# Feruloylated and Nonferuloylated Arabino-oligosaccharides from Sugar Beet Pectin Selectively Stimulate the Growth of *Bifidobacterium* spp. in Human Fecal in Vitro Fermentations

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**ABSTRACT:** The side chains of the rhamnogalacturonan I fraction in sugar beet pectin are particularly rich in arabinan moieties, which may be substituted with feruloyl groups. In this work the arabinan-rich fraction resulting from sugar beet pulp based pectin production was separated by Amberlite XAD hydrophobic interaction and membrane separation into four fractions based on feruloyl substitution and arabino-oligosaccharide chain length: short-chain (DP 2—10) and long-chain (DP 7—14) feruloylated and nonferuloylated arabino-oligosaccharides, respectively. HPAEC, SEC, and MALDI-TOF/TOF analyses of the fractions confirmed the presence of singly and doubly substituted feruloylated arabino-oligosaccharides in the feruloyl-substituted fractions. In vitro microbial fermentation by human fecal samples (*n* = 6 healthy human volunteers) showed a selective stimulation of bifidobacteria by both the feruloylated and the nonferuloylated long-chain arabino-oligosaccharides to the same extent as the prebiotic fructo-oligosaccharides control. None of the fractions stimulated the growth of the potential pathogen *Clostridium difficile* in monocultures. This work provides a first report on the separation of potentially bioactive feruloylated arabino-oligosaccharides from sugar beet pulp and an initial indication of the potentially larger bifidogenic effect of relatively long-chain arabino-oligosaccharides as opposed to short-chain arabino-oligosaccharides.

**KEYWORDS:** prebiotics, arabino-oligosaccharides, feruloyl substitution, hydrophilic interaction chromatography, *Clostridium difficile*

## ■ INTRODUCTION

Sugar beet pulp is a large side stream from the industrial production of sugar. Currently, the main utilization of sugar beet pulp is for cattle feed, but pectin can be extracted for a limited number of applications. In sugar beet pectin the side chains of rhamnogalacturonan I (RGI) are especially rich in arabinan composed of α-(1,5)-linked backbones with a high degree of α-(1,2) and/or α-(1,3) arabinofuranosyl substitutions[1] along with some β-(1,4)-linked galactan. The side chains of sugar beet RGI can be feruloyl substituted either on O-2 in the main backbone of α-(1,5)-linked arabinan, on O-5 in the terminal arabinose,[2] or on O-6 in the main backbone of galactan.[3] The content of ferulic acid can be up to 8.3 mg/g pectin.[4] The feruloyl substitutions can either be present as monomers or form dimers with other side chains through oxidative coupling reactions.[5] Diferulic cross-linking plays an important role in plant texture.[6]

Sugar beet pectin-derived arabino-oligosaccharides with DP 2—6 and DP < 8 have been reported to selectively stimulate bifidobacteria over clostridia, lactobacilli, and bacteroides in in vitro fecal fermentations.[7,8] Moreover, in single-culture studies it has been shown that linear arabino-oligosaccharides may be utilized by *Bifidobacterium adolescentis*, *Bifidobacterium longum*, and *Bacteroides vulgatus*, but not by other bifidobacteria or the tested lactobacilli.[9]

The biological effect of oligosaccharide feruloyl substitution on gut microbiota has so far mainly been investigated for arabino-xylo-oligosaccharides. Feruloylated arabino-xylo-oligosaccharides were shown to stimulate the growth of *Bifidobacterium bifidum* in a single-culture study,[10] xylo-feruloyl-arabinose from grass cell walls has been reported to be metabolized by rat gut microbiota,[11] and recently ferulic acid was found to be released from durum wheat oligosaccharides during digestion by human intestinal microbiota.[12] Moderate feruloyl substitution was shown not to impede the degradation of maize cell wall by human intestinal microbiota.[13]

Probiotic bacteria such as bifidobacteria and lactobacilli have been shown to be able to specifically express feruloyl esterase activity in the presence of feruloylated substrates. *Bifidobacterium* sp., including *B. longum*, thus express feruloyl esterase activity in the presence of wheat bran, rye bran, barley spent grain, and larchwood arabinogalactan,[14] and *Lactobacillus acidophilus* expresses feruloyl esterase activity in the presence of destarched wheat bran.[15] These studies on arabinoxylan-derived oligosaccharides are not directly comparable to feruloyl-substituted arabino-oligosaccharides, because feruloyl substitution in

arabinoxylan is O-5 linked and feruloyl substitutions in arabino-oligosaccharides are mainly O-2 linked. Therefore, these two types of feruloyl-substituted substrates require different types of feruloyl esterases for enzyme-catalyzed ferulic acid removal. It is known that type B feruloyl esterases are active on pectin-derived substrates,[16] but little has been reported with respect to bifidogenic or other prebiotic effects of pectin-derived O-2 ferulic acid—arabinose conjugates. We hypothesized that O-2 feruloyl substitutions on arabino-oligosaccharides derived from sugar beet pectin might exhibit prebiotic effects and, secondly, that the arabino-oligosaccharide backbone chain length might influence the outcome. The purpose of the present study was therefore to first prepare fractions of different arabino-oligosaccharide chain lengths enriched in feruloyl substitutions, and the corresponding nonferulated counterparts, both from sugar beet pectin and then to evaluate the in vitro fermentability of the fractions by human intestinal microbiota.

## ■ MATERIALS AND METHODS

**Substrate.** Sugar beet arabino-oligosaccharides were obtained from Danisco A/S (Nakskov, Denmark). These arabino-oligosaccharides, in the following referred to as "starting material", were supplied as a liquid side stream from the ultrafiltration and diafiltration step in the sequential acid extraction of pectin with nitric acid from sugar beet pulp, involving removal of insoluble cellulose, ultrafiltration, and diafiltration with a 50 kDa cutoff.[4]

**Chemicals.** Ferulic acid, cinnamic acid, sodium hydroxide, sodium acetate, D-glucuronic acid, D-galactose, D-arabinose, D-fucose, L-rhamnose monohydrate, D-galacturonic acid monohydrate, and 2,5-dihydroxy-benzoic acid were obtained from Sigma-Aldrich (Steinheim, Germany). D-Glucose and D-xylose were obtained from Merck (Darmstadt, Germany). Trifluoroacetic acid was from Riedel-deHaën (Seelze, Germany). Arabino-oligosaccharide standards with DP from 2 to 5 were obtained from Megazyme (Bray, Co. Wicklow, Ireland). Acetonitrile was obtained from LGC Promochem (Middlesex, U.K.). Amberlite XAD-2 was obtained from Rohm and Haas Denmark (Copenhagen, Denmark). Fructo-oligosaccharides (FOS) (DP 2—8; OraftiP95) were obtained from Beneo-Orafti (Tienen, Belgium).

