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In Vitro Fermentation of Alternansucrase Raffinose-Derived Oligosaccharides by Human Gut Bacteria

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ABSTRACT: In this work, in vitro fermentation of alternansucrase raffinose-derived oligosaccharides, previously fractionated according to their degree of polymerization (DP; from DP4 to DP10), was carried out using small-scale pH-controlled batch cultures at 37 °C under anaerobic conditions with human feces. Bifidogenic activity of oligosaccharides with DP4−6 similar to that of lactulose was observed; however, in general, a significant growth of lactic acid bacteria *Bacteroides*, *Atopobium* cluster, and *Clostridium histolyticum* group was not shown during incubation. Acetic acid was the main short chain fatty acid (SCFA) produced during the fermentation process; the highest levels of this acid were shown by alternansucrase raffinose acceptor pentasaccharides at 10 h (63.11 mM) and heptasaccharides at 24 h (54.71 mM). No significant differences between the gas volume produced by the mixture of raffinose-based oligosaccharides (DP5−DP10) and inulin after 24 h of incubation were detected, whereas lower gas volume was generated by DP4 oligosaccharides. These findings indicate that novel raffinose-derived oligosaccharides (DP4−DP10) could be a new source of prebiotic carbohydrates.

KEYWORDS: raffinose, oligosaccharides, alternansucrase, prebiotic

1. INTRODUCTION

Prebiotics are "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confer benefits upon host well-being and health".[1] Galacto-oligosaccharides, fructo-oligosaccharides, lactulose, and inulin are widely considered as prebiotics, although a high number of different carbohydrates such as pectic-oligosaccharides, isomalto-oligosaccharides or gentio-oligosaccharides are still under study.[2]

Several works describe the production of oligosaccharides by enzymatic transfer reactions from available and inexpensive resources, such as maltose, cellobiose, lactose, or raffinose, among others.[3,4] The use of alternansucrase [EC 2.4.1.140] isolated from *L. mesenteroides* NRRL B-1355 to obtain oligo- and polysaccharides using sucrose as donor and different low molecular weight carbohydrates as acceptors has been reported.[3,5−7] Oligosaccharides with alternating α-(1→6) and α-(1→3) glycosidic linkages are the resulting products of these enzymatic reactions. Considering the high influence of chemical structure of oligosaccharides to modulate the gut microbiota,[8] this diversity of glycosidic linkages increases the interest about these carbohydrates as potential prebiotics. Moreover, the effect of alternansucrase maltose and gentiobiose acceptor products has recently been evaluated by in vitro studies using mixed cultures, both showing promising bifidogenic activities.[3−7,9]

Raffinose (α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) is found in several vegetables and whole grains and can be easily extracted from cottonseed (a coproduct of cottonseed oil production) and soy whey (a coproduct of soybean oil and soy milk production). The raffinose family of oligosaccharides is constituted by different β-galactosyl derivatives such as the tetrasaccharide stachyose and the pentasaccharide verbascose and can be found in the seeds of many plants such as soybeans and lupines.[10,11] Different studies have demonstrated the low digestibility of these oligosaccharides and their effect on gut microbiota;[12−15] however, they can give rise to the production of undesirable levels of flatulence.[12]

Glucosylation of raffinose via acceptor reactions using alternansucrase has been described.[7] Several novel oligosaccharide structures with different molecular weights (mainly tetra-, penta-, and hexasaccharides) of different glycosidic linkages (including some unusual secondary linkages) and monosaccharide residues (galactopyranosyl, glucopyranosyl, and fructofuranosyl) were obtained.[6] These oligosaccharides showed a bifidogenic effect using pure colonic bacteria but did not support significant growth of coliforms and pathogens.[3] Moreover, these carbohydrates exhibited different effect using pure bacteria in comparison to the alternansucrase maltose or gentiobiose acceptor products, which could be due to the different structures;[3,6] however, to the best of our knowledge, there are no data on the fermentation properties of alternansucrase raffinose acceptor oligosaccharides using fecal bacteria or human-feeding studies. In this work, raffinose acceptor products have been synthesized and fractionated according to their degree of polymerization (DP) and their in vitro fermentation behavior has been tested using human fecal samples. The effect of the different molecular weight oligosaccharides on gut microbiota

