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In Vitro Fermentation of Arabinoxylan-Derived Carbohydrates by Bifidobacteria and Mixed Fecal Microbiota

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Bifidobacterium adolescentis ATCC 15703, *Bifidobacterium breve* ATCC 15700, *Bifidobacterium longum* ATCC 15707, and human fecal microbiota were cultivated in vitro with D-xylose, L-arabinose, xylo-oligosaccharides (XOS), and arabinoxylo-oligosaccharides (AXOS) as carbon sources. The pH, formation of volatile fatty acids, and carbohydrate utilization profiles were followed. In the pure bifidobacteria cultures optical density and in the fecal slurries pressure and H_2 were also detected. A differing substrate preference was observed among the various bifidobacteria strains. *B. adolescentis* grew on XOS, slowly on D-xylose, but not on L-arabinose. In contrast, *B. longum* preferred L-arabinose and did not grow on pure D-xylose or XOS. Both strains were able to utilize AXOS but with differing strategies, since after the cleavage of L-arabinose *B. adolescentis* consumed the XOS formed, whereas *B. longum* fermented the L-arabinose released. *B. breve* grew poorly on all of the substrates provided. A bifidobacterial mixture and the fecal microbiota were able to utilize pure singly substituted AXOS almost completely, but pure AXOS with a doubly substituted xylose residue was fermented only by the fecal microbiota. Thus, AXOS appear to be potential candidates for slowly fermenting prebiotics, but their prebiotic effects may be dependent on the type of arabinose substitution and the presence of other carbohydrates.

KEYWORDS: Arabinoxylan; arabinoxylo-oligosaccharides (AXOS); bifidobacteria; prebiotic; rye; wheat; xylo-oligosaccharides (XOS)

INTRODUCTION

Research on prebiotic compounds and probiotic bacteria has been active for over a decade. Prebiotic compounds are targeted to stimulate the growth of the bifidobacteria and lactobacilli that are recognized as health-promoting probiotics (*1*). Fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), and lactulose are selectively fermented by probiotics and thus can be called prebiotics (*2*). In addition, the prebiotic potentials of several other oligosaccharides have been tested. For example, linear xylo-oligosaccharides (XOS) have shown promising results in in vitro and in vivo studies (*3*,*4*). Linear oligosaccharides may, however, be fermented rapidly in the colon, and thus, oligosaccharides with more complex carbohydrate structures are currently studied to find slower-fermenting prebiotics (*5*,*6*). One potential group is arabinoxylan (AX)-derived arabinoxylo-oligosaccharides (AXOS), which may have varying chemical structures, depending on the xylan source and the degradation method used (*7*).

AX, which consist of a linear (1→4)-β-D-linked xylopyranosyl (β-D-Xyl*p*) backbone, are substituted with α-L-arabinofuranosyl (α-L-Ara*f*) groups at positions *O*-3 and/or *O*-2 of the β-D-Xyl*p* residues. In addition to α-L-Ara*f*, AX may contain some 4-*O*-methyl-α-D-glucopyranosyluronic acid, acetyl, and feruloyl substituents (*8*). The contents and types of substitution vary, depending on the source of AX. The cell walls of wheat and rye endosperms are rich in AX. The ratio of Ara*f* to Xyl*p* is about 0.5 in water-soluble wheat and rye flour AX, but the structure regarding the type of α-L-Ara*f* substitution differs significantly. In water-soluble wheat flour AX, about one-third of the α-L-Ara*f* are (1→3)-linked to monosubstituted β-D-Xyl*p* residues, and two-thirds are (1→2)- and (1→3)-linked to doubly substituted β-D-Xyl*p* (*9*). Rye flour AX has significantly more singly substituted β-D-Xyl*p* residues, since two-thirds of the α-L-Ara*f* are (1→3)-linked to mono- and one-third to doubly substituted β-D-Xyl*p*, respectively (*10*).