**XAD Separation.** XAD separation was performed at room temperature in a 200 mL stirred membrane reactor model 8200 (Millipore, Billerica, MA) equipped with a 100 kDa regenerated cellulose membrane (Millipore), connected to compressed nitrogen for flux regulation. The reactor contents were mixed by magnetic stirring. The starting material was added to the XAD material (washed in 2 volumes of ethanol and 2 volumes of water prior to use) in a 1:1 volume ratio. The suspension was stirred for 1 h, and unbound material was then removed by filtration. In each round, the contact time between the eluent and the XAD was 30 min before filtration. Putative nonferuloylated arabino-oligosaccharides (denoted AOS) were eluted in 4 sample volumes of water, and feruloylated arabino-oligosaccharides (denoted FAOS) were eluted in 5 sample volumes of methanol/water 1:1 (v/v). The methanol/water fraction was dried on a rotary evaporator and resolubilized in deionized water. The AOS and FAOS fractions were subsequently separated according to molecular weight (see below). Residual material was eluted in 3 sample volumes of 100% methanol. This fraction was not used further.

**Membrane Separation.** Separation according to size for both the AOS fraction and the FAOS fraction was performed in a 200 mL stirred membrane reactor model 8200 (Millipore) equipped with a 1 kDa MWCO regenerated cellulose membrane (Millipore), connected to compressed nitrogen for flux regulation. The reactor contents were mixed by magnetic stirring using an RCT basic magnetic stirrer (IKA, Germany). The temperature was maintained at 45 °C. Filtration was performed until the retentate volume was 10% of the sample volume, followed by diafiltration in 1 sample volume of deionized water. After ultra- and diafiltration, all permeates and retentates were concentrated on a rotary evaporator, lyophilized, resolubilized in water, and stored at 4 °C until further use. The permeates enriched in low molecular weight (small) oligosaccharides were denoted SAOS and SFAOS, respectively, and the retentates enriched in high molecular weight (long) oligosaccharides were denoted LAOS and LFAOS, respectively.

**Acid Hydrolysis.** The monosaccharide composition of all fractions was determined by acid hydrolysis with 2 g/L substrate concentration and 2 M trifluoroacetic acid at 121 °C for 2 h followed by lyophilization and resolubilization in deionized water, and the recovery of monosaccharides was determined by performing the same hydrolysis on D-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose, D-xylose, D-galacturonic acid, and D-glucuronic acid.[17] Relative standard deviation values for all measurements were determined for at least three measurements and ranged from 0.5 to 10%.

**Ionic Exchange Chromatography (HPAEC).** Monosaccharide composition and AOS concentrations were analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using a Dionex BioLC system (Dionex Corp., Sunnyvale, CA) equipped with a Dionex CarboPac PA1 analytical column (4 × 250 mm). For analysis of monosaccharides a two-eluent system comprising deionized water and a 500 mM NaOH aqueous solution was used. The monosaccharides were separated by isocratic elution with 25 mM NaOH for 4 min, 10 mM NaOH for 14 min, and 500 mM NaOH for 12 min, followed by regeneration with 25 mM NaOH for 5 min. For analysis of oligosaccharides, a three-eluent system comprising deionized water, a 500 mM NaOH aqueous solution, and a 500 mM sodium acetate aqueous solution was used (modified from ref 18). AOS were eluted isocratically with 25 mM NaOAc for 5 min followed by a linear gradient from 25 to 400 mM NaOAc for 20 min and then isocratically with 400 mM NaOAc for 10 min. The NaOH concentration was maintained at 100 mM throughout the run. The eluent flow was always kept at 1.0 mL/min. Quantification was carried out using external monosaccharide standards: D-fucose, L-rhamnose, D-arabinose, D-galactose, D-glucose, D-xylose, and D-galacturonic acid or di-, tri-, tetra-, and penta-mers of arabino-oligosaccharides. Amounts were expressed as milligrams per gram of dry matter (mg/g) or mole percentage (mol %). The following pulse potentials and durations were used for detection: E1 = 0.1 V, t1 = 400 ms; E2 = −2 V, t2 = 20 ms; E3 = 0.6 V, m3 =10 ms; E4 = −0.1 V, t4 = 60 ms. Data were collected and analyzed with the program Chromeleon 6.80 SP4 Build 2361 software (Dionex Corp., Sunnyvale, CA).

**Size Exclusion Chromatography.** HPSEC was performed using a P680 HPLC pump, an ASI-100 automated sample injector, and an RI-101 refractive index detector (Dionex). Samples were separated on a Shodex SB-806HQGPC column (300 × 8 mm) with a Shodex SB-G guard column (50 × 6 mm) from Showa Denko K.K. (Tokyo, Japan) with 100 mM sodium acetate, pH 6, as mobile phase at a flow rate of 0.5 mL/min; the temperature was maintained at 40 °C. Prior to injection, the sample was diluted in 100 mM sodium acetate to avoid large buffer peaks.[19]

**Phenolics Analysis.** Ferulic acid was released from arabinan structures by saponification by adding NaOH to a final concentration of 1 M followed by incubation at room temperature for 1 h. Subsequently, the pH was adjusted to <3 by adding HCl. Ferulic acid and vanillin were analyzed using RP-HPLC with DAD, Chemstation 1100 series, Hewlett-Packard, and an ODS-L Optimal (250 × 4.6 mm, 5 $\mu$m) column from Capital HPLC. The chromatographic profile consisted of buffer A (5% acetonitrile, 1 mM TFA) and acetonitrile starting with 20% acetonitrile. Running gradient was up to 40% acetonitrile for 35 min and further up to 100% for another 3 min, followed by regeneration to 20% acetonitrile for 2 min. Column temperature was maintained at 40 °C.[20] The amount of *trans*-ferulic acid was detected and quantified at 316 nm

