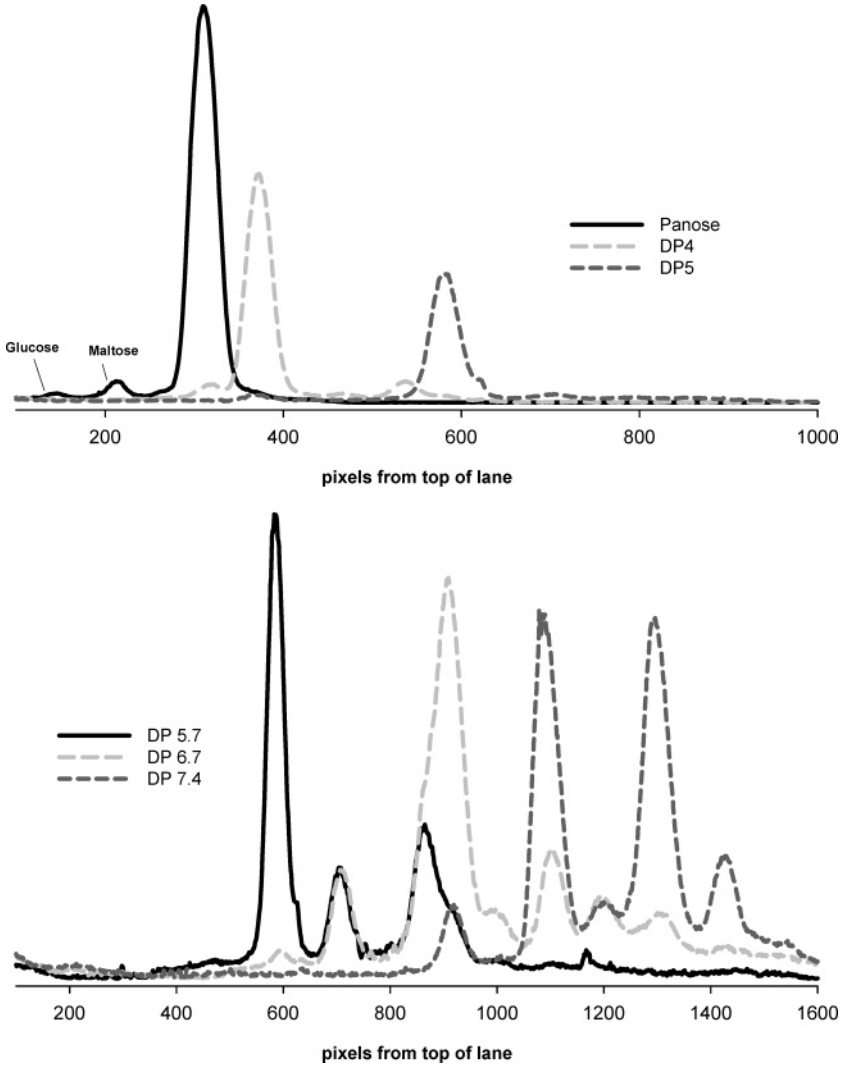

Figure 1. TLC densitogram of each oligosaccharide fraction obtained after separation in gel P2 column: (top) single products, DP 3–5; (bottom) mixed products, DP 5–8. Bio-Gel P2 columns were eluted at room temperature with water. Thin-layer chromatograms were run as previously described (*18*).

have been tested, and also the influence of the molecular weight on their activity has been evaluated.

MATERIALS AND METHODS

Alternansucrase. The alternansucrase was isolated from sucrose-grown cultures of *L. mesenteroides* NRRL B-21297. The cell-free culture fluid was concentrated by ultrafiltration using a 100 000 nominal molecular weight cutoff membrane and dialyzed against 20 mM (pH 5.4) sodium acetate buffer. The only glycansucrase activity detected in this concentrate was alternansucrase (*18*).

Carbohydrates. Glucose, sucrose, and maltose were purchased from Sigma Co. (Poole, U.K.), and FOS (Raftilose P-95) were acquired from Orafti (Tienen, Belgium).