The intestinal microbiota play an important role in the maintainance of health and in the prevention of disease, which is well-recognized (*11*). In the gastrointestinal tract (GIT), there are more than 400 bacterial species, of which the lactic acid bacteria constitute approximately 10%. Gram-positive, anaerobic *Bacteroides*, *Eubacterium*, and *Bifidobacterium* species predominate in the large intestine, but there are also other important groups such as clostridia and lactobacilli (*11*, *12*). The presence of bifidobacteria in the GIT has been associated with beneficial health effects (*13*). The growth of these bacteria is linked to their ability to produce proteins, such as extra- and intracellular hydrolytic

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enzymes, as well as mono- and oligosaccharide transporters involved in the metabolism of nondigestive carbohydrates (*14*).

The genome sequences of *Bifidobacterium longum* NCC 2705 and *B. adolescentis* ATCC 15703 have revealed high levels of proteins related to the utilization of nondigestive polysaccharides and oligosaccharides, including putative glycoside hydrolases (GH) β-D-xylosidases (EC 3.2.1.37) and α-L-arabinofuranosidases (EC 3.2.1.55) for degradation of AXOS (*14*, *15*). Thus far, bifidobacteria are not known at the protein level to produce endo-1,4-β-D-xylanase (EC 3.2.1.8), but one putative xylanase-encoding gene (GH family 8) has been isolated (*16*). Thus, other microorganisms in the GIT are needed for the hydrolysis of polymeric nondigestive AX into oligosaccharides. For example, common intestinal *Bacteroides* bacteria have GH family 10 endo-1,4-β-D-xylanases (*17*). The key enzymes for utilization of AXOS are α-L-arabinofuranosidases. *B. adolescentis* ATCC 15703 harbors genes for two extracellular polymeric xylan-acting α-L-arabinofuranosidases: AXH-m (GH family 51) and AXH-d3 (GH family 43), which are able to release α-L-Ara*f* from mono- and doubly substituted β-D-Xyl*p* residues, respectively (*14*, *16*, *18*). Surprisingly, the *B. longum* NCC 2705 genes annotated into familes GH43 and GH51 have not revealed significant homology to currently known extracellular α-L-arabinofuranosidases (*14*), although several *B. longum* strains were able to grow on rye AX, presumably by releasing and metabolizing α-L-Ara*f* substituents (*19*). Indeed, many other *B. longum* strains evidently have GH family 51 α-L-arabinofuranosidase (*20*). The genome sequences of *Clostridium* and *Lactobacillus* strains have until now revealed no enzymes in GH families 43 and 51 containing α-L-arabinofuranosidases and β-xylosidases (*14*), which further indicates the potential of AXOS as prebiotic oligosaccharides with bifidobacterial selectivity.

Despite several studies suggesting that AXOS and notably various AX hydrolysates are potential prebiotics (*7*, *21*), there is a lack of information on the influence of the detailed molecular structure of the putative prebiotic AXOS on the growth of probiotic bifidobacteria. Here, we report in vitro fermentability of different AXOS by three bifidobacterial strains, *B. adolescentis* ATCC 15703, *B. breve* ATCC 15700, and *B. longum* ATCC 15707, and human fecal microbiota, in particular to examine the influence of specific α-L-Ara*f* substitutions on growth. The AXOS were produced from wheat AX via specific treatment with *Aspergillus aculeatus* GH family 10 endo-1,4-β-D-xylanase, which hydrolyzes the xylosidic linkage before the substituted β-D-Xyl*p*, thus forming AXOS with singly and doubly substituted nonreducing end β-D-Xyl*p* residues (*22*). The aim of this study was also to obtain detailed information on the utilization of individual XOS and AXOS by bifidobacteria, using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis. Monitoring of changes in the composition and structure of complex oligosaccharides during cultivation studies is required to understand their behavior and to further estimate their prebiotic potential.

