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Influence of Disaccharide Structure on Prebiotic Selectivity in Vitro

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To obtain structure−function information of a range of carbohydrates, which are available only in very small quantities, an in vitro fermentation method using 7 mg of carbohydrate, 0.7 mL of basal medium, and 1% (w/v) of fecal bacteria was validated against a pH-controlled batch culture with 150 mL of basal medium and 1.5 g of test carbohydrate. This method was used to determine the influence of different glycosidic linkages and monosaccharide compositions of disaccharides on the selectivity of microbial fermentation. A prebiotic index (PI) was calculated for each disaccharide. Generally, disaccharides with linkages of 1−2, 1−4, and 1−6 generated a high PI score, with kojibiose and sophorose showing the greatest values (21.62 and 18.63, respectively). Apart from 6α-mannobiose, mannose-containing disaccharides gave a low PI due to low numbers of bifidobacteria and lactobacilli and an increase in bacteroides. The structure−function information obtained in this study may lead to a predictive understanding of how specific structures are fermented by the human gut microflora.

KEYWORDS: Prebiotics; in vitro fermentation; disaccharides; glycosidic linkages

INTRODUCTION

In recent times, a rapid development of the prebiotic-based functional food sector has occurred (*1*−*3*). Prebiotics are nondigestible, carbohydrate food components that are selectively fermented in the colon, increasing numbers of bifidobacteria and lactobacilli, which are believed to provide some degree of protection against pathogenic agents (i.e., act as probiotics) (*2*). Moreover, lactic acid and short-chain fatty acids (SCFA) such as acetic, propionic, and butyric acids are desirable products of such fermentation reactions, as they can contribute toward host energy requirements (*4*, *5*).

Many carbohydrates have been reported to exert a prebiotic effect, such as fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and lactulose (*5*−*7*). There is, however, a lack of basic understanding of the mechanisms for this selectively metabolic effect. Studies suggest that probiotic microorganisms possess cell-associated glycosidases, which thereby allow degradation of the prebiotic oligosaccharides (*8*). These liberated monosaccharides can then be taken up by the probiotic rather than other microorganisms in a mixed culture community, such as the human colon. This has been established with respect to metabolism of fructo-oligosaccharides, whereby possession of a β-fructofuranosidase allows bifidobacteria a selective advantage, in mixed culture, when growing upon this particular carbon source (*9*). The operation of this mechanism with other oligosaccharides has not yet been established. This may also explain why oligosaccharides consisting of the same monosaccharides with the same anomeric configuration, but differing only in linkage position have different fermentation properties. One example is maltodextrin (α-1−4 glucans), which is not selectively metabolized, whereas oligodextrans (predominantly α-1−6 glucans) are (*10*).

In vitro fermentation methods using batch cultures of mixed fecal bacteria are useful models for the study of the large intestine microbial functions (*11*, *12*). Anaerobic batch cultures, with or without pH control or stirring, have been exploited (*13*−*18*). A three-stage continuous culture system has also been developed to simulate the physical and nutritional characteristics of the proximal, transverse, and distal colonic regions (*19*, *20*). However, large amounts of oligosaccharides are required for such studies. In an attempt to resolve this problem, Dal Bello et al. (*21*) used 2 mL fermentation systems to study the prebiotic properties of levan-type exopolysaccharides from *Lactobacillus sanfranciscensis*. However, this method was oriented to denaturing gradient gel electrophoresis analysis for microflora composition studies and was not compared to other in vitro fermentation methods previously described in the literature. Moreover, no quantitative data were obtained after 24 h of incubation, when many bacterial changes take place.

The aim of this work was to obtain comparative information on the influence of glycosidic linkages and monosaccharide composition upon selectivity of fermentation of carbohydrate sources that are available only in limited amounts. For this purpose, an in vitro fermentation method allowing the use of small amounts of carbohydrates (7 mg) was developed. The results were compared to those obtained using pH-controlled batch cultures with greater amounts of carbohydrates and volumes (1.5 g and 150 mL, respectively). A range of disaccharides (glucose-, galactose-, fructose-, and mannose-containing

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disaccharides), prebiotic, nonprebiotic, and of unknown activity, were investigated to determine structure−function relationships.