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Table 1. Changes in Bacteria Population (log cell mL^{-1}) after 0, 10, and 24 h of in Vitro Fermentation with Lactulose, Raffinose, and Alternansucrase Raffinose Acceptor Product Oligosaccharides (DP4−DP10)[a]

		bacterial group (log cell mL^{-1})						
treatment	time (h)	total cells population	Bif164	Bac303	Lab158	Chis150	Ato291	Erec482
control[a]	0	9.02 (0.39)[c abcb]	7.85 (0.14)[a]	7.96 (0.03)[a]	6.46 (0.23)[a]	6.76 (0.21)[a]	7.57 (0.34)[a]	7.55 (0.08)[ab]
	10	8.88 (0.13)[a]	8.11 (0.05)[ab]	8.41 (0.09)[bc]	7.05 (0.33)[b]	7.00 (0.44)[abc]	7.98 (0.34)[abcd]	7.46 (0.11)[a]
	24	9.46 (0.05)[cd]	8.33 (0.20)[bc]	8.59 (0.02)[cde]	7.24 (0.11)[bcde]	7.70 (0.30)[de]	8.08 (0.02)[bcde]	7.97 (0.36)[bc]
lactulose	10	9.33 (0.12)[bcd]	9.21 (0.08)[h]	8.48 (0.10)[bc]	7.06 (0.14)[b]	7.23 (0.16)[abcd]	7.65 (0.06)[ab]	7.55 (0.06)[ab]
	24	9.73 (0.08)[bcd]	8.85 (0.12)[h]	8.52 (0.06)[bc]	7.22 (0.03)[b]	7.73 (0.06)[abcd]	7.53 (0.03)[ab]	7.95 (0.03)[ab]
DP3 (raffinose)	10	9.21 (0.42)[abcd]	8.87 (0.59)[defgh]	8.21 (0.71)[ab]	7.21 (0.62)[bcd]	6.79 (0.65)[ab]	7.68 (0.67)[abc]	7.51 (0.96)[ab]
	24	9.17 (0.59)[abcd]	8.57 (0.59)[cde]	8.50 (0.46)[bcd]	7.37 (0.58)[bcdef]	6.93 (0.66)[abc]	7.81 (0.59)[abcd]	7.61 (0.56)[ab]
DP4	10	9.19 (0.07)[abcd]	8.95 (0.10)[efgh]	8.52 (0.04)[bcde]	7.52 (0.08)[bcdef]	6.96 (0.34)[abc]	8.12 (0.36)[de]	7.51 ± 0.07[ab]
	24	9.50 (0.02)[d]	9.10 (0.02)[gh]	8.99 (0.05)[fg]	7.70 (0.04)[f]	7.09 (0.18)[abc]	8.35 (0.02)[e]	7.86 (0.20)[abc]
DP5	10	9.29 (0.23)[abcd]	9.07 (0.17)[gh]	8.53 (0.34)[bcde]	7.18 (0.23)[bcd]	7.06 (0.16)[abc]	8.18 (0.06)[de]	7.87 (0.17)[abc]
	24	9.51 (0.02)[d]	8.99 (0.07)[fgh]	8.86 (0.05)[efg]	7.66 (0.07)[def]	7.21 (0.18)[abcd]	8.09 (0.16)[cde]	7.88 (0.13)[abc]
DP6	10	9.22 (0.19)[abcd]	8.84 (0.28)[defgh]	8.46 (0.11)[bc]	7.08 (0.10)[b]	7.79 (0.11)[e]	8.04 (0.19)[bcde]	7.79 (0.13)[abc]
	24	9.50 (0.03)[d]	8.88 (0.08)[defgh]	8.72 (0.01)[cdef]	7.70 (0.09)[ef]	7.23 (0.21)[abcd]	7.78 (0.12)[abcd]	7.98 (0.12)[bc]
DP7	10	8.95 (0.13)[ab]	8.57 (0.21)[cde]	8.51 (0.07)[bcde]	7.17 (0.23)[bc]	7.31 (0.43)[bcde]	8.05 (0.30)[bcde]	7.63 (0.14)[ab]
	24	9.53 (0.02)[d]	8.70 (0.28)[cdefg]	8.76 (0.12)[cdef]	7.68 (0.05)[ef]	6.97 (0.15)[abc]	8.06 (0.05)[bcde]	7.93 (0.04)[abc]
DP8	10	9.18 (0.58)[abcd]	8.49 (0.15)[cd]	8.53 (0.09)[bcde]	7.34 (0.42)[bcdef]	6.98 (0.13)[abc]	7.91 (0.23)[abcde]	7.85 (0.10)[abc]
	24	9.50 (0.03)[d]	8.65 (0.34)[cdef]	8.85 (0.07)[defg]	7.66 (0.06)[def]	7.03 (0.11)[abc]	7.98 (0.07)[abcde]	7.85 (0.19)[abc]
DP9	10	9.18 (0.56)[abcd]	8.39 (0.07)[bc]	8.56 (0.23)[bcde]	7.31 (0.27)[bcdef]	7.32 (0.52)[cde]	8.00 (0.23)[abcde]	7.57 (0.08)[ab]
	24	9.50 (0.01)[d]	8.54 (0.28)[cd]	9.18 (0.05)[g]	7.62 (0.10)[cdef]	7.10 (0.08)[abc]	7.99 (0.03)[abcde]	8.11 (0.18)[c]
DP10	10	8.93 (0.14)[ab]	8.48 (0.04)[bcd]	8.68 (0.11)[cdef]	7.36 (0.57)[bcdef]	7.03 (0.26)[abc]	7.88 (0.15)[abcd]	7.64 (0.32)[abc]
	24	9.45 (0.03)[cd]	8.72 (0.21)[cdefg]	9.15 (0.09)[g]	7.64 (0.05)[def]	7.23 (0.24)[abcd]	7.89 (0.08)[abcd]	7.89 (0.12)[abc]