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**Table 1. 16S rRNA Primers for Real Time PCR**

| target gene | forward primer (5′–3′) | reverse primer (5′–3′) | efficiency (%) | ref |
|---|---|---|---|---|
| *Bifidobacterium* spp. | cgc gtc ygg tgt gaa ag | ccc cac atc cag cat cca | 101 | 32 |
| *Lactobacillus* spp. | agc agt agg gaa tct tcc a | cac cgc tac aca tgg ag | 95 | 33, 34 |
| *Firmicutes* phylum | gga gya tgt ggt tta att cga agc a | agc tga cga caa cca tgc ac | 99 | 35 |
| *Bacteroidetes* phylum | gga rca tgt ggt tta att cga tga t | agc tga cga caa cca tgc ag | 100 | 35 |
| total bacteria | act cct acg gga ggc agc agt | gta tta ccg cgg ctg ctg gca c | 101 | 34 |

**Table 2. Mass and Ferulic Acid Distributions in Fractions from XAD Separation**

| | dry matter (mass %) | ferulic acid ($\mu$mol/g) | ferulic acid (mol %) |
|---|---|---|---|
| starting material | 100.0 | 36.2 | 100.0 |
| unbound | 47.9 | 6.1 | 8.1 |
| water | 28.7 | 0.7 | 0.5 |
| MeOH/water | 21.3 | 143.9 | 84.8 |
| MeOH | 1.8 | 12.0 | 0.6 |
| loss | 0.4 | | 6.1 |

using an authentic external standard and expressed as micromoles per gram of dry matter ($\mu$mol/g). *cis*-Ferulic acid and vanillin were detected and recognized at 316 nm but quantified at 280 nm according to response factors as described by Waldron et al.[21]

**HILIC Chromatography.** Separation of feruloylated AOS based on hydrophilic interaction was performed using an ÄKTA purifier 100 workstation equipped with a UV-900 monitor, a pH/C monitor, and a Frac-950 fraction collector, equipped with an TSK-GEL Amide-80 column (250 × 4.6 mm i.d., 5 $\mu$m, Tosoh Bioscience), all controlled by UNICORN software. The temperature was maintained at 55 °C using a Thermasphere HPLC column heater/chiller (Phenomenex). The elution system consisted of acetonitrile and deionized water. The column was equilibrated with 80% acetonitrile for 2 column volumes (CV), isocratic elution at 80% for 2 CV, followed by a linear gradient at 2% acetonitrile/CV. Feruloylated compounds were detected and quantified by UV absorption at 316 nm using ferulic acid as external standard. Prior to injection, the sample was adjusted to 80% acetonitrile, centrifuged briefly for removal of precipitate, and filtered through a 0.22 $\mu$m nylon filter.

**Mass Spectrometry.** One microliter of sample followed by 0.5 $\mu$L of matrix solution (20 mg/mL 2,5-dihydroxybenzoic acid 70% acetonitrile and 0.1% trifluoroacetic acid/water) was added to an Opti-TOF 384 well plate. For fast crystallization, the sample was dried under a lamp and an additional 0.5 $\mu$L of matrix solution added. The samples were analyzed on a 4800 Plus MALDI TOF/TOF (AB Sciex) mass spectrometer. The instrument was operated in reflectron, positive ion mode. Acceleration voltage was 20 kV. Depending on the sample analyzed, laser intensity and number of laser shots were varied to obtain optimal spectra. The mass range was set to 100–3500 Da. For all MS/MS data, air was used as collision gas. The MS and MS/MS data were exported as a text file using DataExplorer (version 4.6), and each spectrum was smoothed, labeled, and analyzed manually employing M/Z (Genomic Solutions). For annotation of MS/MS spectra, the nomenclature suggested by Doman and Costello[22] was applied.

**Subjects and Fecal Samples.** Fecal samples were obtained from six healthy volunteers (mean age = 40.7 ± 8.5 years). None of the participants had been treated with antibiotics for at least 3 months before participation and had no history of gastrointestinal disorder. Whole stools were collected in airtight containers, immediately stored at 4 °C, and processed within 12 h. Feces (200 mg wet weight) were collected for DNA extraction, and additional fecal samples for in vitro fermentation

were prepared by homogenization in 50% glycerol (1:1 dilution in deionized water) in an anaerobic cabinet (containing 10% $H_2$, 10% $CO_2$, and 80% $N_2$) and stored at −80 °C until further analysis.

**In Vitro Fermentation.** A small-scale in vitro fermentation method was used to assess the fermentability of the oligosaccharides on human fecal samples principally as reported previously.[23] FOS was applied as a standard with known bifidogenic effect. Oligosaccharides or FOS were added to an autoclaved minimal basal medium to give a final concentration of 5 g (dry matter)/L in a reaction volume of 2 mL. The solutions were reduced in an anaerobic cabinet overnight. The minimal basal medium contained, per liter, 2 g of peptone water, 1 g of yeast extract, 0.1 g of NaCl, 0.04 g of $K_2HPO_4$, 0.04 g of $KH_2PO_4$, 0.01 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $CaCl_2 \cdot 2H_2O$, 2 g of $NaHCO_3$, 0.5 g of bile salts, 0.5 g of L-cysteine hydrochloride, 0.005 g of hemin, 10 $\mu$L of vitamin $K_1$ (0.02 mM), 2 mL of Tween 80, and 1 mL of 0.05% (w/v) resazurin solution. A 10% (w/v) fecal slurry was prepared by mixing the feces stored in 50% glycerol with degassed PBS. The reduced minimal medium samples with added oligosaccharides were inoculated at a final concentration of 1% (w/v) feces. Each fermentation experiment for the fecal sample of each healthy volunteer was carried out in triplicate to give 6 × 3 fermentations that were incubated at 37 °C for 24 h in an anaerobic cabinet.