MATERIALS AND METHODS

Carbohydrates. Cellobiose (4-*O*-β-D-glucopyranosyl-D-glucose), gentiobiose (6-*O*-β-D-glucopyranosyl-D-glucose), isomaltose (6-*O*-α-D-glucopyranosyl-D-glucose), lactose (4-*O*-β-D-galactopyranosyl-D-glucose), lactulose (4-*O*-β-D-galactopyranosyl-D-fructose), maltose (4-O-α-D-glucopyranosyl-D-glucose), melibiose (6-O-α-D-galactopyranosyl-D-glucose), sophorose (2-*O*-β-D-glucopyranosyl-D-glucose), sucrose (2-*O*-α-D-glucopyranosyl-β-D-fructofuranoside), α,α-trehalose (1-*O*-α-D-glucopyranosyl-α-D-glucopyranoside), α,β-trehalose (1-*O*-α-D-glucopyranosyl- β-D-glucopyranoside), β,β-trehalose (1-*O*-β-D-glucopyranosyl-β-D-glucopyranoside), and turanose (3-*O*-α-D-glucopyranosyl-D-fructose) were obtained from Sigma Chemical Co. (Poole, U.K.); leucrose (5-*O*-α-D-glucopyranosyl-D-fructose) and palatinose (6-*O*-α-D-glucopyranosyl-D-fructose) were obtained from Fluka (Gillingham, U.K.); maltulose (4-*O*-α-D-glucopyranosyl-D-fructose) was from Aldrich Chemical Co. (Gillingham, U.K.); 3α-galactobiose (3-*O*-α-D-galactopyranosyl-D-galactose), 4β-galactobiose (4-*O*-β-D-galactopyranosyl-D-galactose), 6β-galactobiose (6-*O*-β-D-galactopyranosyl-D-galactose), laminaribiose (3-*O*-β-D-glucopyranosyl-β-D-glucose), 3α-mannobiose (3-*O*-α-D-mannopyranosyl-D-mannose), 4α-mannobiose (4-*O*-α-D-mannopyranosyl-D-mannose), and nigerose (3-*O*-α-D-glucopyranosyl-D-glucose) were acquired from Dextra Laboratories (Reading, U.K.). Trehalulose (1-*O*-α-D-glucopyranosyl-D-fructose) was a gift from Dr. W. Wach from Südzucker AG, Mannheim, Germany. Kojibiose (2-*O*-α-D-glucopyranosyl-D-glucose) was a gift from Hayashibara Corp. (Okayanna, Japan). 2α-Mannobiose (2-*O*-α-D-mannopyranosyl-D-mannose) and 6α-mannobiose (6-*O*-α-D-mannopyranosyl-D-mannose) were synthesized in the laboratory (*22*, *23*). Fructo-oligosaccharides (FOS) [Raftilose P-95, degree of polymerization (DP) 2−8] were acquired from Orafti (Tienen, Belgium).

Small-Scale in Vitro Fermentation. Due to the very small amounts of disaccharides available for further studies, a small-scale in vitro fermentation method was optimized. Carbohydrates (7 and 10 mg) were dissolved in autoclaved nutrient basal medium to give a final concentration of 1% (w/v). This basal medium contained, 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 samples were inoculated with 70 and 100 μL, respectively, 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.) in an anaerobic cabinet (containing 10% H_2, 10% CO_2, and 80% N_2). The donor had no history of gastrointestinal disorders and had avoided probiotics, prebiotics, and antibiotics for 3 months prior to the study. Each fermentation experiment was carried out in triplicate and incubated at 37 °C. One sample was prepared without any carbohydrate addition, and acted as a control. All additions, inoculations, and incubations were carried out inside the anaerobic cabinet. Samples (100 μL) were removed after 0, 6, 12, and 24 h of fermentation for enumeration of bacteria, and the optimum time of incubation was tested. When the best conditions were chosen, samples (700 μL) were removed for enumeration of bacteria and SCFA and lactic acid analysis.

pH-Controlled Batch Cultures. Water-jacketed fermenters were filled with 135 mL of prereduced basal growth medium and inoculated with 15 mL of fecal slurry. Lactulose was added separately before inoculation, to give a final concentration of 1% (w/v). Fecal slurries were prepared by homogenizing 10% (w/v) freshly voided human feces in 0.1 M PBS, pH 7.3 (Oxoid). Each vessel was magnetically stirred and maintained under anaerobic conditions with oxygen-free nitrogen. Culture pH was controlled automatically during the fermentation at 6.8 and temperature maintained at 37 °C (*18*). All fermentation experiments were carried out in triplicate.

Enumeration of Bacteria. Bacteria were counted using fluorescent in situ hybridization (FISH). In brief, 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. (*16*, *17*) 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 4′,6-diamidino-2-phenylindole (DAPI) for total cell counts. The bacterial groups studied were chosen to represent those most predominant in the human colon. Therefore, probes used for each bacteria, previously validated by different authors, were Bif164, specific for *Bifidobacterium* (*24*), Bac303, specific for *Bacteroides* (*25*), His150, specific for *Clostridium* (*histolyticum* subgroup; *26*), EREC482, specific for *Eubacterium* (*Clostridium coccoides*−*Eubacterium rectale* group; *26*), and Lab158, specific for *Lactobacillus/Enterococcus* (*27*). Samples were then filtered onto 0.2 μm pore size filters (Millipore Corp., Watford, U.K.) and filters mounted in SlowFade (Molecular Probes, Leiden, The Netherlands) onto clean microscope slides. Cells were counted using a Nikon Eclipse E400 fluorescent microscope. A minimum of 15 random fields were counted in each slide.

Analysis of SCFA and Lactic Acid. Samples were centrifuged at 13000*g* for 5 min, and 20 μL was injected onto an HPLC system (Hewlett-Packard HP1050 series) equipped with UV detection 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 rate was 0.6 mL min^{-1}. Detection was carried out at 210 nm, and data acquired using Chem Station for LC3D software (Agilent Technologies). Quantification of the samples was obtained through calibration curves of acetic, propionic, butyric, and lactic acids in concentrations between 0.5 and 100 mM.

Prebiotic Index (PI). To obtain a general quantitative comparative measure of changes in bacterial populations and to compare the influence of the different linkages and monosaccharide composition on the selectivity of fermentation, a PI was calculated. The PI has been previously reported in the literature as a relationship between changes in the "beneficial" and "undesirable" elements within the microflora, all of them related to their starting levels (*10*, *28*). The equation used was therefore as follows: $PI = \alpha + \beta + \gamma - \delta - \epsilon$, where $\alpha =$ (*Bif*12/*Bif*0)/total; $\beta =$ (*Lac*12/*Lac*0)/total; $\gamma =$ (*Erec*12/*Erec*0)/total; $\delta =$ (*Bac*12/*Bac*0)/total; $\epsilon =$ (*His*12/*His*0)/total; total = total count (12 h)/total count (0 h); *Bif*12 = bifidobacterial count at 12 h; *Bif*0 = bifidobacterial count at 0 h, etc.