[a] A control sample without carbohydrate source is also included. [b] Different letters indicate significant differences ($P \leq 0.05$) for each bacterial group and for each time of fermentation including 0 h. [c] Standard deviation in parentheses ($n = 3$).

and the production of short chain fatty acids and gas have also been evaluated.

2. MATERIALS AND METHODS

2.1. Enzymes. Alternansucrase [EC 2.4.1.140] was isolated from sucrose-grown cultures of *Leuconostoc mesenteroides* NRRL B-21297 as previously described.[3] Enzymes were concentrated by ultrafiltration and dialyzed against 20 mM sodium acetate buffer (pH 5.4) and used without further purification.

2.2. Carbohydrates. Raffinose pentahydrate, lactulose, and sucrose were purchased from Sigma-Aldrich Corp (St. Louis, MO, United States). Inulin (Raftiline HP) was obtained from Orafti (Tienen, Belgium). The tested oligosaccharides were synthesized as described the Section 2.3.

2.3. Production of Oligosaccharides. Acceptor reactions were carried out as previously described.[7] In brief, reactions were performed at room temperature in 20 mM, pH 5.4, sodium acetate buffer containing 0.01% (w/v) sodium azide. Raffinose (38 g) was suspended as a slurry in 250 mL of buffer into which 87 g of sucrose had been dissolved. Alternansucrase solution (15 mL, containing 3.5 U/mL) was added, and the reaction mixture was gently stirred at ∼22 °C for 5 days. During that time, all of the raffinose eventually dissolved, presumably due to glucosylation. After sucrose consumption, the reaction mixture was mixed with 1.5 volumes of ethanol to precipitate the glucans, which were removed by centrifugation. The supernatant fluid was nanofiltered with a 500 Da membrane cutoff; the retentate was refrigerated for 3 days, and the crystallized raffinose was removed by suction filtration. The clarified solution was fractionated by size exclusion chromatography over a 7.5 cm × 150 cm column of Bio-Rad P-2 gel. The fractions were separated according to their DP, obtaining samples from DP4 to DP10.

2.4. In Vitro Fermentation. Fecal samples were obtained from three healthy donors (one male, two female; age 25−30 years old) without any known metabolic or gastrointestinal disorders. Samples were collected and kept in an anaerobic cabinet for 15 min and then diluted (1/10 w/v) with phosphate buffer (0.1 M, pH 7.4) and homogenized in a stomacher for 2 min.

Gently stirred pH-controlled fermenters (5 mL of working volume) were filled with basal nutrient medium (2 g/L peptone water, 2 g/L yeast extract, 0.1 g/L NaCl, 0.04 g/L K_2HPO_4, 0.04 g/L KH_2PO_4, 0.01 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $CaCl_2 \cdot 6H_2O$, 2 g/L $NaHCO_3$, 2 mL of Tween 80, 0.02 g/L hemin, 10 μL of vitamin K1, 0.5 g/L cysteine HCl, and 0.5 g/L bile salts, pH 7.0) and gassed overnight with nitrogen (99.99% purity). Test substrates (oligosaccharides with different degree polymerization obtained from alternansucrase raffinose acceptor reaction, raffinose, and lactulose as positive control) were dissolved in this medium to give a final concentration of 1% (w/v), and each vessel was inoculated with 500 μL of faecal slurry Additionally, a vessel without any substrate was used as negative control. The temperature was kept at 37 °C at the pH between 6.7 and 6.9. Samples (1 mL) were taken at 0, 10, and 24 h for fluorescent in situ hybridization (FISH) and short chain fatty acid (SCFA) analyses.

2.5. Enumeration of Bacteria by Fluorescent in Situ Hybridization. Samples obtained from fermenters were diluted (1/4) in 4% w/v paraformaldehyde and fixed for 6 h at 4 °C. The bacteria were then centrifuged at 1500*g* for 10 min, washed twice with phosphate-buffered saline (PBS 0.1M, pH 7.0), resuspended in a mixture of cold PBS and 99% ethanol (1:1; v/v), and stored for at least 1 h at −20 °C. The samples were diluted with PBS, in order to obtain an appropriate number of cells to count after hybridization. Hybridization was carried out following the method proposed by Martin-Pelaez et al.[16] using 16S rRNA-targeted oligonucleotide probes labeled with Cy3.