MATERIALS AND METHODS

Carbohydrates. D-Xylose was purchased from Fluka (Buchs, Switzerland), L-arabinose was from Acros Organics (Geel, Belgium), a mixture of XOS was from Wako Pure Chemical Industries (Osaka, Japan), and a mixture of FOS (used as a reference substrate) was from BioCare (Birmingham, United Kingdom). High-viscosity AX from rye (*Secale cereale* L.) flour (RAX) and medium-viscosity AX from wheat (*Triticum aestivum* L.) flour (WAX) were purchased from Megazyme (Bray, Wicklow, Ireland). In sulfuric acid hydrolysis (*23*), the total carbohydrate content of RAX was 92 wt % with a monosaccharide composition of 65 wt % xylose, 30 wt % arabinose, and 5 wt % glucose. The carbohydrate yield of WAX after total enzymatic hydrolysis (*24*) was 84 wt % with 64 wt % xylose and 36 wt % arabinose. The remainder contained ash, moisture, proteins, and negligible amounts of other sugars. The RAX and WAX hydrolysates were prepared by degradation with the commercial Shearzyme (CDN00220, Novozymes A/S, Bagsvaerd, Denmark), which contains the *A. aculeatus* GH 10 endo-1,4-β-D-xylanase as the main enzyme (49,100 nkat/mL) (*23*). The WAX and RAX (5 g/L) in milli-Q-water (pH 5.5) (Milli-Q-plus, Millipore, Billerica, MA) were incubated with Shearzyme (20000 nkat/g of AX) at 40 °C for 24 h, after which the reaction was terminated by boiling the solutions for 10 min. Pure water was preferred as a solvent to avoid any buffer salt in the later fermentations. A mixture of D-xylose, xylobiose, and AXOS with different lengths was obtained. The WAX and RAX hydrolysates were used in the growth experiments as such.

Pure AXOS with nonreducing end-terminal α-L-Ara*f* (1→2) or α-L-Ara*f* (1→3) monosubstituted β-D-Xyl*p* residues and α-L-Ara*f* (1→2) (1→3) doubly substituted β-D-Xyl*p* residues were obtained from RAX and WAX as reported earlier (*22*, *23*). The pure AXOS used in the growth experiments were α-L-Ara*f*-(1→2)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl and α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl, referred to later in the text as A^2XX and $A^{2+3}XX$, respectively. These and α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→4)-D-Xyl (A^3X) and α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-D-Xyl ($A^{2+3}X$) were used as standards in the HPAEC-PAD analysis for identification and quantification of the AXOS formed. AXOS abbreviations are according to Fauré et al. (*25*). The xylobiose, xylotriose, and xylotetraose were purchased from Megazyme.

Pure Culture Fermentation Experiments. The bacterial strains used were *B. adolescentis* ATCC 15703 (DSM 20083), *B. breve* ATCC 15700 (DSM 20213), and *B. longum* ATCC 15707 (DSM 20219) purchased from DSMZ (Braunschweig, Germany). The bacteria were maintained at −80 °C as glycerol stock solutions. The bacterial suspensions were thawed, and the bacteria were propagated by anaerobically inoculating 2 mL of the solutions into 50 mL bottles with 25 mL of *Bifidobacterium* medium (DSMZ, medium 58). The strains were then cultivated two or three times in fresh, nutrient-rich *Bifidobacterium* medium by inoculating 0.5 mL from the previous solution into the next solution. The bacteria were later inoculated (0.5 mL) into 15 mL glass tubes with 10 mL of nutrient-poor BA medium containing 5 g/L FOS (described below) and cultivated anaerobically at 37 °C, until an optical density (OD; 600 nm) of 1 was attained, after which 0.5 mL was inoculated further for growth studies on AX-derived carbohydrates.

In the fermentation samples with restricted carbohydrate source, the strains were cultured anaerobically in BA medium as previously described by Angelidaki et al. (*26*) with the modification that yeast extract (1 g/L; Merck, Darmstadt, Germany) was added to the medium. The pH was 6.9−7.0, and the medium was dispensed (2.5 mL in 15 mL glass tubes) and autoclaved at 121 °C for 20 min. The vitamin solution was prepared according to Wolin et al. (*27*), sterile-filtered, and added prior to inoculation. The medium was not reduced with Na_2S. The carbohydrates were dissolved in sterilized water and filtrated (0.45 μm syringe filters) into sterile bottles. The purified oligosaccharides (AXOS) were not filtrated because of the partial loss of the sample during the filtration, but the solutions were heated twice up to 100 °C to sterilize them. The final substrate concentration was 4 g/L for D-xylose, L-arabinose, XOS, and AX hydrolysates (starting material weighed prior to hydrolysis). In the fermentation experiments where purified AXOS were used as a sole carbon source, a mixture of three bifidobacteria species (*B. adolescentis*, *B. breve*, and *B. longum*) was used to reduce the number of experiments with the limited quantity of purified AXOS. The final substrate concentrations for A^2XX and $A^{2+3}XX$ were 2.3 and 2.9 g/L, respectively. FOS (4 g/L) was used as a substrate in all cultivation experiments as a positive control. The media were inoculated with a culture (7% v/v) in the late exponential growth phase to a final volume of 3 mL. The cultures were incubated in the dark without shaking at 37 °C for 140 h (6 days). The fermentations on purified AXOS were carried out in duplicate and all of the other fermentations in triplicate.