Statistical Analysis. Statistical analysis was performed using SPSS for Windows, version 11.5. Differences between bacterial counts using the two different in vitro fermentation methods were tested for significance using paired *t* test. Univariate analysis of variance (ANOVA) and Tukey test were also used to determine significant differences for each bacterial population and SCFA composition among the different carbohydrate sources used in this study. Differences were considered to be significant when $P < 0.05$. These results have been indicated with a lettering system in figures and tables.

RESULTS

Validation of the Method. Figure 1 shows the bacterial population after 0, 6, 12, and 24 h of incubation of lactulose using small-scale batch cultures and pH-controlled batch cultures. No significant differences were seen between the methods except for clostridia at 12 h and bifidobacteria at 24 h.

The largest changes in bacterial population, for all groups enumerated, took place during the first 6 h of incubation. Also, an increase was detected in total anaerobes and bifidobacteria up until 12 h of incubation, although it was not statistically significant. Bacteroides, clostridia, and eubacteria counts did not alter between 6 and 12 h. However, a decrease in

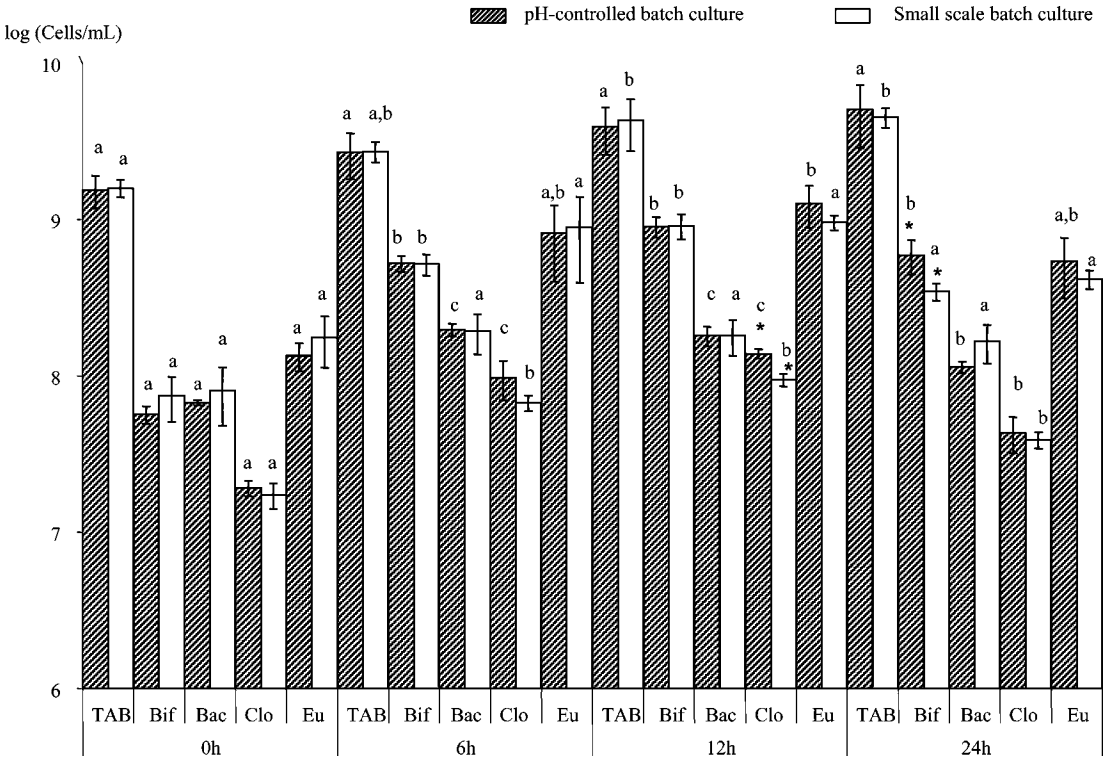

Figure 1. Bacterial population (log 10 cells/mL) at 0, 6, 12, and 24 h using small-scale batch cultures and pH-controlled batch cultures with lactulose. Different letters indicate significant differences ($P < 0.05$) for each bacterial genus and for each method. * indicates significant differences ($P < 0.05$) between both methods. TAB, total anaerobic bacteria; Bif, *Bifidobacterium*; Bac, *Bacteroides*; Clo, *Clostridium* (*histolyticum* subgroup); Eu, *Eubacterium* (*E. rectale* group). Error bars represent standard error.

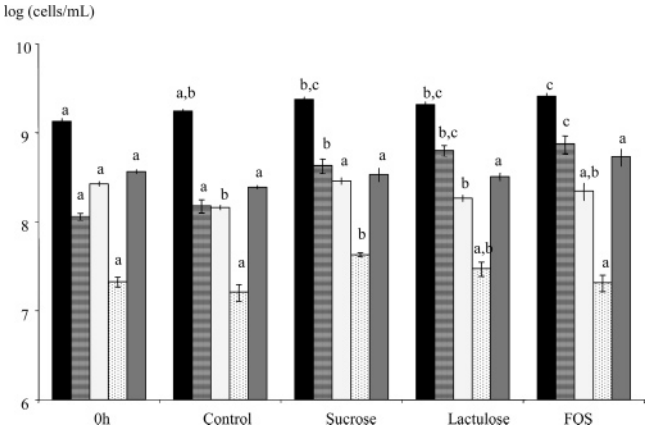

Figure 2. Bacterial population changes (log 10 cells/mL) in small-scale cultures with each carbohydrate used (7 mg) during 12 h of fermentation. Different letters indicate significant differences ($P < 0.05$) for each bacterial genus. The bars represent, from left to right within each grouping, total anaerobic bacteria, *Bifidobacterium*, *Bacteroides*, *Clostridium* (*histolyticum* subgroup), and *Eubacterium* (*E. rectale* group). Error bars represent standard error.

bifidobacteria, clostridia, and eubacteria was observed at 24 h of incubation.