Table 2. SCFA and Lactic Acid Concentration (mM) after 0, 10, and 24 h of in Vitro Fermentation with Lactulose, Raffinose, and Alternansucrase Raffinose Acceptor Product Oligosaccharides[a]

		acids (mM)			
	time (h)	lactic	acetic	propionic	butyric
control	0	0.00^{ab}	0.00^{a}	0.00^{a}	0.00^{a}
	10	0.00^{a}	9.24 (2.68)^{b}	0.00^{a}	0.00^{a}
	24	0.00^{a}	11.06 (1.99)^{bc}	0.00^{a}	0.00^{a}
lactulose	10	9.55 (0.43) ^{cd}	26.75 (1.00)^{ef}	1.81 (0.04)^{abc}	0.00^{a}
	24	0.00^{a}	27.83 (6.72)^{ef}	11.12 (2.04)^{i}	0.00^{a}
raffinose	10	23.06 (2.09)^{e}	30.35 (10.68)^{fg}	1.39 (0.16)^{abc}	0.00^{a}
	24	0.00^{a}	49.65 (3.28)^{ih}	1.96 (0.24)^{abc}	1.86 (0.49)^{b}
DP4	10	2.00 (0.35)^{b}	22.15 (5.04)^{de}	1.40 (0.77)^{abc}	0.00^{a}
	24	0.00^{a}	46.07 (2.73)^{h}	7.02 (2.73)^{fg}	1.42 (0.26)^{bc}
DP5	10	7.24 (0.23)^{c}	63.11 (18.03)^{j}	4.86 (0.34)^{de}	0.90 (0.59)^{ab}
	24	0.00^{a}	46.09 (8.40)^{h}	7.50 (2.81)^{fgh}	2.87 (1.82)^{d}
DP6	10	0.00^{a}	36.04 (2.08)^{g}	1.47 (1.15)^{abc}	0.00^{a}
	24	0.00^{a}	48.16 (1.87)^{h}	9.48 (0.85)^{hi}	5.44 (1.81)^{e}
DP7	10	0.00^{a}	17.53 (1.82)^{cd}	3.35 (1.29)^{cd}	0.00^{a}
	24	0.00^{a}	54.71 (3.13)^{i}	8.94 (0.81)^{gh}	1.16 (0.47)^{b}
DP8	10	0.00^{a}	13.06 (2.09)^{c}	2.71 (1.89)^{bc}	0.00^{a}
	24	0.00^{a}	27.41 (1.81)^{ef}	7.97 (2.52)^{gh}	0.67 (0.07)^{ab}
DP9	10	0.00^{a}	11.96 (2.25)^{bc}	1.36 (0.49)^{ab}	0.00^{a}
	24	0.00^{a}	25.22 (4.39)^{def}	5.62 (0.67)^{ef}	0.67 (0.30)^{ab}
DP10	10	3.96 (1.20)^{b}	10.15 (1.99)^{bc}	2.35 (0.26)^{bc}	0.00^{a}
	24	0.00^{a}	30.74 (8.22)^{j}	18.81 (2.33)^{j}	2.33 (1.72)^{cd}

[a] A control sample without carbohydrate source is also included. [b] Different letters indicate significant differences ($P \leq 0.05$) for each acid and for each time of fermentation. [c] Standard deviation in parentheses ($n = 3$).

Probes, all commercially available (Sigma), were as follows: Bif164, specific for *Bifidobacterium*,[17] Bac303, specific for *Bacteroides*,[18] Chis150 for the *Clostridium histolyticum* group clusters I, II,[19] Erec482 for the *Clostridium coccoides*—*Eubacterium rectale* group,[19] Lab158 for *Lactobacillus*—*Enterococcus* group,[20] and Ato291 for the *Atopobium* cluster.[20] For total counts, the nucleic acid stain 4,6-diamino-2-phenylindole (DAPI) was used. Cells were counted using a fluorescent microscope (Nikon Eclipse, E400), and DAPI-stained cells were examined under ultraviolet light. A minimum of 15 random fields of view were counted per sample.

2.6. Analysis of SCFAs and Lactic Acid. The samples from the fermenters were centrifuged at 13 000*g* for 10 min to remove all insoluble particles, and the produced lactic, acetic, propionic, and butyric acids were quantified using a BioRad HPX-87H HPLC column (Watford, U.K.) at 50 °C, with a 0.005 mM H_2SO_4 as mobile phase, in isocratic mode, at a flow rate of 0.6 mL/min.[21]

2.7. Gas Production. Fermentations of alternansucrase raffinose acceptor oligosaccharides of DP4 and a mixture of DP5—DP10 were carried out in batch tubes and set up as previously described in the Section 2.4. Gas production of lactulose, raffinose, and inulin was also determined for comparative purposes. Appropriate blank tubes without substrate were also included in the experiment. Batch tubes were capped with gas impermeable butyl rubber septa, sealed with aluminum caps in an anaerobic cabinet, and kept at 37 °C with constant agitation. The produced gas volume was measured every 3 h until a maximum of 24 h of fermentation by inserting a pressure sensor fitted with a sterile needle into the rubber septum and recording the pressure using a manometer. After measurements, the headspace of each tube was allowed to equilibrate with the atmosphere of the anaerobic cabinet. Gas production (milliliters) was calculated using a calibration curve with air (gas volume vs pressure) and expressed as gas produced from the substrate minus gas produced from the tubes without substrate. The gas production experiments were performed in quadruplicate for each donor/substrate/mixture.