**Extraction of Bacterial DNA.** DNA was extracted from fecal and fermentation samples using the QIAamp Stool DNA Mini Kit (Qiagen, Hilden, Germany) with a bead-beater step in advance, as described by Leser et al.[24] The purified DNA was stored at −20 °C until use.

**Real-Time PCR Assay Conditions.** Amplification and detection of purified bacterial DNA by real-time PCR were performed with the ABI-Prism 7900 HT from Applied Biosystems using optical grade 384-well plates. Primers specifically targeting 16S rRNA gene sequences of the *Bacteroidetes* and *Firmicutes* phyla and the *Lactobacillus* and *Bifidobacterium* genera were included in the qPCR analysis (Table 1). The amplification reactions were carried out in a total volume of 11 $\mu$L containing 50 $\mu$L of EXPRESS SYBR GreenER qPCR SuperMix (Invitrogen A/S, Taastrup, Denmark), 400 nM of each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany), 2 $\mu$L of template DNA, and nuclease-free water (Qiagen) purified for PCR. The amplification program consisted of one cycle at 50 °C for 2 min; one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s and 60 °C for 1 min; and finally one cycle of melting curve analysis for amplicon specificity at 95 °C for 15 s and 60 °C for 20 s with increasing ramp rate by 2% until 95 °C for 15 s.

**Real-Time PCR Data Handling.** The relative quantity of gene targets encoding 16S rRNA of the bacterial taxa were calculated using the approximation $2^{-Ct}$. Ct is the threshold cycle calculated by the ABI software as the PCR cycle, where the amplifications signal exceeds the selected threshold value, also set by the software. The amounts of bacterial specific DNA targets were normalized to total bacteria DNA targets to correct for differences in total DNA concentration between individual samples. Standard curves were created using serial 10-fold dilutions of bacterial DNA extracted from one of the fermentation samples for all primer sets. Analysis of the standard curves allowed verification of PCR efficiency for the chosen PCR conditions (Table 1). All results were calculated as means of duplicate determinations.

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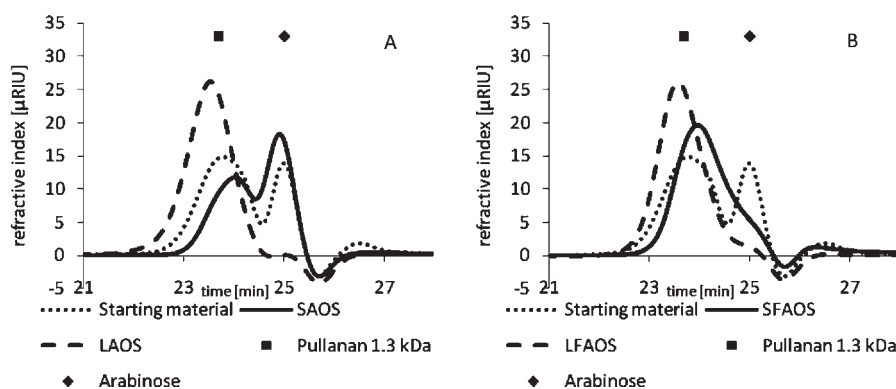
**Figure 1.** Size exclusion chromatography SAOS and LAOS (A) and SFAOS and LFAOS (B), both in comparison with the original starting material, arabinose, and 1.3 kDa pullanan standard.

**Statistics.** Statistical analysis was performed using GraphPad PRISM v5.03. One-way ANOVA and Tukey's multiple-comparison test were used to determine significant differences among bacteria populations for the in vitro fermentation. The quantitative PCR measurements were log-transformed before statistical analysis to obtain normal distribution of the data. Tests were considered to be statistically significant if $P$ values lower than 0.05 were obtained.

**Bioscreen.** The ability of the different fractions to function as a sole carbon source for the potential pathogen *Clostridium difficile* was assessed in a pure culture fermentation study. *C. difficile* was obtained from Deutche Sammlung Microorganismes and grown in reinforced clostridial media for preculturing and tryptic soy broth without dextrose (both from Bacto, BD, Franklin Lakes, NJ) for the experiment. Bacterial growth in a 1% w/v substrate solution was measured with the automatic Bioscreen C system as described by Mäkeläinen et al.[25] and measured as the area under the growth curve ($OD_{600} \times$ min) obtained from the Bioscreen data. This method also takes the speed of growth initiation into account, compared to just evaluating the end absorbance.

## ■ RESULTS AND DISCUSSION

**Separation Based on Feruloyl Substitution.** The original starting material, which comprised a ferulic acid content of 36 $\mu$mol/g (Table 2), was separated into feruloylated (FAOS) and nonferuloylated (AOS) oligosaccharides by the XAD separation. The unbound fraction (representing the part of the starting material unable to bind to the XAD material) represented 48% of total sample load and had a ferulic acid concentration of 6 $\mu$mol/g. The recovered water fraction, containing the AOS fraction, accounted for 29% of total sample load and had a ferulic acid concentration of 0.7 $\mu$mol/g, whereas 21% of total sample load was found in the MeOH/water fraction, which had a ferulic acid concentration of 144 $\mu$mol/g. The MeOH/water fraction accounted for 85% of the total ferulic acid load, and thus contained the FAOS fraction. The remaining 2% of sample load was found in the MeOH fraction with a ferulic acid concentration of 12 $\mu$mol/g and trace amounts of vanillin. No further analysis of this fraction was conducted.