Counts of selected bacteria at 12 h in small-scale cultures using sucrose, lactulose, and FOS are shown in **Figure 2**. Bifidobacteria significantly increased with these three carbohydrates. The clostridial population slightly increase with the three samples; however, this response was significant only when sucrose was fermented.

These treatments were also carried out using 10 mg of carbohydrates. No differences in metabolic profiles were observed between 7 and 10 mg; therefore, the smallest amount (7 mg) was selected for subsequent experiments (10 mg data are not shown). Lower amounts were not feasible as the volumes would have been too small for subsequent analysis.

Fermentation of Disaccharides. Although it is well-known that many disaccharides are hydrolyzed and absorbed in the upper human gastrointestinal tract, their simple structure and commercial availability make them appropriate carbohydrates to study the influence of glycosidic linkages upon prebiotic properties.

Table 1 shows changes in bacterial population following 12 h of incubation of a fecal inoculum with FOS (as a reference to an established prebiotic) and glucose-, galactose-, and mannose-containing disaccharides. Disaccharides with 1−2 linkages (kojibiose and sophorose) resulted in the highest bifidobacterial populations (8.83 and 8.89 log, respectively), significantly higher even than those resulting from FOS ($P < 0.05$). Apart from α,α-trehalose, β,β-trehalose, and gentiobiose, which did not present any significant change in bifidobacteria population, the rest of the glucosyl-glucoses showed a significant increase in bifidobacteria, displaying, in most of the cases, similar behavior to FOS (8.34 log). Generally, a slight increase was detected in bacteroides and eubacteria with the glucosyl-glucose disaccharides, although these changes were not statistically significant. The greatest values in clostridia during incubation with glucosyl-glucoses were found for linkages 1−4 (maltose and cellobiose), followed by linkages 1−1 (α,β-trehalose and β,β-trehalose), whereas incubation on disaccharides with linkages 1−6 and 1−3 led to a slight decrease at that time. Maltose was the glucose-containing disaccharide that presented the highest lactobacilli population and gentiobiose that with the lowest value.

Bifidobacterial populations increased with all of the galactose-containing disaccharides, although no significant differences were determined. 6β-Galactobiose and melibiose (disaccharides with linkages 1−6) showed the greatest values (8.44 and 8.40

Table 1. Bacterial Populations (Log 10 Cells per Milliliter) in Small-Scale Batch Cultures at 12 h Using Glucosyl-glucose, Galactosyl-galactose, and Mannosyl-mannose Disaccharides[a]

	total bacteria	*Bifidobacterium*	*Bacteroides*	*Clostridium*	*Eubacterium*	*Lactobacillus*	PI score
0 h	9.35 (0.05)abcd	7.62 (0.02)a	8.35 (0.05)ab	7.32 (0.07)abc	8.29 (0.05)ab	7.22 (0.05)ab	
control, 12 h	9.20 (0.01)abc	7.60 (0.07)a	8.30 (0.06)a	7.31 (0.05)cdef	8.23 (0.07)a	7.10 (0.02)ab	1.02
FOS	9.29 (0.07)abcd	8.34 (0.05)dfg	8.53 (0.07)ab	7.51 (0.11)defg	8.46 (0.06)abcd	7.72 (0.07)abc	7.64
α,α-trehalose	9.24 (0.03)abcd	7.82 (0.04)abc	8.42 (0.07)ab	7.20 (0.06)bcdef	8.42 (0.05)abcd	7.01 (0.03)ab	2.10
α,β-trehalose	9.10 (0.05)a	8.34 (0.08)def	8.38 (0.10)ab	7.75 (0.09)fg	8.29 (0.12)ab	7.23 (0.08)abc	6.28
β,β-trehalose	9.25 (0.07)abcd	7.68 (0.04)a	8.30 (0.08)a	7.62 (0.17)efg	8.45 (0.03)abcd	7.57 (0.13)abc	2.22
kojibiose	9.32 (0.03)abcd	8.83 (0.08)gh	8.43 (0.08)ab	7.11 (0.07)abcde	8.62 (0.06)abcd	7.51 (0.30)abc	21.62
sophorose	9.38 (0.06)abcd	8.89 (0.04)h	8.43 (0.04)ab	7.22 (0.03)bcdef	8.63 (0.09)abcd	7.19 (0.08)ab	18.63
nigerose	9.47 (0.03)cd	8.59 (0.02)efgh	8.73 (0.06)b	7.04 (0.19)abcde	8.64 (0.04)abcd	7.48 (0.14)abc	8.13
laminaribiose	9.37 (0.07)abcd	8.25 (0.17)cdef	8.52 (0.08)ab	6.83 (0.15)abc	8.68 (0.05)bcd	6.88 (0.12)a	5.60
maltose	9.36 (0.06)abcd	8.20 (0.06)cde	8.62 (0.06)ab	7.96 (0.01)g	8.32 (0.06)abc	7.92 (0.09)bc	3.71
cellobiose	9.30 (0.05)abcd	8.48 (0.03)efgh	8.39 (0.03)ab	7.78 (0.02)fg	8.46 (0.10)abcd	7.57 (0.09)abc	8.19
isomaltose	9.37 (0.05)abcd	8.67 (0.03)fgh	8.47 (0.15)ab	6.71 (0.07)abc	8.38 (0.06)abcd	6.94 (0.07)ab	10.86
gentiobiose	9.14 (0.02)ab	7.98 (0.03)abcd	8.40 (0.12)ab	6.93 (0.11)abcd	8.32 (0.09)abc	6.84 (0.17)a	3.70
3α-galactobiose	9.38 (0.06)abcd	8.15 (0.13)bcde	8.66 (0.05) ab	6.95 (0.00)abcd	8.75 (0.05)d	7.08 (0.19)ab	4.13
4β-galactobiose	9.10 (0.04)a	8.36 (0.06)def	8.65 (0.05)ab	7.04 (0.08)abcde	8.69 (0.07)bcd	7.80 (0.13)abc	17.39
6β-galactobiose	9.37 (0.01)abcd	8.44 (0.04)defgh	8.72 (0.06)b	6.97 (0.04)abcd	8.72 (0.10)cd	7.67 (0.35)abc	10.70
lactose	9.34 (0.04)abcd	8.20 (0.17)cde	8.59 (0.04)ab	7.30 (0.14)bcdef	8.59 (0.09)abcd	7.43 (0.18)abc	5.75
melibiose	9.46 (0.07)cd	8.40 (0.12)defg	8.59 (0.07)ab	7.24 (0.11)bcdef	8.69 (0.09)bcd	7.56 (0.07)abc	6.81
2α-mannobiose	9.43 (0.03)cd	7.71 (0.13)ab	8.76 (0.09)b	6.57 (0.20)a	8.42 (0.11)abcd	7.14 (0.53)ab	–0.52
3α-mannobiose	9.34 (0.08)abcd	7.60 (0.12)a	8.71 (0.01)ab	6.59 (0.20)a	8.56 (0.10)abcd	6.82 (0.20)a	0.99
4α-mannobiose	9.52 (0.06)d	7.63 (0.10)a	8.49 (0.11)ab	6.81 (0.11)abc	8.24 (0.08)a	6.97 (0.09)ab	0.49
6α-mannobiose	9.41 (0.07)bcd	7.70 (0.03)ab	8.55 (0.05)ab	6.69 (0.02)ab	8.33 (0.04)abc	8.20 (0.09)c	8.86