The growth of bifidobacteria was observed, using the following methods: The OD was measured immediately after inoculation, at 15 h, and thereafter every 24 h for 6 days (140 h), the pH was measured at the end of the growth period, and random samples were examined microscopically to exclude contamination of the bacterial cultures. Volatile fatty

acids (VFA) and the carbohydrate contents remaining after the growth period were analyzed, using gas chromatography (GC) and HPAEC-PAD, respectively, as described later.

Fecal Microbiota Fermentation Studies. The human feces samples were obtained from a single healthy donor on four occasions. In each case, the fecal samples were used fresh. The samples were mixed in sterilized anaerobic 0.1 M PBS buffer (phosphate-buffered saline; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 dissolved in 800 mL of distilled water) to make a 10% (w/v) slurry. The BA medium and vitamin solution were prepared as described earlier and dispensed (1.5 mL in 10 mL glass tubes and 7.7 mL in 30 mL glass bottles for the 0-samples) and autoclaved at 121 °C for 20 min. The medium was reduced with $Na_2S \cdot 9H_2O$ (0.3 mg/mL) before inoculation. The feces slurry was transferred to BA medium (10% v/v) to a final volume of 2 mL (10 mL for the 0-samples). The final substrate concentrations were as described in the pure culture experiments. The samples were incubated in the dark at 37 °C without shaking for 48 h. The fermentations on purified and defined AXOS were carried out in duplicate and all of the other fermentations in triplicate. The separate reference fermentations without added carbohydrates were also carried out by fecal slurries in 10 mL volumes.

Growth was observed by measuring the change in pH, pressure, and VFA, and the formation of hydrogen gas was examined after the incubation. The hydrogen content was analyzed by GC 82-22 gas chromatograph (Mikrolab, Aarhus, Denmark) equipped with thermal conductivity detection. From the fermentation experiments, 0.5 mL gas samples were taken and injected into the GC system. The pressure was measured by a pressure transducer coupled to a digital display. The remaining carbohydrate contents were analyzed, using HPAEC-PAD after the 48 h incubation period.

GC Method for VFA Analysis. Prior to quantification of VFA, the samples were acidified to pH < 2 with 17% H_3PO_4 and then analyzed using GC (Hewlett-Packard 5890 series II; Hewlett-Packard, Palo Alto, CA) with a flame ionization detector and a capillary column (Hewlett-Packard FFAP 30 m; inner diameter, 0.53 mm; film, 1 μm). The temperatures of the injector and detector were 175 and 200 °C, respectively. The temperature gradient was as follows: The initial temperature was 110 °C, ramped at 5 °C/min to 140 °C, maintained at 140 °C for 6 min, ramped at 45 °C/min to 200 °C, and maintained at 200 °C for 3 min. The injection volume of the sample was 10 μL. The quantified VFA were analyzed as acids: acetic, propionic, iso-butyric, butyric, iso-valeric, and valeric acids. The standards used in the VFA analyses were the same acids as mentioned above with concentrations of 1−40 mM. Later, these compounds are referred to as VFA although they appear as acid anions (acetate, propionate, iso-butyrate, butyrate, iso-valerate, and valerate) at physiological pH.