**Separation Based on Molecular Weight.** The AOS and FAOS fractions were both separated according to size and subsequently analyzed with respect to average molecular weight, content of monosaccharides and short oligosaccharides, monosaccharide composition, and ferulic acid content. Size exclusion chromatography (Figure 1) showed the original starting material to have a dual distribution, with one peak at 25 min corresponding to monomers and a larger broader peak around 1.0 kDa. For the nonferulated fractions (Figure 1A) LAOS showed one
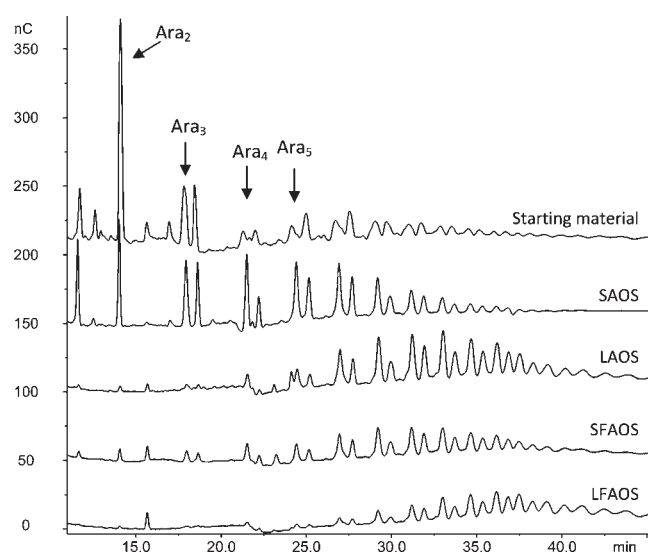


**Figure 2.** HPAEC elution profiles with electrochemical detection. Elution of monomers is not shown. Linear $\alpha$-1,5 arabinooligosaccharides DP 2−5 are marked.

homogeneous peak above 1.0 kDa and SAOS showed two peaks, both below 1.0 kDa. For the feruloylated fractions, the larger fraction LFAOS had a shape and size similar to those of LAOS, whereas SFAOS showed a broader distribution and had only a shoulder that corresponds to the presence of lower amounts of monomers, compared to the starting material (Figure 1B).

**Carbohydrate Composition.** The starting material and the four final fractions, SAOS, LAOS, SFAOS, and LFAOS, were investigated with respect to content of monosaccharides, shorter linear arabino-oligosaccharides, and monosaccharide composition using HPAEC directly on the fractions and on fractions subjected to acid hydrolysis. HPAEC analysis of the original starting material showed that approximately 125 mg/g was monosaccharides (mainly arabinose, glucose, and fructose) and 18 mg/g was made up of linear $\alpha$-1,5 arabinooligosaccharides DP 2−5. For SAOS the amount of monosaccharides was 15 mg/g, and linear arabinooligosaccharides constituted 17 mg/g. For LAOS, SFAOS, and LFAOS the amounts of monosaccharides were significantly lower. These results indicated that the majority of monomers did not bind to the XAD and therefore could be assumed to be in the unbound fraction.

HPAEC (Figure 2) revealed pairs of peaks, which may be indicative of branched arabino-oligosaccharides because branched arabino-oligosaccharides do not coelute with linear arabino-oligosaccharides.[26] HPAEC of SFAOS showed elution of a broad peak with a retention time comparable to that of monomers, but no identification was possible (Figure 3). The broad peak with heavy tailing indicated coelution of various smaller compounds, presumably feruloylated arabinose and arabinobiose. The monosaccharide analysis after acid hydrolysis revealed that besides at least 80% AOS, all of the samples contained traces of galactose, rhamnose, and galacturonic acid normally found in RGI (Table 3). Glucose was found mainly in the SAOS fraction. Fucose, xylose, and glucuronic acid were detected in the starting material and the high molecular weight fractions (LAOS and LFAOS) in trace amounts (<0.1 mol %, data not shown). The presence of these sugars could indicate the presence of trace amounts of RGII and/or xyloglucan.

**Ferulic Acid Distribution.** Analysis of the *trans*-ferulic acid content of each fraction revealed that the ferulic acid present in the water fraction from XAD separation, after the membrane separation, primarily was found in the high molecular fraction, whereas the distribution of ferulic acid in the SFAOS and LFAOS fractions was in the same order of magnitude (Table 3). Besides *trans*-ferulic acid, minor amounts of *cis*-ferulic acid were found in the range of 0.2–2.8 $\mu$mol/g in all fractions. Also, minor amounts of vanillin were detected, mainly in the SFAOS fraction.

Feruloylated arabino-oligosaccharides SFAOS and LFAOS were further analyzed using hydrophilic separation (Figure 4). Each peak was collected and analyzed by MALDI-TOF and TOF/TOF. Series of arabino-oligosaccharides with a single feruloyl substitution with DP 2–10 and series with double feruloyl substitutions DP 7–14 were detected (Table 4). No
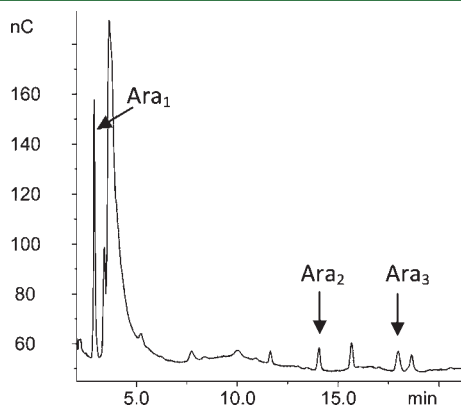


**Figure 3.** HPAEC elution profile of SFAOS with electrochemical detection. Arabinose and linear $\alpha$-1,5 arabinooligosaccharides DP 2–3 are marked.

ferulic dimers were detected in the analyzed fractions, even though reverse phase analysis indicated the presence of putative dimers (data not shown). No feruloyl substitutions on galactose residues were detected. MS/MS analysis confirmed the presence of monosubstituted and double-substituted arabino-oligosaccharides, but MS/MS data did not give sufficient results for complete determination of the degree of branching or the exact point of feruloyl substitution on each type of molecule, because molecules with different substitution patterns would have the same fragmentation pattern. As exemplified by MS/MS fragmentation of a single feruloylated DP 5 (Figure 5) from fraction V (Figure 4; Table 4), at least two different molecules were present in the fraction. In the fragmentation nomenclature the suffix "F" describes whether a feruloyl substitution is present on the ion or not. Arabinose moieties are numbered from the reducing end. Because MS/MS analysis was performed in positive mode, sodium adducts were formed. The fragmentation pattern lacked any trace of ferulated $Y_1$, $Z_1$, and $X_0$ ions, indicating that feruloyl substitution did not occur at the reducing end. The presence of both feruloylated and nonferuloylated $Y_2$ and $^{1,4}X_2$ ions ($m/z$ 481, 509, 305, and 333, respectively) indicated that feruloyl substitution occurred at arabinose 2 in one of the molecule types. The presence of $^{1,4}X_4F$ ($m/z$ 773) would indicate that no O-2 or O-3 feruloyl substitutions were present at the nonreducing end, but the presence of $^{0,3}X_4$ ($m/z$ 641) indicated feruloyl substitution at O-5 at the nonreducing end. It was not possible to obtain ions confirming any feruloyl substitution at arabinose 3 or 4. The fragmentation pattern thus clearly indicated the presence of at least two different molecules, one type with feruloyl substitution at the O-5 at the nonreducing end and one type with feruloyl substitution at O-2 at arabinose 2. For simplicity, only linear structures were considered, and the presumably branched nature of sugar beet arabinan is not considered. Besides arabino-oligosaccharides singly substituted with ferulic acid, also arabino-oligosaccharides doubly substituted with ferulic acid were observed. The exact structures could not be elucidated by MS/MS due to an even higher complexity and diversity than the singly substituted arabino-oligosaccharides (data not shown).