Acceptor Reaction Conditions. Acceptor reactions were carried out at room temperature in 20 mM (pH 5.4) sodium acetate buffer containing 0.01% sodium azide, by using the cell-free culture fluid concentrate described above (*18*). Reactions were terminated when all sucrose had been consumed, typically after 24−48 h. The effect of substrate concentrations on yields of various products has been discussed previously and will be studied in greater detail in subsequent work. The quantitative analysis of the products used here is shown in **Figure 1**.

Characterization of Oligosaccharides. Synthesized oligosaccharides were separated using a Bio-Gel P2 (fine mesh) column (5 × 150 cm), eluted with water under gravity flow. Each fraction was detected by thin-layer chromatography (TLC) as previously described by Côté and Robyt (*17*). Samples were also characterized using a Bruker Daltonics Omniflex MALDI-TOF mass spectrometer. Aqueous solutions 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.

Bacteria. *Bifidobacterium* (*B.*) *bifidum* (DSM 20456), *Bifidobacterium longum* (DSM 20219), *Bifidobacterium angulatum* (DSM 20098), *Escherichia* (*E.*) *coli* (DSM 30083), *Eubacterium* (*Eu.) limosum* (DSM 20543), *Clostridium* (*C.*) *perfringens* (DSM 756), *Clostridium coccoides* (DSM 935), *Lactobacillus* (*L.*) *gasseri* (DSM 20243), *Bacteroides* (*Bac.*) *ovatus* (DSM 1896), *Bacteroides fragilis* (DSM 2151), and *Enterococcus* (*Ent.*) *faecalis* (DSM 20478) were obtained from DSMZ (Braunschweig, Germany).

Pure Culture Growth Treatments. Bacteria were grown on basal medium containing, per liter, 2 g of peptone water (Oxoid Ltd., Basingstoke, U.K.), 2 g of yeast extract (Oxoid), 0.1 g of NaCl, 0.04 g of K_2HPO_4, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 6H_2O$, 2 g of $NaHCO_3$, 0.005 g of hemin (Sigma), 0.5 g of L-cysteine hydrochloride (Sigma), 0.5 g of bile salts (Oxoid), 2 mL of Tween 80, 10 μL of vitamin K (Sigma), and 4 mL of 0.025% (w/v) resazurin solution. The medium was boiled and placed into an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2; Don Whitley, U.K.). Once cooled, it was dispensed in 9 mL volumes to Hungate tubes, which were sealed inside the cabinet to maintain the anaerobic conditions. The Hungate tubes were then autoclaved, and 1 mL of glucose solution was inoculated using 0.22 μm filters to achieve a final concentration of 1% (w/v). Each bacterium was inoculated and incubated at 37 °C until the highest optical density (OD) was achieved; 0.1 mL samples of these inocula were injected

Table 1. Growth Rates and Maximum OD (A_{650nm}) of Selected Gut Bacteria on 1% Carbohydrates