HPAEC-PAD Method for Carbohydrate Analysis. The HPAEC-PAD system was equipped with an SSI pulse equalizer (Scientific Systems, Inc., model LP 21; State College, PA), two Waters 515 HPLC pumps, a PC Waters pump control module, and a cooling Waters 717 autosampler using Millenium^{32} software (Waters Corp., Milford, MA) for instrument control and data handling. The analytical CarboPac PA-100 column (250 mm × 4 mm, i.d.) and the guard column PA-100 (25 mm × 3 mm, i.d.) (Dionex, Sunnyvale, CA) were maintained at 30 °C. The analyses were carried out using the oligosaccharide method with 100 mM NaOH−1 M NaOAc gradient at a flow rate of 1 mL/min, as described in Rantanen et al. (*23*). A Decade detector with a gold electrode (Antec Leyden, Zoeterwoude, The Netherlands) was used in pulse mode at 30 °C. The samples were filtered using a 0.45 μm Acrodisc syringe filter with nylon membrane (Pall Corp., Ann Arbor, MI), and the injection volume was 10 μL. A mixture of D-xylose, xylobiose, xylotriose, and xylotetraose (all in concentrations of 25 μg/mL) was used as an external standard to monitor the stability of the separation. Commercial L-arabinose, D-xylose, and XOS were used for identification and quantification of the respective saccharides in the hydrolysates and samples after fermentations. Identification and quantification of AXOS were done using pure A^3X, A^2XX, $A^{2+3}X$, and $A^{2+3}XX$ as standards.

DNA Extraction, Purification, Polymerase Chain Reaction (PCR), and Terminal Restriction Fragment Length Polymorphism (t-RFLP). At the beginning of the incubation period and after 48 h, 1 mL samples were taken from the fecal samples for DNA extraction. At the same time points, 4 mL samples were taken from the reference fermentations (0-samples without added carbon source). The samples were centrifuged for 2 min at 10000*g* at 4 °C, after which the supernatant was disposed and the pellet was suspended in 1 mL of 0.01 M PBS buffer, and this treatment was repeated three times. After the third centrifugation, the pellet was suspended in 1.5 mL of ASL buffer (stool lysis buffer; Qiagen, GmbH, Hilden, Germany) and bead-beaten (0.1 mm zirconia/silica beads) for 3 min, followed by centrifugation at 16000*g* at 4 °C for 3 min. The subsequent DNA purification was carried out following the Qiagen kit instructions for "DNA purification from stool samples" (Qiagen, Valencia, CA).

The bifidobacterial 16S rRNA genes were amplified by a PCR using a bifidobacteria-specific primer set. The total volume of the PCR mixture was 50 μL and contained milli-Q water, reaction buffer, Mg^{2+} (25 mM), dNTP (2 mM), forward primer [25 pmol/μL; TET-Bifido 144f NF, MWG-Biotech AG, Ebersberg, Germany, 5′-CCGGAATAGCTCCTGGAAAC-3′, based on the Pbi F1 primer described by Roy and Sirois (*28*)], reverse primer [25 pmol/μL; rP2, MWG-Biotech-AG, 5′-ACGGCTACCTTGTTACGACTT-3′ (*29*)], bovine serum albumin (BSA; 2%), polymerase, and template. The PCR (iCycler Thermal Cycler; Bio-Rad, Hercules, CA) was started with an initial denaturation step at 94 °C for 5 min, followed by 30 cycles comprising denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, elongation at 72 °C for 90 s, and final extension at 72 °C for 5 min. Agarose gel (1%) was loaded with the PCR products, and gel electrophoresis was run for 30 min at 80 V (Bio-Rad/PowerPac 300 power supply), and the bands on the gel were visualized using the Bio-Rad Gel Doc 2000 documentation system (darkroom cabinet, UV transilluminator, camera, and software). The band containing the PCR product of interest was purified using gel extraction according to the Gel-Out procedure (A&A Biotechnology, Gdynia, Poland).

After the PCR, the 16S rRNA gene products were terminally fluorescent-labeled. This PCR product (10 μL) was digested with 1 μL of enzyme *Mbo*I (10 U/μL; Fermentas International Inc., Burlington, Ontario, Canada) for 6 h at 37 °C, after which the enzyme was inactivated at 65 °C for 20 min. The labeled fragments were analyzed by electrophoresis in an ABI PRISM 373 DNA sequencer in Gene-Scan mode, and the fragment sizes were determined using DNA fragment standards (GS-500 ROX and GS-1000 ROX; PE Biosystems, Foster City, CA). t-RFLP analyses were used together with a specific *Bifidobacterium* primer set to demonstrate the population changes in the fecal samples on addition of the xylan-derived carbohydrates.