[a] Sample at 0 h, control, FOS, lactose, and melibiose are also included. Different letters indicate significant differences ($P < 0.05$) for each bacterial genus among the different carbohydrate sources. Standard error is given in parentheses.

Table 2. Bacterial Populations (Log 10 Cells per Milliliter) in Small-Scale Batch Cultures after 12 h Using Glucosyl-fructose Disaccharides and Prebiotic Index (PI) Scores for These Samples[a]

	total bacterial	*Bifidobacterium*	*Bacteroides*	*Clostridium*	*Eubacterium*	*Lactobacillus*	PI score
0 h	9.30 (0.08)ab	8.12 (0.07)ab	8.07 (0.03)a	7.70 (0.05)a	8.09 (0.13)a	7.26 (0.07)a	
control, 12 h	9.23 (0.05)a	8.08 (0.05)b	8.13 (0.04)ab	7.74 (0.06)ab	8.14 (0.06)a	7.37 (0.08)ab	1.23
lactulose	9.70 (0.05)d	8.82 (0.03)d	8.41 (0.03)def	8.06 (0.04)def	8.59 (0.08)bc	8.17 (0.04)de	4.66
trehalulose	9.61 (0.03)cd	8.40 (0.02)bc	8.29 (0.03)bcd	8.04 (0.04)bcd	8.73 (0.10)bc	7.96 (0.06)cde	3.40
sucrose	9.36 (0.07)abc	8.68 (0.06)cd	8.36 (0.05)cdef	7.92 (0.01)cdef	8.45 (0.02)ab	8.15 (0.06)de	8.62
turanose	9.69 (0.09)d	8.23 (0.02)ab	8.49 (0.02)ef	7.68 (0.08)ef	8.70 (0.06)bc	7.88 (0.09)cd	2.30
maltulose	9.60 (0.06)cd	8.72 (0.03)d	8.51 (0.06)f	8.01 (0.02)f	8.84 (0.05)c	8.23 (0.02)e	6.77
leucrose	9.56 (0.03)bcd	8.18 (0.14)ab	8.32 (0.06)cde	7.66 (0.13)cde	8.57 (0.01)bc	7.94 (0.10) cde	3.55
palatinose	9.17 (0.06)a	8.23 (0.06)ab	8.21 (0.04)abc	7.86 (0.03)abc	8.53 (0.05)bc	7.65 (0.05)bc	4.65

[a] Sample at 0 h, control, and lactulose are also included as reference. Different letters indicate significant differences ($P < 0.05$) for each bacterial genus among the different carbohydrate sources. Standard error is given in parentheses.

log, respectively). Generally, bacteroides, eubacteria, and lactobacilli showed a slight increase with all of the galactose-containing disaccharides, although the increase reached statistical significance only for eubacteria.

In contrast to glucose- and galactose-containing disaccharides, no changes were detected in bifidobacteria with mannose-containing disaccharides after 12 h of treatment. Mannobioses did not sustain growth of clostridia and maintained the eubacterial population at the inoculum level. The *Lactobacillus* population increased with only 6α-mannobiose, reaching levels of 8.20 log.