	glucose		DP3		DP4		DP5		DP5.7		DP6.7		DP7.4	
	rate[a]	max OD	rate	max OD	rate	max OD	rate	max OD	rate	max OD	rate	max OD	rate	max OD
B. longum	0.25 (0.08)[b]	1.12 (0.04)	0.02 (0.00)	0.88 (0.03)	0.02 (0.00)	0.89 (0.02)	0.02 (0.00)	0.83 (0.02)	0.01 (0.00)	0.47 (0.07)	ng[c]	ng	ng	ng
B. bifidum	0.15 (0.04)	1.00 (0.05)	0.07 (0.04)	0.88 (0.03)	0.01 (0.00)	0.60 (0.03)	ng	ng	ng	ng	ng	ng	ng	ng
B. angulatum	0.26 (0.03)	1.15 (0.05)	0.32 (0.04)	1.35 (0.01)	0.29 (0.06)	1.13 (0.18)	0.30 (0.05)	1.31 (0.09)	0.33 (0.04)	1.37 (0.02)	0.06 (0.03)	0.66 (0.05)	ng	ng
E. coli	0.39 (0.07)	0.93 (0.01)	0.32 (0.05)	0.36 (0.03)	0.27 (0.04)	0.26 (0.01)	0.26 (0.01)	0.29 (0.01)	0.31 (0.03)	0.25 (0.01)	0.27 (0.02)	0.24 (0.01)	ng	ng
C. coccoides	0.56 (0.07)	1.16 (0.04)	0.26 (0.04)	1.17 (0.01)	0.36 (0.04)	0.45 (0.01)	0.35 (0.07)	0.64 (0.02)	0.41 (0.09)	0.34 (0.05)	0.35 (0.02)	0.28 (0.02)	0.23 (0.09)	0.28 (0.02)
C. perfringes	0.52 (0.05)	1.58 (0.07)	0.34 (0.04)	1.39 (0.08)	0.30 (0.06)	0.87 (0.17)	0.28 (0.03)	1.06 (0.07)	0.29 (0.05)	0.51 (0.02)	0.31 (0.04)	0.43 (0.02)	0.36 (0.03)	0.39 (0.02)
Bac. ovatus	0.56 (0.03)	1.16 (0.08)	0.20 (0.03)	1.15 (0.04)	0.47 (0.05)	0.46 (0.04)	0.22 (0.04)	0.67 (0.02)	0.49 (0.17)	0.34 (0.02)	0.34 (0.06)	0.31 (0.02)	0.39 (0.23)	0.27 (0.01)
Bac. fragilis	0.52 (0.02)	1.19 (0.01)	0.31 (0.05)	1.16 (0.01)	0.60 (0.07)	0.47 (0.04)	0.53 (0.12)	0.63 (0.06)	0.43 (0.11)	0.43 (0.04)	0.62 (0.21)	0.30 (0.03)	ng	0.28 (0.01)
Ent. faecalis	0.36 (0.05)	1.22 (0.04)	0.30 (0.03)	1.12 (0.02)	0.27 (0.03)	0.45 (0.02)	0.27 (0.00)	0.78 (0.04)	0.78 (0.20)	0.45 (0.04)	0.19 (0.03)	0.40 (0.03)	0.42 (0.15)	0.28 (0.03)
Eu. limosum	0.22 (0.06)	1.81 (0.02)	0.06 (0.02)	0.80 (0.04)	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng
L. gasseri	0.04 (0.00)	0.70 (0.03)	0.01 (0.00)	0.10 (0.02)	ng	ng	ng	ng	ng	ng	ng	ng	ng	ng

[a] (μ, h^{-1}) according to Baranyi and Roberts (*20*). [b] Standard deviation in parentheses. [c] No growth.

aseptically into each tube and incubated at 37 °C. Each species was grown on each maltose-acceptor oligosaccharide in triplicate. A blank tube without any addition of carbohydrates was also inoculated for each bacterial strain in triplicate to take into account any carry-over of glucose.

The OD of the cultures was measured at 650 nm at different time points until the highest value was achieved. Growth of each microorganism was calibrated to relate absorbance with cell number. Growth rates were calculated using DMFit version 2.0 software, using the model of Baranyi and Roberts (*20*). Acid production from each bacterial species during the incubation period was checked by measuring pH.

In Vitro Fermentation Method. In vitro fermentation was carried out following the method of Sanz et al. (*21*). Ten milligrams of carbohydrates was dissolved in autoclaved nutrient basal medium to give a final concentration of 1% (w/v). Samples were inoculated with 100 μL of fecal slurry, which was prepared by homogenizing fresh human feces from a healthy donor (10%, w/v) in phosphate-buffered saline (PBS; 8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, and 0.2 g/L KH_2HPO_4) (pH 7.3) (Oxoid) with a manual homogenizer (Fisher, Loughborough, U.K.) inside an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2). Each fermentation experiment was carried out in triplicate and incubated at 37 °C. One sample was prepared without any carbohydrate addition as a control. All additions, inoculations, and incubations were carried out inside an anaerobic cabinet. Samples (200 μL) were removed after 0, 12, and 24 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 (FISH). 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 washed twice with filtered PBS and 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 by Rycroft et al. (*22*) using the appropriate genus-specific 16S rRNA-targeted oligonucleotide probes labeled with the fluorescent dye Cy3 (MWG Biotech, Ebersberg, Germany) for the different bacteria or the nucleic acid stain DAPI (4′,6-diamidino-2-phenylindole) for total cell counts. The probes used for each bacteria, previously validated by different authors, were Bif164, specific for *Bifidobacterium* (*23*); Bac303, specific for *Bacteroides* (*24*); His150, for *Clostridium* (*histolyticum* subgroup; *25*); EREC482, for *Eubacterium* (*C. coccoides*−*Eu. rectale* group; *25*); and Lab158, for *Lactobacillus*/*Enterococcus* (*26*). The samples were then filtered onto 0.2 μm pore size filters (Millipore Corp., Watford, U.K.) and cells were counted using a Nikon Eclipse E400 fluorescent microscope. A minimum of 15 random fields were counted in each slide.