RESULTS

Analysis of the Oligosaccharide Mixtures. The oligosaccharide mixtures used as substrates were analyzed with HPAEC-PAD, and their mono- and oligosaccharide profiles were defined. The commercial XOS mixture contained mainly xylobiose (26 wt %) and smaller amounts of xylotriose (8 wt %) and xylotetraose (3 wt %) (**Figure 1a**). Thus, in this mixture, only 37% of the weighed amount was detected as linear XOS. In addition to the XOS identified, the commercial XOS product contained some longer unidentified oligosaccharides with retention times between 30 and 35 min. Some of these may indeed have been AXOS, as Moura et al. reported the presence of AXOS in the commercial XOS product (Xylo-oligo 95P) (*30*).

The AXOS hydrolysates were obtained by enzymatically degrading water-soluble rye and wheat flour AX. These two AX were selected, due to their structural differences in Ara*f* substitution patterns. High endo-1,4-β-D-xylanase dosage and long hydrolysis times were used to obtain efficient hydrolysis to "limiting" AXOS. The major AXOS peaks in the hydrolysates were identified and quantified, using previously isolated, structurally known AXOS as standards (*22*, *23*). The identified and quantified carbohydrates in the RAX hydrolysate were 14 wt % and in the WAX hydrolysate 20 wt % (**Figure 1b**,**c**). Both the RAX and the WAX hydrolysates contained similar amounts of xylose, xylobiose, and A^3X but differed clearly in the levels of doubly substituted AXOS, since the WAX hydrolysate had more

$A^{2+3}X$ and $A^{2+3}XX$. The difference in short-chain AXOS formed from RAX and WAX reflects their structural differences. As seen from the low amount of quantified products, the hydrolysates also contained longer AXOS not detected by the HPAEC-PAD method used.

Growth of Pure Bifidobacterial Cultures. Three bifidobacteria species, *B. adolescentis* ATCC 15703, *B. breve* ATCC 15700, and *B. longum* ATCC 15707, were selected for the fermentation studies. These species probably differ in their capability for growing on AX-derived carbohydrates. FOS was used as the positive control carbon source. Various markers (OD, pH, VFA, and consumption of carbohydrates) were followed to define the growth of the bacteria. The OD was followed for 140 h, although in most cases growth reached the stationary phase at 60 h. Long fermentation times were used, due to the possible prolongation of the lag phase, since small-scale and nutrient-poor media were used in the cultivations. The samples were examined microscopically to ensure that there was no contamination. The bifidobacterial strains tested were considered able to utilize the carbohydrate provided when the growth was detected in minimum one of the test tubes, even though the parallel samples did not grow in all cases. Growth of the three bifidobacterial species studied on the carbohydrates provided is shown in **Table 1**.