The PI results are also shown in **Table 1**. Kojibiose and sophorose showed the greatest PI values (21.62 and 18.63, repectively) followed by 4β-galactobiose (17.39). Isomaltose, 6β-galactobiose, 6α-mannobiose, and nigerose also presented higher values than FOS (7.64). Apart from 6α-mannobiose, mannosyl-mannoses showed a very low PI due to a small number of bifidobacteria and lactobacilli and an increase in bacteroides.

Counts of selected bacteria at 12 h in small-scale cultures using glucosyl fructose disaccharides are shown in **Table 2**. Significant differences among these samples are shown. Lactulose (4-O-β-D-galactopyranosyl-D-fructose), an established prebiotic, was also included in this study. An increase in total bacteria was observed in all of the batch culture systems, except with palatinose. A significant increase in bifidobacteria was seen with lactulose, sucrose, and maltulose, with lactulose displaying the greatest value. All of the fructose-containing disaccharides significantly increased numbers of lactobacilli, with lactulose, sucrose, and maltulose showing the highest values (8.17, 8.15, and 8.23 log, respectively). Generally, bacteroides and eubacteria populations significantly increased with all of the disaccharides tested, except for palatinose for bacteroides and sucrose for eubacteria. Significant changes in clostridia populations were also seen.

PI values for fructose-containing disaccharides are shown in **Table 2**. Sucrose showed the highest value (8.62) followed by maltulose, lactulose, and palatinose, whereas turanose presented the lowest one.

Table 3 shows the values obtained for lactic acid and SCFA production during incubation with glucose-, galactose-, and mannose-containing disaccharides. Gentiobiose- and galactose-containing disaccharides, apart from 3α-galactobiose, presented the greatest content of the total amount of these acids mainly due to the high concentrations of lactic and acetic acids. Lactic acid was not produced during the incubation with mannobioses;

Table 3. Lactic Acid, Short-Chain Fatty Acid, and Total (Lactic + Acetic + Propionic + Butyric) Concentrations (Millimolar) Produced after 12 h of Fermentation Using Glucosyl-glucose, Galactosyl-galactose, and Mannosyl-mannose Disaccharide[a]

	lactic acid	acetic acid	propionic acid	butyric acid	total
0 h	0.00a	1.41 (0.04)a	0.00a	0.00a	1.41 (0.04)a
control, 12 h	0.00a	10.92 (0.08)b	4.93 (0.12)b	3.89 (0.05)b	19.74 (0.19)b
FOS	18.41 (0.20)e	33.09 (0.38)g	6.30 (0.04)bcdef	6.90 (0.13)cde	64.70 (0.60)fg
α,α-trehalose	0.00a	26.98 (0.83)ef	5.54 (0.12)bc	22.27 (0.77)k	54.79 (1.68)ef
α,β-trehalose	20.86 (0.53)ef	39.35 (1.70)h	6.79 (0.39)cdefgh	8.89 (0.00)def	72.93 (1.33)gh
β,β-trehalose	0.00a	28.61 (0.55)efg	9.68 (0.03)i	17.77 (0.67)j	56.07 (1.23)ef
kojibiose	11.79 (0.64)d	45.48 (1.54)ij	7.98 (0.19)h	12.45 (0.55)gh	77.71 (2.39)h
sophorose	8.91 (0.54)cd	39.38 (0.63)h	10.82 (0.32)lj	14.59 (0.57)hi	73.69 (0.51)gh
nigerose	23.49 (0.21)f	40.73 (1.33)h	6.16 (0.35)bcde	8.68 (0.53)def	79.05 (2.19)h
laminaribiose	9.18 (1.04)d	30.12 (0.93)fg	7.71 (0.58)fgh	14.21 (0.80)hi	53.43 (6.50)ef
maltose	35.77 (0.37)gh	43.99 (0.38)hi	5.38 (0.06)bc	6.59 (0.55)bcd	91.73 (0.44)i
cellobiose	12.46 (0.52)d	19.49 (0.95)c	7.80 (0.15)gh	16.87 (0.99)ij	52.47 (3.99)e
isomaltose	2.17 (0.40)ab	49.68 (0.86)jk	9.44 (0.16)i	8.77 (0.25)def	70.06 (1.62)gh
gentiobiose	43.96 (1.55)l	53.26 (1.14)kl	5.53 (0.41)bc	7.01 (0.13)cde	107.92(1.83)j
3α-galactobiose	5.13 (0.18)bc	41.16 (0.75)hi	7.28 (0.18)efgh	17.47 (0.13)j	71.03 (1.23)gh
4β-galactobiose	36.68 (0.39)h	55.12 (0.72)l	6.38 (0.15)cdefg	7.84 (0.09)def	106.02(1.34)j
6β-galactobiose	32.33 (0.69)g	61.54 (0.76)m	7.12 (0.24)defgh	9.60 (0.41)ef	110.59(1.34)j
lactose	46.40 (0.77)i	56.26 (1.02)l	5.80 (0.28)bcd	7.93 (0.04)def	116.39(2.49)j
melibiose	35.91 (2.68)gh	61.46 (0.40)m	6.38 (0.39)cdefg	10.16 (1.23)fg	113.92(0.66)j
2α-mannobiose	0.00a	30.83 (0.16)fg	13.46 (0.18)k	6.49 (0.03)bcd	50.78 (0.25)de
3α-mannobiose	0.00a	24.72 (0.27)de	10.36 (0.18)ij	5.06 (0.15)bc	40.14 (0.32)cd
4α-mannobiose	0.00a	18.80 (0.28)c	11.65 (0.24)j	4.42 (0.06)bc	34.87 (0.41)c
6α-mannobiose	4.08 (0.09)b	20.59 (0.15)cd	10.80 (0.30)ij	17.43 (0.19)j	52.89 (0.49)e

[a] Sample at 0 h, control, FOS, lactose, and melibiose are also included. Different letters indicate significant differences ($P < 0.05$) for acid among the different carbohydrate sources. Standard error is given in parentheses.