Analysis of SCFA. Samples were centrifuged at 13000*g* for 5 min, and 20 μL was injected onto the 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 × 300 mm, Bio-Rad) maintained at 50 °C. The eluent was 0.005 mmol L^{-1} 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 of acetic, propionic, butyric, and lactic acids in concentrations between 0.5 and 100 mM. The detection limit was calculated for each compound following the method of Foley and Dorsey (*27*). Values of 0.03, 0.05, 0.05, and 0.06 mM were obtained for acetic, propionic, butyric, and lactic acid, respectively.

Statistical Analysis. Statistical analysis was performed using SPSS for Windows, version 11.5. Univariate analysis of variance (ANOVA) and the Tukey test were also used to determine significant differences among the bacteria populations using the different oligosaccharides. The differences were considered to be significant when $P < 0.05$.

RESULTS

Oligosaccharide Characterization. Figure 1 shows the densitogram obtained by TLC analysis of the oligosaccharide fractions separated using a gel filtration column. Fractions 1, 2, and 3 are mainly composed of DP3, DP4, and DP5 maltose-acceptor products, respectively. In previous studies (*17*) only one trisaccharide was found from the acceptor reaction of alternansucrase with sucrose and maltose, being identified as panose [*O*-α-D-glucopyranosyl-(1−6)-*O*-α-D-glucopyranosyl-(1−4)-D-glucopyranose]. Two tetrasaccharides were identified as *O*-α-D-glucopyranosyl-(1−3)-*O*-α-D-glucopyranosyl-(1−6)-*O*-α-D-glucopyranosyl-(1−4)-D-glucopyranose and 6^2-*O*-α-isomaltosylmaltose. Fraction 4 was found to contain approximately 40 wt % of DP5 and 60% wt % of DP6, with trace amounts of DP7. It will be identified here as DP5.7 according to its number-average molecular weight, as measured by reducing value. Fraction 6 was mainly composed of DP5 (15 wt %), DP6 (50 wt %), DP7 (20 wt %), and DP8 (15 wt %). It is referred to as DP6.7, according to its reducing-value number-average molecular weight. Fraction 7 was DP7 (35 wt %), DP8 (48 wt %), and DP9 (15 wt %), and it is referred to as DP7.4, from its reducing-value number-average molecular size. These results were also confirmed by MALDI-TOF analysis. It should be pointed out that average DP determinations derived from reducing-value number-average molecular weight and DP from MALDI-TOF did not agree exactly, as they each measure different properties. Furthermore, because the structures of products greater than DP5 are not yet known, the identity of TLC spots cannot be known for certain and can only be used as an estimate of DP.

Pure Culture Growth Treatments. Pure cultures were selected to represent the main constituents of the gut microflora, and their evolution with each maltose-acceptor product was studied. **Table 1** shows the rate (μ, h^{-1}) and the maximum OD achieved for the growth of the selected gut bacteria on each oligosaccharide fraction and glucose as a control. *B. bifidum* grew only on glucose, DP3 and DP4, whereas *B. longum* grew on most of the oligosaccharides; however, the rates were very

Table 2. Changes in Bacterial Population (Log Cell per Milliliter) after 12 and 24 h of in Vitro Fermentation Incubation with Maltose-Acceptor Products[a]