The sample preparation prior to HILIC separation might have favored the recovery of lower DPs, because the adjustment of acetonitrile concentration to 80% caused some of the sample to precipitate. MALDI-TOF analysis of the SFAOS and LFAOS precipitates showed traces of feruloylated arabino-oligosaccharides with sizes up to DP 22 and 30, respectively (data not shown). Still, the HILIC separation gave information about the relative abundance of each size of oligomers based on the area of each peak. SFAOS consisted mainly of oligosaccharides with DP 1–5, whereas LFAOS had a broader distribution, with some oligosaccharides below 1 kDa being present based on both HPSEC and HILIC data.

**Table 3. Mass Distribution, Ferulic Acid Content, and Monosaccharide Composition of Fractions from Membrane Separation**

| | | | mol % | | | | | |
|---|---|---|---|---|---|---|---|---|
| | mass distribution (mg/g starting material) | ferulic acid content ($\mu$mol/g) | rhamnose | arabinose | galactose | glucose | galA | fructose |
| starting material | 1000 | 36.2 | 1.13 | 85.16 | 1.95 | 6.78 | 1.63 | 2.77 |
| SAOS | 196.9 | 1.7 | 1.75 | 82.15 | 1.62 | 13.18 | 0.70 | 0.61 |
| LAOS | 40.1 | 66.0 | 0.99 | 86.68 | 3.44 | 0.75 | 7.07 | 0.09 |
| SFAOS | 67.2 | 186.5 | 1.47 | 92.63 | 3.14 | 0.69 | 1.17 | 0.25 |
| LFAOS | 148.7 | 247.3 | 0.72 | 95.98 | 1.77 | 0.29 | 0.82 | 0.00 |
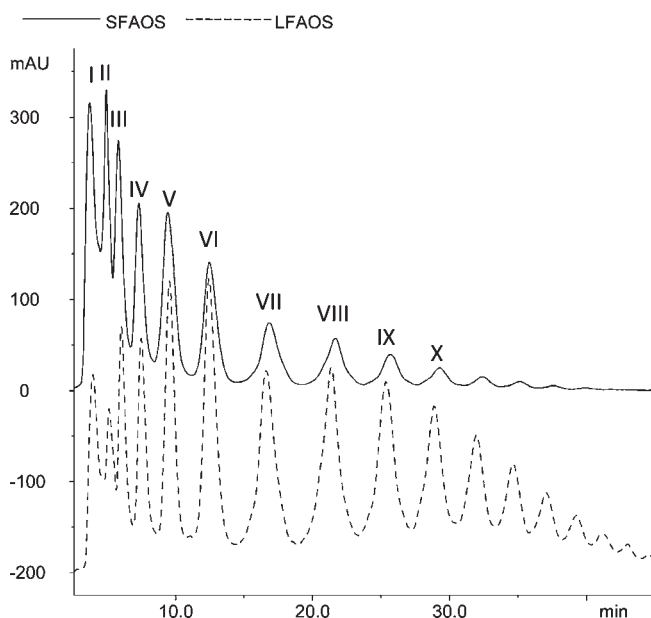
**Figure 4.** HILIC separation profile with UV detection at 316 nm. For peak identities refer to Table 4.

**Table 4. Peak Identities from HILIC Separation (Fig 4)[a]**

| peak | $ara_nFA$ | $ara_nFA_2$ |
| --- | --- | --- |
| I | | |
| II | DP2 | |
| III | DP3 | |
| IV | DP4 | |
| V | DP5 | DP7 |
| VI | DP6 | DP8 |
| VII | DP6, DP7 | DP9 |
| VIII | DP7, DP8 | DP10 |
| IX | DP8, DP9 | DP11, DP12 |
| X | DP9, DP10 | DP13, DP14 |

[a] Underscore indicates the mass with highest intensity in the MALDI-TOF analysis (MALDI-TOF data not shown).

The HILIC separation method is capable of detection of ferulated arabino-oligosaccharides from DP 1 to at least DP 15—20. Analysis of LAOS did not show any molecules within this size frame (data not shown), despite this fraction containing significant amounts of ferulic acid (Table 3). According to the HPAEC analysis, LAOS did contain oligosaccharides with an approximate size of DP 5—10, but apparently the feruloyl substitution was present on oligosaccharides with higher DP than DP 20. (The term "oligosaccharides" is used for consistency.) The fact that only the relatively larger oligosaccharides were feruloyl substituted justifies the appearance of ferulated molecules in the water fraction, because the overall hydrophilicity of, for example, an arabino-oligosaccharide with DP 20 and a single feruloyl substitution would still fractionate into the water fraction.