Table 4. Lactic Acid, Short-Chain Fatty Acid (SCFA), and Total (Lactic + Acetic + Propionic + Butyric) Concentrations (Millimolar) Produced after 12 h of Fermentation Using Fructose-Containing Disaccharide[a]

	lactic acid	acetic acid	propionic acid	butyric acid	total SCFA
0 h	0.00a	0.94 (0.06)a	1.70 (0.15)a	0.00a	2.65 (0.17)a
control, 12 h	0.00a	14.14 (0.02)b	4.48 (0.02)c	3.28 (0.01)b	21.89 (0.01)b
lactulose	30.67 (0.46)c	51.41 (0.54)g	4.61 (0.06)c	7.35 (0.45)cd	94.04 (1.45)f
trehalulose	0.00a	27.82 (0.08)d	4.16 (0.02)b	9.36 (0.03)e	38.22(3.21)c
sucrose	19.05 (0.16)b	33.02 (0.10)e	5.25 (0.03)d	6.41 (0.28)c	61.94 (0.39)e
turanose	0.00a	34.15 (0.45)f	6.53 (0.03)f	7.85 (0.25)d	48.54 (0.65)d
maltulose	0.00a	21.06 (0.21)c	4.74 (0.01)c	17.28 (0.08)f	43.08 (0.14)cd
leucrose	0.00a	30.11 (0.63)e	5.81 (0.01)e	6.85 (0.08)cd	42.76 (0.66)cd
palatinose	0.00a	26.06 (0.30)d	3.99 (0.00)b	10.02 (0.19)e	40.07 (0.49)c

[a] Different letters indicate significant differences ($P < 0.05$) for acid among the different carbohydrate sources. Standard error is given in parenthess.

however, these samples gave the highest content of propionic acid. α,α-Trehalose showed the highest value of butyric acid statistically significant from the rest. Lactic acid and SCFA values obtained during incubation with fructose-containing disaccharides are shown in **Table 4**. Lactic acid was detected only during lactulose and sucrose incubations, reaching levels of 30.67 and 19.05 mM, respectively. Maltulose fermentation gave the highest concentration of butyric acid, and lactulose showed the highest production of acetic acid. Propionic acid presented low values for all of the fermentation studies, although significant differences were found among the samples.

DISCUSSION

The development of a small-scale in vitro fermentation method is important for testing a wide range of carbohydrates to obtain structure−function information, especially when many carbohydrates can only be obtained in only very small amounts. In addition, attempts to develop novel, enhanced prebiotics require testing of carbohydrates that are initially available only in small quantities. This method was established and compared to higher volume pH-controlled batch cultures. Although generally no significant differences were found between both methods, variations at longer times of incubation (24 h) were slightly greater, mainly for bifidobacteria and bacteroides. This could possibly be explained by the effect of pH on the function of different enzymes during the fermentation of carbohydrates (*29*). However, the statistical treatment demonstrates that both methods can be applied to in vitro studies.

By taking the comparison of both methods into consideration, the effect of fermentation time was also tested. Numbers of total anaerobic bacteria and bifidobacteria increased up until 12 h of fermentation. Following this time, some bacteria began to decrease. For this reason, 12 h of treatment was chosen for the subsequent experiments. This sampling time has been previously used by other authors with different carbohydrate sources such as FOS, gentio-oligosaccharides, and maltodextrins (*17*).

The small-scale fermentation method was also validated using some carbohydrates (sucrose, lactulose, and FOS, **Figure 2**) for which prebiotic properties had been previously studied by other authors using different in vitro fermentation systems (*16*, *29*). As previously described (*6*), FOS and lactulose increased bifidobacterial populations, and the results obtained for the different carbohydrates tested were consistent with those found in the literature.

Once the method had been optimized, quantitative comparisons of the effect of the glycosidic linkages and monosaccharide

Table 5. Summary of the Effects of Glycosyl Linkage and Monosaccharide Composition on Relative Prebiotic Index among the Disaccharides Tested

	disaccharide	prebiotic index
effect of linkage	α-glucobioses	1–2 > 1–6 > 1–3 > 1–4 > 1–1
	β-glucobioses	1–2 > 1–4 > 1–3 > 1–6 > 1–1
	α-galactobioses and melibiose	1–6 > 1–3
	β-galactobioses and lactose	1–4 > 1–6
	α-mannobioses	1–6 > 1–3 > 1–4 > 1–2
	α-fructobioses	1–2 > 1–4 > 1–6 > 1–5 > 1–1 >1–3
effect of monosaccharide composition	α-1–2 linkage	Glu-glu > Glu-fru > Man-man
	α-1–3 linkage	Glu-glu > Gal-gal > Glu-fru > Man-man
	α-1–4 linkage	Glu-fru > Glu-glu > Man-man
	β-1–4 linkage	Gal-gal > Glu-glu > Gal-glu > Gal-fru
	α-1–6 linkage	Glu-glu > Man-man > Gal-glu > Glu-fru
	β-1–6 linkage	Gal-gal > Glu-glu
	1–1 linkage	α, β Glu-glu > α Glu-fru > α, α Glu-glu = β,β Glu-glu

composition of each disaccharide on their fermentation properties were studied.