	time (h)	control	FOS	DP3	DP4	DP5	DP5.7	DP6.7	DP7.3
total bacteria	0	9.56 (0.18)[b]a							
	12	9.31 (0.07)a	9.42 (0.04)a	9.55 (0.07)a	9.45 (0.14)a	9.60 (0.09)a	9.48 (0.04)a	9.45 (0.11)a	9.45 (0.23)a
	24	9.61 (0.07)abd	9.97 (0.06)bc	9.43 (0.12)ad	9.26 (0.01)d	9.81 (0.18)ac	9.77 (0.08)ac	9.63 (0.22)a	9.58 (0.12)ad
Bifidobacteria	0	7.84 (0.19)a							
	12	8.06 (0.07)ab	8.46 (0.20)bc	8.89 (0.11)cd	8.55 (0.23)bd	8.60 (0.10)cd	8.56 (0.08)bd	8.56 (0.35)bd	7.95 (0.19)a
	24	7.83 (0.13)a	8.34 (0.14)bc	8.63 (0.08)b	8.34 (0.17)bd	8.48 (0.11)b	8.39 (0.14)b	8.59 (0.18)b	7.86 (0.10)acd
Bacteroides	0	8.51 (0.02)ab							
	12	8.32 (0.14)a	8.60 (0.03)ab	8.34 (0.09)a	8.40 (0.08)ac	8.78 (0.16)b	8.54 (0.01)ab	8.58 (0.06)ab	8.68 (0.08)bc
	24	8.20 (0.13)a	8.36 (0.08)ac	8.50 (0.06)ab	8.66 (0.22)bc	8.55 (0.11)bc	8.38 (0.10)ac	8.72 (0.10)b	8.57 (0.06)bc
Clostridia	0	7.87 (0.07)ab							
	12	7.28 (0.13)b	7.72 (0.14)a	7.39 (0.08)bc	7.61 (0.24)ab	7.94 (0.37)ac	8.14 (0.13)a	7.74 (0.47)ab	7.78 (0.16)ab
	24	7.83 (0.13)a	8.00 (0.13)a	7.95 (0.24)a	8.08 (0.25)a	8.21 (0.14)a	8.12 (0.13)a	8.20 (0.13)a	8.22 (0.11)a
Eubacteria	0	8.58 (0.05)a							
	12	8.54 (0.07)a	8.70 (0.06)a	8.51 (0.06)a	8.46 (0.05)a	8.66 (0.17)a	8.66 (0.14)a	8.74 (0.14)a	8.42 (0.20)a
	24	8.27 (0.09)a	8.48 (0.26)a	8.38 (0.23)a	8.46 (0.07)a	8.58 (0.10)a	8.50 (0.13)a	8.30 (0.25)a	8.50 (0.14)a
Lactobacillus	0	7.44 (0.27)ab							
	12	7.14 (0.08)a	7.57 (0.18)a	7.29 (0.18)a	7.69 (0.37)a	7.35 (0.09)a	7.57 (0.09)a	7.53 (0.36)a	7.37 (0.11)a
	24	7.21 (0.19)a	7.77 (0.01)ab	7.71 (0.08)ab	7.41 (0.12)ab	7.94 (0.07)b	7.96 (0.15)b	7.94 (0.43)b	7.63 (0.24)ab

[a] A control sample without carbohydrate source is also included. Different letters indicate significant differences ($P < 0.05$) for each bacterial genus and for each time of fermentation. [b] Standard deviation in parentheses.

low, with no growth on DP6.7 and DP7.4. *B. angulatum* grew in all of the oligosaccharides tested, except DP7.4. Moreover, this species grew better on DP5 and DP5.7 than on DP4, and it reached even higher OD values than on glucose (1.15, 1.31, and 1.37 for glucose, DP5, and DP5.7, respectively). In general, other bacteria presented the highest OD values for glucose, DP3, and DP5, whereas *L. gasseri* and *Eu. limosum* failed to grow with most of the oligosaccharides.

Acid production from each species during the incubation with the oligosaccharide fractions was also evaluated. The lowest pH values were detected for glucose and DP3, reaching values of 3.90 and 4.28 for *L. gasseri* and *B. angulatum*, respectively. DP 5.7, DP6.7, and DP7.4 did not present changes in their pH during the incubation process.

Batch Culture Fermentations. Table 2 shows the bacterial populations after 0, 12, and 24 h of incubation of a fecal inoculum with FOS and maltose-acceptor products. In general, levels of total bacteria were maintained during the fermentation process for all of the oligosaccharides tested; only FOS gave rise to a significant increase at 24 h of incubation, whereas incubation on DP4 led to a significant decrease at that time. Generally, a decrease was observed in bifidobacteria, bacteroides, and eubacteria between 12 and 24 h of fermentation. This effect had been previously observed by other authors using different carbohydrate sources (*8*, *22*).