2.8. Statistical Analyses. Statistical analyses were performed using Statistica for Windows version 6 (2002) by Statsoft Inc. (Tulsa, United States). Differences between bacterial counts and SCFA concentration were tested using a one-way ANOVA test, followed by a least significant difference (LSD) test as a posthoc comparison of means ($P < 0.05$).

3. RESULTS AND DISCUSSION

3.1. Effect of Raffinose-Derived Oligosaccharides Fermentation on Human Gut Microbiota. Chemical structures determined by NMR of alternansucrase raffinose acceptor oligosaccharides have been described by Côté et al.[7] Fractions of different molecular weights of these oligosaccharides were used to study their effect on gut microbiota.

Changes in human faecal bacterial populations at inoculation and 10 and 24 h of in vitro fermentation of the different oligosaccharide fractions are shown in Table 1. Lactulose was also included in this study. No significant variations were detected in total cell counts at 10 h of incubation compared with the initial inoculum for all the samples, whereas an increase in total bacteria populations was observed for the DP4—DP9 oligosaccharides at 24 h of fermentation. For DP7 and DP10, significant differences were found in total bacteria between 10 and 24 h of fermentation. This increment can be due to the fermentation of these carbohydrates not only by the bacteria studied in this work but also by other bacterial species. A similar increment was also found during the fermentation of other carbohydrate sources such as

gentio-oligosaccharides[22] and xylo-oligosaccharides[23] between 5 and 24 h of fermentation.

Comparing to the bacterial populations found in the negative control vessel at 0 h of incubation, a significant increase of Bif164 populations was observed for the raffinose acceptor products of DP4−6 at both 10 and 24 h. These bacterial counts were similar to that produced for lactulose fermentation. Oligosaccharides of DP7 and DP8 also showed a significant increase of Bif164 population at 10 h; however, changes were not significant for these carbohydrates at 24 h and for DP9 and DP10 oligosaccharides at both incubation times. On the other hand, significant changes in Lab158 were not detected, except for DP4 at 24 h. Previously, Holt et al.[3] reported the bifidogenic activity of a mixture of these raffinose-based oligosaccharides (95% of DP4, < 5% of DP3, and <5% of DP5 and higher) using pure cultures, whereas no growth of lactobacilli was observed; however, the effect of the degree of polymerization was not studied. Taking into account the molecular weight of oligosaccharides, similar results has been reported by Sanz et al.[4] for alternansucrase maltose-acceptor oligosaccharides. For these carbohydrates, the highest increase in bifidobacteria populations was also obtained for DP 4, 5, and 6, whereas a nonsignificant increase in bifidobacteria was observed for oligosaccharides of DP8−DP11. Moreover, the Lab158 population did not increase during incubation. Similarly, gentiooligosaccharides obtained by alternansucrase reaction (DP4−6) showed a high bifidogenic effect.[9]

A significant increase in Bac303 populations was detected for DP4, DP9, and DP10 at 24 h, whereas, in general, no significant differences among all carbohydrates studied were detected for Chis150, Ato291, and Erec482. It is worth noting the relatively high value of Chis150 found for DP6 at 10 h of incubation.

The molecular weight of oligosaccharides is very likely relevant to their prebiotic activity, and although some studies suggest that carbohydrates with higher degree of polymerization could reach the more distal regions of the gut where most of disorders take place,[24−26] lower molecular weight oligosaccharides present higher bifidogenic activity. However, more studies are required with oligosaccharides of higher DP to confirm this behavior.

3.2. Effect of Raffinose-Derived Oligosaccharides Fermentation on Lactic Acid and SCFA Production. It is known that lactic acid and SCFA are produced by almost all intestinal bacteria by the fermentation of nondigestible carbohydrates. Table 2 shows the concentration of these acids produced by the assayed oligosaccharides. Acetic acid was the most abundant acid detected in all studied samples. On the contrary, lactic acid was only observed after 10 h of fermentation of raffinose, lactulose, DP5, DP10, and DP4 oligosaccharides. Lactic and acetic acid are considered products of the fermentation pathway of *Bifidobacterium* genus; however, lactic acid is converted into other SCFA by different bacteria genus, which could be a possible cause of the decline of this acid during fermentation.[27,28]

Regarding propionic acid concentration, the highest values were detected after 24 h of fermentation of raffinose-based oligosaccharides of DP10 that also showed a significant increase in the Bac303 population. This genus is partially responsible for the production of this acid.[29] Previous works reported that an increase of propionic acid and a decrease of acetic acid amount could be an indicator of the hypolipidemic effect of some prebiotics.[30,31]

Butyric acid was only detected at 24 h of fermentation, and the highest values were found for DP6 oligosaccharides, followed by DP5 and DP10. Although butyric acid is considered an end product of the fermentation pathways of the bacteria enumerated by Chis150 and Erec482, a significant increase of these genera was not found in the studied samples.