**In Vitro Fermentation.** Quantitative real-time PCR from in vitro fermentations showed that fermentation on SAOS, LAOS, LFAOS, and the starting material selectively increased the density of *Bifidobacterium* spp. significantly ($P < 0.05$, $P < 0.01$ $P < 0.001$, and $P < 0.05$, respectively) when compared to the original fecal sample (Figure 6). The densities of bifidobacteria after fermentation of the high molecular weight fractions, LAOS and LFAOS, were not significantly different from the densities obtained by fermentation of FOS, which is considered to be the "golden standard" within the field of prebiotics. This result confirmed that the induced growth was due to the arabino-oligosaccharides and not a result of the presence of monomers. The finding that arabino-oligosaccharides are bifidogenic is in agreement with a recently patented discovery stating that branched arabino-oligosaccharides with DP 2—15 are bifidogenic.[27] However, the patent[27] stated that sugar beet derived arabinose-rich pectin oligosaccharides do not exert prebiotic effects in vitro. This statement may be due to the fact that mainly homogalacturonan sugar beet pectin oligosaccharides were evaluated in the patent as opposed to the arabino-oligosaccharides described in the present work, which consisted of only 1—7% galacturonic acid and 87—96% arabinose (Table 3). Fermentation of the LAOS and LFAOS fractions did not yield significantly different results,

indicating that the size of the oligosaccharides was more important for selective bacterial stimulation than the amount of feruloyl substitutions. The findings that the high molecular weight fractions were more bifidogenic than the low molecular weight fractions further elaborates on the findings reported by Al-Tamimi et al.[8] which indicated that the low molecular weight fractions were more selective for bifidobacteria than arabinan. In that study[8] only arabino-oligosaccharides up to DP 8 were tested, and some fractions appeared to contain mainly monosaccharides. The fact that feruloyl substitution was no hindrance to bifidogenic metabolism was in good correlation with the results by Funk et al.[13] They found that human intestinal microbial communities were able to degrade maize cell wall material regardless of ferulate levels.

No significant changes were seen in the densities of *Lactobacillus* spp. and *Firmicutes* after fermentation. The relative amount of *Bacteroidetes* decreased significantly ($P < 0.001$) for all tested fractions, including FOS, as compared to the inoculum (Figure 6). This decrease was most likely due to an increase in other types of bacteria. The relative balance between *Firmicutes* and *Bacteroidetes* is believed to play a role in obesity risk, both with respect to developing obesity as shown by introducing "obese microbiota" in germ-free mice[28] and by the observation that the relative levels of *Firmicutes* and *Bacteroidetes* change, that is, the relative levels of *Bacteroidetes* increase even though the levels of *Firmicutes* are still dominant, when the diet for obese humans is restricted.[29] In the study presented in this paper the relative level of *Firmicutes* remained unchanged and the relative level of *Bacteroidetes* decreased significantly, thereby altering the relative balance toward a higher *Firmicutes/Bacteroidetes* ratio. These data suggest that all of the oligosaccharide fractions, including FOS, a commercial prebiotic, may increase the risk of obesity and maybe, in turn, the risk of developing the metabolic syndrome associated with obesity. However, it is not clear if the extent of change observed after the in vitro fermentations has any significance, as the available in vivo evidence of an effect of change in the *Firmicutes/Bacteroidetes* ratio was recorded over a longer period, namely 1 year, and the change was accompanied by a change in body weight (in humans).[29] Clearly, the data therefore require further investigation.

Besides the positive bifidogenic effects of the feruloylated arabino-oligosaccharides, it was investigated whether these substrates could be fermented by an opportunistic intestinal pathogen, *C. difficile*. *C. difficile* infection is linked to consumption of antibiotics, which disrupt the normal intestinal microbiota, allowing *C. difficile*
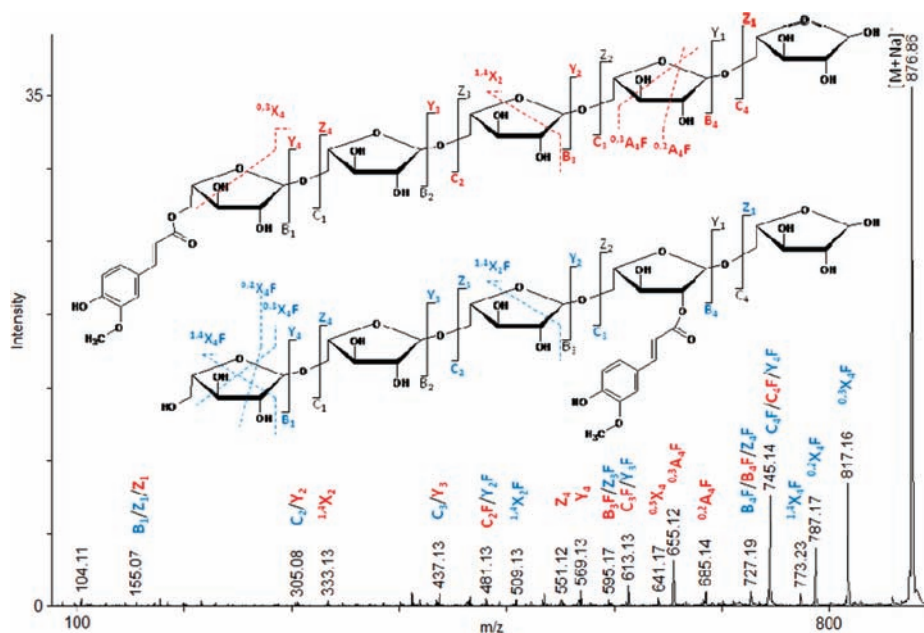
**Figure 5.** MS/MS high-energy CID spectrum of a sodium adduct of ara$_5$FA (fraction V, Figure 4), illustrating the fragmentation pattern and nomenclature, and two different proposed structures.
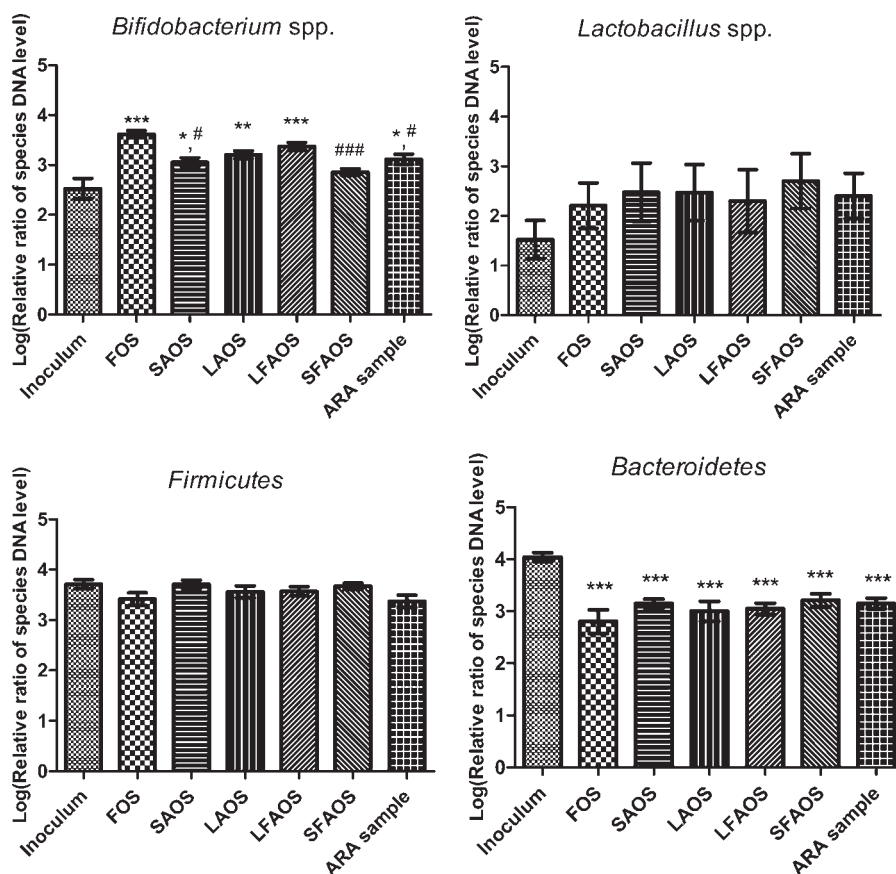