Except for DP7.4, all of the oligosaccharides resulted in a significant increase in bifidobacteria population. However, none of them showed any variation in the lactobacillus number at 12 h of incubation, and a significant increase was detected only on DP5, DP5.7, and DP6.7 after 24 h.

Bacteroides, clostridia, and eubacteria populations did not present significant differences during the whole process compared with the sample at 0 h. Nevertheless, in some cases variations in the number of bacteria among the different molecular weight oligosaccharides were found. For example, the production of bacteroides was significantly higher for DP5 than for DP3 and DP4.

To obtain a general quantitative measure of all these changes in bacterial population, a prebiotic index (PI) was calculated as

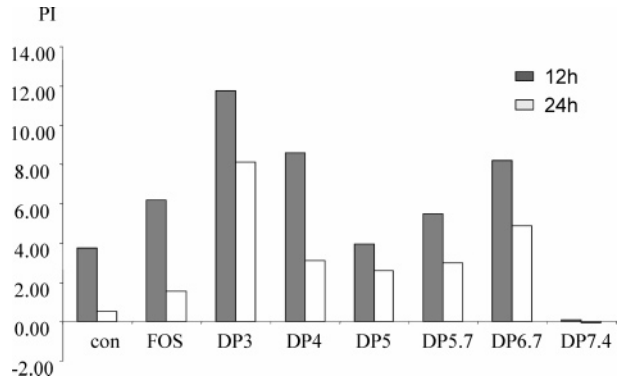

Figure 2. Prebiotic index (PI) scores from batch cultures at 12 and 24 h and 37 °C using FOS and maltose-acceptor oligosaccharides.

previously described in the literature (*21*, *28*, *29*). This measure can be considered as a relationship between the growth of the "beneficial" fecal bacteria (bifidobacteria, lactobacillus, and eubacteria) and the "undesirable" ones (clostridia and bacteroides), related to the changes of the total number of bacteria. **Figure 2** shows the obtained results. Values at 12 h of incubation were higher than those at 24 h in all of the samples. DP3 presented the highest value at 12 h followed by DP4 and DP6.7. The lowest PI was shown by DP7.4, corresponding to a negative number, due to the low number of bifidobacteria and lactobacilli and an increase in bacteroides. The same behavior was detected at 24 h. Except for DP7.4, all of the maltose-acceptor products presented greater values than FOS at 24 h.

Table 3 shows the values obtained for SCFA. DP5 and DP5.7 presented the highest contents of lactic and acetic acids, which are fermentation products of the bifidus pathway. The highest formic acid content was detected in DP3, whereas DP7.4 showed the greatest propionic acid production. All of the samples presented similar values of butyric acid, which varied between 3.35 and 5.64 mM for 12 h of incubation and between 4.20 and 6.80 mM at 24 h.

Table 3. Short-Chain Fatty Acid Concentrations (Millimolar) Produced by Oligosaccharide Fermentation[a]