3.3. Effect of Raffinose-Derived Oligosaccharides Fermentation on Gas Production. An undesirable effect of raffinose intake is the high production of gas during its fermentation in the lower intestine with the concomitant liberation of carbon dioxide, hydrogen, and methane, which causes flatulence.[32] In this study, the gas production during fermentation of alternansucrase raffinose acceptor products was measured. Figure 1 shows the total gas produced after 24 h of fermentation of raffinose, DP4, and a mixture of DP5−DP10 of raffinose acceptor oligosaccharides. No significant differences were observed between gas values produced by lactulose and raffinose and between this last carbohydrate and DP4 raffinose-based

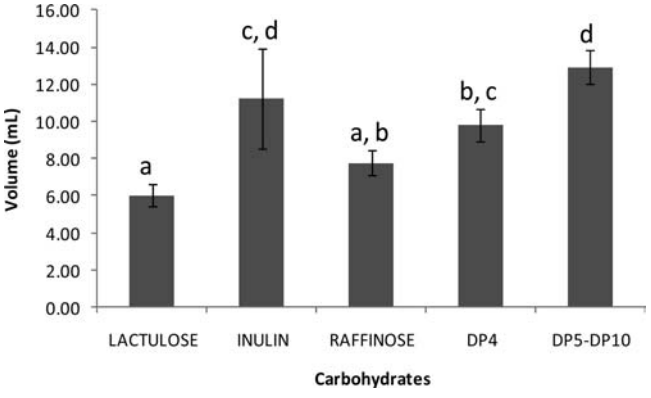

Figure 1. Total gas produced after 24 h of fermentation with lactulose, inulin, raffinose, alternansucrase raffinose acceptor tetrasaccharides (DP4), and a mixture of alternansucrase raffinose acceptor penta-, hexa-, hepta-, octa-, nona-, and decasaccharides (DP5−DP10).

Table 3. Rate of Gas Production (mL h^{-1}) Registered at Different Times of in Vitro Fermentation (from 3 to 24 h) with Lactulose, Inulin, Raffinose, Alternansucrase Raffinose Acceptor Tetrasaccharides (DP4), and a Mixture of Penta-, Hexa-, Hepta-, Octa-, Nona-, and Deca- (DP5−DP10) Alternansucrase Raffinose Acceptor Products

	rate of gas production (mL h^{-1})							
substrate/time (h)	3	6	9	12	15	18	21	24
lactulose	0.49 (0.02) [baa]	0.57 (0.19)[a]	0.26 (0.02)[a]	0.28 (0.06)[ab]	0.18 (0.04)[a]	0.15 (0.02)[a]	0.05 (0.03)[a]	0.02 (0.03)[a]
inulin	0.89 (0.14)[bc]	0.71 (0.24)[ab]	0.55 (0.05)[b]	0.38 (0.04)[a]	0.48 (0.14)[b]	0.32 (0.17)[b]	0.24 (0.08)[b]	0.19 (0.14)[b]
raffinose	1.19 (0.04)[d]	0.70 (0.20)[ab]	0.30 (0.03)[a]	0.12 (0.03)[a]	0.08 (0.06)[a]	0.13 (0.01)[a]	0.04 (0.03)[a]	0.02 (0.03)[a]
DP4	1.11 (0.22)[cd]	0.85 (0.20)[ab]	0.75 (0.23)[bc]	0.34 (0.11)[a]	0.20 (0.08)[a]	0.13 (0.02)[a]	0.08 (0.03)[a]	0.08 (0.04)[ab]
DP5−DP10	0.66 (0.11)[ab]	0.95 (0.14)[b]	0.93 (0.14)[c]	0.69 (0.06)[c]	0.61 (0.03)[b]	0.41 (0.11)[b]	0.20 (0.04)[b]	0.13 (0.04)[ab]

[a] Different letters indicate significant differences ($P \leq 0.05$) among substrates for each time of fermentation. [b] Standard deviation in parentheses ($n = 4$).

oligosaccharides; however, a higher gas volume was produced by the mixture of DP5−DP10 with no significant differences with volume produced by commercial inulin.