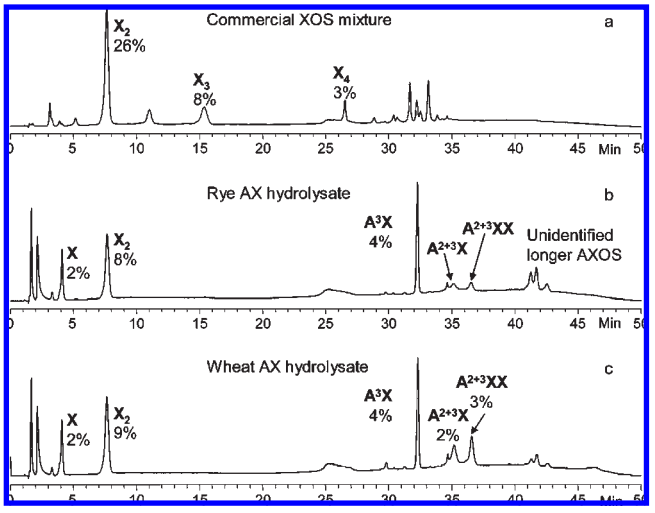

Figure 1. HPAEC-PAD chromatograms of (**a**) commercial XOS mixture, (**b**) rye AX hydrolysate, and (**c**) wheat AX hydrolysate. A = arabinose, X = xylose, X_2 = xylobiose, X_3 = xylotriose, A^3X = α-L-Ara*f*-(1→3)-β-D-Xyl*p*-(1→4)-D-Xyl, $A^{2+3}X$ = α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-D-Xyl, $A^{2+3}XX$ = α-L-Ara*f*-(1→2)-[α-L-Ara*f*-(1→3)]-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-D-Xyl. The % represents quantified wt % of the XOS mixture and AX hydrolysates.

In the samples with OD > 0.4, lowered pH was also detected. In these samples, the pH decreased from 7 to 4−4.5. The concentrations of six different VFA were analyzed, but changes were seen only in acetate content. With a significant increase in OD and a decrease in pH, acetate was also formed during the fermentation. The samples with no evident growth had acetate concentrations below 10 mM. In the samples where growth was detected, the acetate concentrations increased to 15−30 mM, and the acetate production coincided with a decrease in pH.

The three bifidobacteria species differed clearly in their ability to utilize pure pentoses, since D-xylose and L-arabinose supported the growth of *B. adolescentis* and *B. longum*, respectively, whereas *B. breve* showed no growth on these two monosaccharides (**Table 1**). The growth of *B. longum* on L-arabinose was strong, while *B. adolescentis* was able to grow slowly on xylose for the first 60 h but was still presumably in the slow exponential growth phase, and at 140 h, the growth was strong. Some changes between these two time points were also seen in the growth of *B. breve* on RAX hydrolysate and in the utilization of WAX hydrolysate by *B. adolescentis* (**Table 1**). Differences between *B. adolescentis*, *B. breve*, and *B. longum* were also observed when the bifidobacteria were cultivated on XOS and AX hydrolysates (**Table 1**). A commercial XOS mixture supported only the growth of *B. adolescentis*, whereas both *B. adolescentis* and *B. longum* showed low but clear growth on RAX and WAX hydrolysates, while *B. breve* was able to grow slowly on RAX hydrolysate.

Consumption of Carbohydrates. Utilization of the carbohydrates after the 140 h fermentation was followed, using HPAEC-PAD chromatography to gain better insight into the fate of mono- and oligosaccharides during fermentation. The samples with all of the other markers of growth detected also showed clear reductions in the substrate carbohydrates. *B. adolescentis* consumed almost all xylose when this was the single substrate. In the XOS mixture, *B. adolescentis* utilized all of the xylobiose, xylotriose, and xylotetraose, leaving some xylose (80 μg/mL) that was presumably formed from the XOS. In the RAX and WAX hydrolysates, *B. adolescentis* was able to utilize all of the xylobiose and oligosaccharide A^3X (**Figure 2b**). However, the L-arabinose released from the AXOS (93 μg/mL in the RAX hydrolysate and 265 μg/mL in the WAX hydrolysate) and some xylose (90 and 135 μg/mL, respectively) were left unfermented. More xylose was found after than before the fermentation (originally 56 μg/mL in the RAX hydrolysate and 62 μg/mL in the WAX hydrolysate), indicating slow fermentation of xylose by *B. adolescentis*.

B. longum also effectively utilized all of the A^3X from the RAX and WAX hydrolysates (**Figure 2c**), and neither free L-arabinose nor D-xylose was detected after fermentation. On the other hand, xylobiose in the hydrolysates was not consumed by *B. longum*. There were 268 μg/mL xylobiose in the RAX hydrolysate and

Table 1. Growth of the Three Bifidobacterial Species[a]

	growth of					
	B. adolescentis ATCC 15703		*B. breve* ATCC 15700		*B. longum* ATCC 15707	
substrate	60 h	140 h	60 h	140 h	60 h	140 h
D-xylose	+	+++	−	−	−	−
L-arabinose	−	−	−	−	+++	+++
XOS	+++	+++	−	−	−	−
RAX hydrolysate	+	+	−	+	+	+
WAX hydrolysate	+	++	−	−	+	+
FOS	+++	+++	++	++	+++	+++

[a] Scale: OD < 0.1 (−, no growth), 0.1−0.39 (+, mild growth), 0.4−0.69 (++, moderate growth), and 0.7−1 (+++, strong growth). Acetate content, pH, and consumption of carbohydrates in the samples were also measured and considered in defining growth intensities. RAX hydrolysate, rye AX hydrolysate; and WAX hydrolysate, wheat AX hydrolysate.