	time (h)	control	FOS	DP3	DP4	DP5	DP5.7	DP6.7	DP7.3
lactic acid	0	2.26 (0.04)[b]							
	12	1.33 (0.10)	10.67 (0.38)	3.53 (0.24)	10.56 (0.58)	21.04 (1.78)	20.67 (0.48)	1.68 (0.07)	1.62 (0.12)
	24	1.43 (0.21)	13.95 (0.38)	8.12 (0.14)	15.69 (1.37)	31.00 (1.28)	26.43 (0.46)	1.86 (0.02)	1.76 (0.11)
formic acid	0	0.00							
	12	0.00	4.45 (0.14)	7.01 (0.45)	5.64 (0.40)	1.20 (0.15)	1.28 (0.01)	2.14 (0.24)	0.00
	24	0.00	5.53 (0.57)	8.45 (0.35)	5.30 (0.36)	0.86 (0.13)	1.36 (0.08)	3.23 (0.21)	0.03 (0.00)
acetic acid	0	0.00							
	12	13.12 (0.04)	24.05 (0.98)	26.65 (0.69)	34.72 (1.07)	43.15 (3.01)	44.01 (0.63)	25.21 (3.01)	20.20 (0.50)
	24	15.08 (0.03)	31.97 (1.34)	34.23 (3.63)	44.65 (5.05)	55.60 (2.26)	51.76 (0.59)	30.09 (0.45)	23.56 (0.62)
propionic acid	0	0.85 (0.04)							
	12	3.90 (0.20)	1.57 (0.06)	1.84 (0.15)	2.17 (0.14)	2.66 (0.16)	3.01 (0.35)	5.07 (0.93)	6.82 (0.86)
	24	4.58 (0.25)	1.77 (0.29)	1.83 (0.17)	1.89 (0.07)	2.78 (0.19)	3.09 (0.29)	4.53 (0.18)	7.94 (0.25)
butyric acid	0	0.00							
	12	3.55 (0.28)	3.35 (0.05)	4.42 (0.04)	5.64 (0.53)	4.82 (0.36)	5.63 (0.41)	4.62 (0.33)	4.56 (0.15)
	24	4.73 (0.29)	4.20 (0.78)	5.48 (1.05)	5.73 (0.32)	6.80 (1.09)	4.85 (0.17)	5.17 (0.04)	5.80 (0.31)

[a] A control sample without carbohydrate source is also included. [b] Standard deviation in parentheses.

DISCUSSION

To study the prebiotic effect of new compounds it is important to determine the evolution of the individual and mixed bacterial population in the presence of the carbohydrate source. The behavior of each bacterial species in the utilization of a carbohydrate source is different in pure and mixed cultures. Fermentation in the gut can be thought of as a global process, which comprises different bacterial pathways. The end products of one species can be used as a substrate by others, and some microorganisms benefit from substrates that they are not able to ferment directly (*1*). Therefore, in this study a combination of pure and mixed culture was used to study the potential prebiotic properties of the maltose-acceptor products.

There was an influence of DP on fermentation in pure culture. Most of the bacteria failed to grow or grew poorly on the higher oligosaccharides (DP6.7 and DP7.3) and showed the greatest growth on DP3. Moreover, DP5 and DP5.7 maltose-acceptor products could be more selective than DP4. Previous studies have demonstrated that oligosaccharides with lower molecular weight presented higher prebiotic activity: Some lactobacillus species metabolized only the trisaccharides and tetrasaccharides in FOS and could not metabolize pentasaccharide (*30*). Trisaccharide and tetrasaccharide fractions of galacto-oligosaccharides have been found to increase *B. lactis* more than glucose or disaccharides (*6*).

In mixed cultures this behavior was different. Bifidobacteria grew well on all of the oligosaccharides except DP7.4, whereas bacteroides and clostridia did not present significant changes. All of the oligosaccharides, apart from DP7.4, presented selectivities similar to that of FOS. At 12 h the PI values were lower for DP5 and DP5.7 than for FOS; however, values at 24 h were higher for all oligosaccharides.

The influence of molecular weight in mixed cultures was also detected. Higher prebiotic indices were detected on DP3 and DP4 at 12 h, corresponding to behavior seen in pure culture. From DP5 PI values increased until DP6.7; however, DP7.4 presented considerably lower PI values due to the low population level of bifidobacteria attained during the fermentation. Differences in carbohydrate composition and structure between DP6.7 and DP7.4 (mainly composed of hexasaccharides and octasaccharides, respectively) can influence their availability for bacteria. However, more studies are required with oligosaccharides of higher DP to confirm and understand this trend.

The generation of end-products depends on the type and quantity of the fermentation source (*31*). Acetate and lactate are fermentation end-products of the bifidus pathway, whereas butyrate is a major product of clostridia and eubacteria. Formic acid can also be an end-product of the reaction, in which pyruvate is converted to formic acid instead of lactic acid. Surprisingly, DP5 and DP5.7, which did not show high PI values compared with the other oligosaccharides, presented the greatest production of lactic and acetic acid, whereas DP3 and DP4, with the highest PI values, showed the most elevated formic acid content.

These studies demonstrate that alternan oligosaccharides obtained from the reaction using maltose as an acceptor carbohydrate can be considered as potential prebiotics from the point of view of fermentation selectivity. It is not currently known whether these oligosaccharides are digestible by humans.

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