Considering the rate of gas production (Table 3), similar values were obtained for lactulose and DP5−DP10 oligosaccharides during the first three hours of incubation; however, significant differences in gas production were observed in these samples from 6 to 21 h. Gas production of the oligosaccharide mixture only differed from inulin at 9 and 12 h of incubation. In general, gas production of DP4 raffinose-based oligosaccharides was not significantly different from those of raffinose and lactulose during the whole incubation period. Moreover, it only differed from the gas produced by lactulose at 3 and 9 h of incubation.

In conclusion, the bifidogenic activity of alternansucrase raffinose acceptor oligosaccharides with molecular weights from DP4 to DP6 has been indicated by in vitro studies using human feces, showing a high dependence of the molecular weight on the bifidogenic activity and confirming the results previously reported for gentioligosaccharides and maltooligosaccharides obtained by alternansucrase reaction. Although further in vivo studies should be conducted, these carbohydrates could be considered as a new and alternative source of prebiotics. In addition, these oligosaccharides generated a similar volume of gas as commercial inulin, with the exception of DP4 oligosaccharides, which showed lower gas generation, similar to those produced by raffinose and lactulose.

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REFERENCES

(1) Gibson, G. R.; Probert, H. M.; Loo, J. V.; Rastall, R. A.; Roberfroid, M. B. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* **2004**, *17*, 259–275.

(2) Rastall, R. A. Functional oligosaccharides: application and manufacture. *Annu. Rev. Food Sci. Technol.* **2010**, *1*, 305–339.

(3) Holt, S. M.; Miller-Fosmore, C. M.; Côté, G. L. Growth of various intestinal bacteria on alternansucrase-derived oligosaccharides. *Lett. Appl. Microbiol.* **2005**, *40*, 385–390.

(4) Sanz, M.; Côté, G. L.; Gibson, G. R.; Rastall, R. A. Influence of glycosidic linkages and molecular weight on the fermentation of maltose-based oligosaccharides by human gut bacteria. *J. Agric. Food Chem.* **2006**, *54*, 9779–9784.

(5) Côté, G. L.; Sheng, S. Penta-, hexa-, and heptasaccharide acceptor products of alternansucrase. *Carbohydr. Res.* **2006**, *341*, 2066–2072.

(6) Côté, G. L. Acceptor products of alternansucrase with gentiobiose. Production of novel oligosaccharides for food and feed and elimination of bitterness. *Carbohydr. Res.* **2009**, *344*, 187–190.

(7) Côté, G. L.; Dunlap, C. A.; Vermillion, K. E. Glucosylation of raffinose via alternansucrase acceptor reactions. *Carbohydr. Res.* **2009**, *344*, 1951–1959.

(8) Sanz, M.; Gibson, G. R.; Rastall, R. A. Influence of disaccharide structure on prebiotic selectivity in vitro. *J. Agric. Food Chem.* **2005**, *53*, 5192–5199.

(9) Sanz, M. L.; Côté, G. L.; Gibson, G. R.; Rastall, R. A. Selective fermentation of getiobiose-derived oligosaccharides by human gut bacteria and influence of molecular weight. *FEMS Microbiol. Ecol.* **2006**, *56*, 383–388.

(10) Peterbauer, T.; Richter, A. Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. *Seed Sci. Res.* **2001**, *11*, 185–197.

(11) Martinez-Villaluenga, C.; Frias, J.; Vidal-Valverde, C. Functional lupin seeds (*Lupinus albus* L. and *Lupinus luteus* L.) after extraction of alpha-galactosides. *Food Chem.* **2006**, *98*, 291–299.

(12) Rackis, J. J. Oligosaccharides of food legumes: alpha-galactosidase activity and the flatus problem. In *Physiological Effects of Food Carbohydrates*; Jeanes, A., Hodege, J., Eds.; American Chemical Society, Washington, D.C., 1975; Vol. 15, pp 207−222.

(13) Gibson, G. R.; Roberfroid, M. B. Dietary modulation of the human colonic microbiota − Introducing the concept of prebiotics. *J. Nutr.* **1995**, *125*, 1401–1412.

(14) Gopal, P. K.; Sullivan, P. A.; Smart, J. B. Utilisation of galacto-oligosaccharides as selective substrates for growth by lactic acid bacteria including *Bifidobacterium lactis* DR10 and *Lactobacillus rhamnosus* DR20. *Int. Dairy J.* **2001**, *11*, 19–25.

(15) Martinez-Villaluenga, C.; Frias, J.; Vidal-Valverde, C.; Gomez, R. Raffinose family of oligosaccharides from lupin seeds as prebiotics: Application in dairy products. *J. Food Prot.* **2005**, *68*, 1246–1252.

(16) Martin-Pelaez, S.; Gibson, G. R.; Martin-Orue, S. M.; Klinder, A.; Rastall, R. A.; La Ragione, R. M.; Woodward, M. J.; Costabile, A. In vitro fermentation of carbohydrates by porcine faecal inocula and their influence on *Salmonella typhimurium* growth in batch culture systems. *FEMS Microbiol. Ecol.* **2008**, *66*, 608–619.