Significant differences in the populations of bacteria on incubation with glucosyl-glucose disaccharides were seen. Apart from maltose and α,α-trehalose, α-glucosyl-glucose disaccharides presented higher PI indices than the β isomers. Bifidobacteria can produce glycosidases such as β-D-glucosidases, α-D-galactosidases, or β-D-galactosidases and other enzymes located on the cell surfaces that are able to catalyze the hydrolysis of carbohydrates (*30*). Therefore, α-D-glucosidases could better hydrolyze the glycosidic linkages than β-D-glucosidases. A particular behavior was observed for the different isomers of trehalose. α,α-Trehalose and β,β-trehalose presented low PI values, whereas their isomer α,β-trehalose, showed an increase in bifidobacteria population similar to FOS giving a high PI.

The effect of glucosidases on the hydrolysis of disaccharides with linkages 1−2 (kojibiose and sophorose), which presented the highest selectivity toward desirable colonic bacteria, was favored over the other linkages. Previous studies (*31*) have also demonstrated the growth of *Bifidobacterium*, *Lactobacillus*, and *Eubacterium* during the incubation of kojibiose with pure bacteria; however, growth of *Clostridium* and *Bacteroides*, this last species at lower levels, was also detected. Nevertheless, bacterial metabolism of carbohydrates is different in pure and mixed cultures, and the end products of one species can be used as a substrate by others (*2*). Kojioligosaccharides were utilized by fewer strains but more selectively than kojibiose (*31*). Digestibility of these carbohydrates was also tested. Kojibiose was hydrolyzed by small intestinal enzymes; however, kojioligosaccharides were not. Other studies have demonstrated that α-(1−2) glucosidic linkages were partially resistant to the action of enzymic hydrolysis throughout the digestive tract of germfree rats (*32*).

Isomaltose, nigerose, and cellobiose also resulted in a higher PI than FOS. Recently, studies have demonstrated that cellobiose is well fermented in human large intestine by carrying out tolerance tests and breath hydrogen excretion tests (*33*) and also is not hydrolyzed by human small intestinal enzymes, reaching the colon undigested. (*34*, *35*). These data combined with our results could indicate that this disaccharide can be considered as a potential prebiotic.

The bifidobacterial population did not increase in the presence of mannose-containing disaccharides. However, the high PI value found in 6α-mannobiose was due to the increase of lactobacilli when utilizing this carbohydrate. Studies carried out by Asano et al. (*36*) suggested a potential beneficial effect of its isomer, 4β-mannobiose, on the growth of pure bacteria and also stated (*37*) the resistance of this disaccharide and the β-mannooligosaccharides to the action of different enzymes and determined SCFA production during fermentation with fecal bacteria. However, in this study 4α-mannobiose did not generate any prebiotic effect.

Considering the fructose-containing disaccharides, sucrose resulted in the highest PI. It is the only compound of this series that is a glucopyranosyl-fructofuranoside. Possibly, the high increase in the number of bifidobacteria for sucrose compared with other disaccharides may be due to the presence of two nonreducing ends, allowing degradation by α-glucosidases and β-fructosidases. Maltulose (4-*O*-α-D-glucopyranosyl-D-fructose) also generated a high PI, which was greater than that of lactulose (4-*O*-β-D-galactopyranosyl-D-fructose). Glucosyl-fructose disaccharides with glycosidic linkages 1−1, 1−3, and 1−5 (trehalulose, turanose, and leucrose, respectively) gave the lowest PI.

With respect to the monosaccharide composition, for α linkages, glucosyl-glucoses presented higher PI than galactosyl-galactoses. However, for β linkages the behavior was the opposite. Apart from α 1−4 linkages, fructose-containing disaccharides caused lower PI than the corresponding glucosyl-glucoses or galactosyl-galactoses.

Table 5 summarizes the conclusions obtained from the PI values for all of the disaccharides tested using the in vitro culture system.

SCFA are generated from the fermentation of carbohydrates, and they have beneficial effects on human health. Lactic acid was also determined in this study. Acetate and lactate are typical fermentation end products of the bifidus pathway, whereas butyrate is a major product of clostridia and eubacteria. Lactic acid was not detected during α,α-trehalose, β,β-trehalose, and mannobioses fermentation, carbohydrates that did not allow any increase in bifidobacteria. For fructose-containing disaccharides, large amounts of lactic and acetic acids (**Table 4**) were produced during lactulose and sucrose fermentations, the two carbohydrates that resulted in a high number of bifidobacteria (8.82 and 8.68 log recorded, respectively). However, lactic acid was not detected from maltulose fermentation, which also generated a large increase in bifidobacteria (8.72 log). Nevertheless, this carbohydrate showed the highest numbers of eubacteria that do produce butyric acid.

Due to the very restricted quantities of carbohydrate available, the fermentation volumes were kept to an absolute minimum, necessitating the use of one fecal donor. This does, of course, restrict the scope of the conclusions drawn. The study has, however, identified several promising leads for future investigation, most notably the 1−2-linked gluco-oligosaccharides kojibiose and sophorose. Biotechnological approaches to the large-scale manufacture of 1−2-linked oligosaccharides can be applied. The approach taken here should lead to a more

predictive understanding of how specific structures are fermented by the gut microflora, including materials presently available only in low amounts. Although many disaccharides are metabolizable by humans, the results presented here can facilitate the development of novel oligosaccharides that can be beneficial to gut health.

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