**Figure 6.** Relative quantities of target genes in samples from original fecal bacterial communities and after fermentation of oligosaccharides by these communities. Target genes encoded 16S rRNA from *Bifidobacterium* spp., *Lactobacillus* spp., *Bacteroidetes*, and *Firmicutes*. Fecal samples were obtained from the six healthy volunteers. The bars represent the average ± SEM of the response from six volunteers. Asterisks indicate a significant difference between target density in the original community and in the fermented samples: $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***. Pound signs indicate a significant difference between target density after fermentation of FOS and after fermentation of the oligosaccharides: $P < 0.05$, #; $P < 0.01$, ##; $P < 0.001$, ###.

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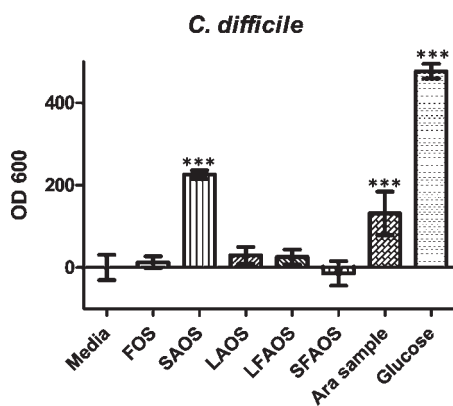
**Figure 7.** Induced growth of *Clostridium difficile* expressed as area under the curve. The bars represent the average ± SEM of 10 fermentations. Asterisks indicate a significant difference between media and the fermentation of the oligosaccharides: $P < 0.05$, *; $P < 0.01$, **; $P < 0.001$, ***.

similarly narrow DP profiles) with and without feruloyl substitutions. The availability of more well-defined structures, or of oligosaccharide mixtures having very narrow chain-length profiles and substitutions, could make it possible to examine and develop an improved understanding of the functionality and possible bioactive role of feruloyl-substituted arabino-oligosaccharides and potentially provide a new base for upgrading of sugar beet pulp to valuable functional food ingredients.

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### ■ ABBREVIATIONS USED

DP, degree of polymerization; DM, dry matter; SAOS, small arabino-oligosaccharides; LAOS, long arabino-oligosaccharides; SFAOS, small ferulated arabino-oligosaccharides; LFAOS, long ferulated arabino-oligosaccharides.

to establish itself and induce disease.[30] Over the past decade an increase in cases of *C. difficile*-associated diarrhea has been observed. Only a limited number of antibiotics are available for treatment of *C. difficile* infections. Currently, vancomycin or metronidazole is recommended for treatment, and many patients suffer from relapse following infections.[31]

Single-culture fermentations of *C. difficile* showed that glucose (positive control), starting material, and SAOS were able to support bacterial growth, but FOS, LAOS, SFAOS, and LFAOS did not sustain growth (Figure 7). Similar to what was seen in the mixed fermentations, it was not possible to distinguish between the effects of the feruloylated and the nonferuloylated high molecular weight fraction in the monoculture fermentations. It may be speculated that the starting material and SAOS supported growth of *C. difficile* due to the high concentrations of monosaccharides such as arabinose and glucose relative to oligosaccharides present in these samples. It should be noted that single-culture studies do not take substrate competition and possible positive or negative effects of secondary metabolites from competing microbiota into account. The data obtained deserve further investigation in mixed fecal fermentations. Nevertheless, the lack of induced growth of *C. difficile* provides a good indication that the tested compounds will not stimulate this species in vivo. Both fecal fermentations and single-culture experiments showed similar results for both LAOS and LFAOS. Although the LFAOS tended to elicit a higher selective stimulation of bifidobacteria than LAOS, the results from these two fractions were not significantly different. The effects of these two high molecular weight fractions differed significantly from their low molecular weight counterparts. The membrane separation procedure applied in this experimental setup provided a fast, but crude, method for an initial separation based on size. As we reported recently,[23] even a single DP change in oligosaccharide chain length (of homogalacturonides) may elicit a differential response in fecal fermentations. The data therefore provide an incentive to evaluate the effects of even more defined structures with respect to the role of feruloyl substitution and chain length for prebiotic response. The HILIC separation procedure showed great potential in separating the feruloylated arabino-oligosaccharides according to chain length. Post treatment with feruloyl esterases could finally generate similar structures (or oligosaccharide mixtures having

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