(17) Langendijk, P. S.; Schut, F.; Jansen, G. J.; Raangs, G. C.; Kamphuis, G. R.; Wilkinson, M. H. F.; Welling, G. W. Quantitative fluorescence in-situ hybridization of *Bifidobacterium* spp with genus-specific 16S ribosomal-RNA-targeted probes and its application in fecal samples. *Appl. Environ. Microbiol.* **1995**, *61*, 3069–3075.

(18) Manz, W.; Amann, R.; Ludwig, W.; Vancanneyt, M.; Schleifer, K. H. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. *Microbiol. U.K.* **1996**, *142*, 1097–1106.

(19) Franks, A. H.; Harmsen, H. J. M.; Raangs, G. C.; Jansen, G. J.; Schut, F.; Welling, G. W. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. *Appl. Environ. Microbiol.* **1998**, *64*, 3336–3345.

(20) Harmsen, H. J. M.; Elfferich, P.; Schut, F.; Welling, G. W. A 16S rRNA-targeted probe for detection of *Lactobacilli* and *Enterococci* in faecal samples by fluorescent in situ hybridization. *Microb. Ecol. Health Dis.* **1999**, *11*, 3–12.

(21) Sanz, M.; Polemis, N.; Morales, V.; Corzo, N.; Drakoularakou, A.; Gibson, G. R.; Rastall, R. A. In vitro investigation into the potential prebiotic activity of honey oligosaccharides. *J. Agric. Food Chem.* **2005**, *53*, 2914–2921.

(22) Rycroft, C. E.; Jones, M. R.; Gibson, G. R.; Rastall, R. A. Fermentation properties of gentio-oligosaccharides. *Lett. Appl. Microbiol.* **2001**, *32*, 156–161.

(23) Rycroft, C. E.; Jones, M. R.; Gibson, G. R.; Rastall, R. A. A comparative in vitro evaluation of the fermentation properties of prebiotic oligosaccharides. *J. Appl. Microbiol.* **2001**, *91*, 878–887.

(24) Wiele, T. v. d.; Boon, N.; Possemiers, S.; Jacobs, H.; Verstraete, W. Inulin-type fructans of longer degree of polymerization exert more

pronounced in vitro prebiotic effects. *J. Appl. Microbiol.* **2007**, *102* (2), 452–460.

(25) Gibson, G. R. From probiotics to prebiotics and a healthy digestive system. *J. Food Sci.* **2004**, *69* (5), M141–M143.

(26) Roberfroid, M.; Gibson, G. R.; Hoyles, L.; McCartney, A. L.; Rastall, R.; Rowland, I.; Wolvers, D.; Watzl, B.; Szajewska, H.; Stahl, B.; Guarner, F.; Respondek, F.; Whelan, K.; Coxam, V.; Davicco, M. J.; Leotoing, L.; Wittrant, Y.; Delzenne, N. M.; Cani, P. D.; Neyrinck, A. M.; Meheust, A. Prebiotic effects: metabolic and health benefits. *Br. J. Nutr.* **2010**, *104*, S1–S63.

(27) Bourriaud, C.; Robins, R. J.; Martin, L.; Kozlowski, F.; Tenailleau, E.; Cherbut, C.; Michel, C. Lactate is mainly fermented to butyrate by human intestinal microbiotas but inter-individual variation is evident. *J. Appl. Microbiol.* **2005**, *99*, 201–212.

(28) Hughes, S. A.; Shewry, P. R.; Gibson, G. R.; McCleary, B. V.; Rastall, R. A. In vitro fermentation of oat and barley derived β-glucans by human faecal microbiota. *FEMS Microbiol. Ecol.* **2008**, *64*, 482–493.

(29) Macy, J.; Probst, I. The biology of gastrointestinal bacteroides. *Annu. Rev. Microbiol.* **1979**, *33*, 561–594.

(30) Salazar, N.; Ruas-Madiedo, P.; Kolida, S.; Collins, M.; Rastall, R.; Gibson, G.; de los Reyes-Gavilán, C. G. Exopolysaccharides produced by *Bifidobacterium longum* IPLA E44 and *Bifidobacterium animalis* subsp. lactis IPLA R1 modify the composition and metabolic activity of human faecal microbiota in pH-controlled batch cultures. *Int. J. Food Microbiol.* **2009**, *135*, 260–267.

(31) Delzenne, N. M.; Kok, N. Effects of fructans-type prebiotics on lipid metabolism. *Am. J. Clin. Nutr.* **2001**, *73*, 456S–458S.

(32) Muzquiz, M.; Burbano, C.; Pedrosa, M. M.; Folkman, W.; Gulewicz, K. Lupins as a potential source of raffinose family oligosaccharides − Preparative method for their isolation and purification. *Ind. Crops Prod.* **1999**, *9* (3), 183–188.