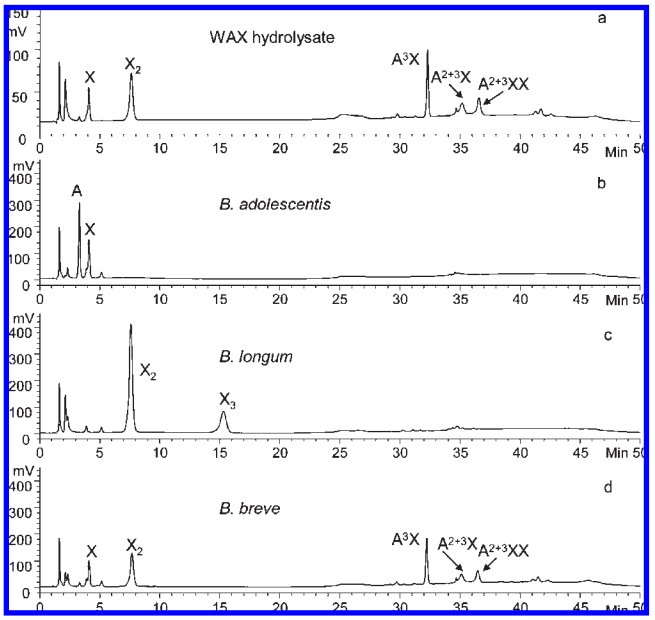

Figure 2. Carbohydrate analysis by HPAEC-PAD (**a**) before fermentation and (**b**) after a 140 h fermentation by *B. adolescentis* ATCC 15703, (**c**) *B. longum* ATCC 15700, and (**d**) *B. breve* ATCC 15707. Abbreviations used for saccharides are as in **Figure 1**.

316 μg/mL xylobiose in the WAX hydrolysate before the fermentation and 975 and 1115 μg/mL xylobiose, respectively, after the fermentation. Xylotriose was also found in the sample after the *B. longum* cultivation, indicating that *B. longum* was able to remove and ferment L-arabinose from AXOS but was not able to consume the xylobiose and xylotriose released. *B. breve* poorly fermented all of the AX-derived substrates provided, based on OD and the other growth markers measured. The only clear change was found in the content of xylobiose in the RAX hydrolysate before (268 μg/mL) and after (98 μg/mL) the *B. breve* fermentation. Thus, the data imply that growth of this organism may in fact not be significantly promoted by AXOS (**Figure 2d**).

Growth of Bifidobacteria Mixture on Pure AXOS. The fermentation of pure singly and doubly substituted AXOS was further studied by cultivating a mixture of three bifidobacteria (*B. adolescentis*, *B. longum*, and *B. breve*) with A^2XX and $A^{2+3}XX$. FOS was used as the control substrate. Separate cultivations with pure bifidobacteria could not be carried out due to the limited amount of pure AXOS. The growth of the bacteria in a mixture was strong after 60 h when A^2XX and FOS were used as carbon sources. However, some A^2XX was left (31 μg/mL), and low amounts of L-arabinose, D-xylose, xylobiose, and xylotriose were also found (10, 13, 8, and 12 μg/mL, respectively) after a 60 h fermentation, indicating slow complete utilization of pure A^2XX with the bifidobacteria mixture (**Figure 3**). No growth was detected in either of the parallel samples when a more complex AXOS, $A^{2+3}XX$, was used as a carbon source for the bifidobacteria mixture.

Fermentation with Fecal Microbiota. In fecal samples, there are many different bacterial species and strains, and thus, the complete fermentation of various carbohydrate compounds is likely to occur. However, it becomes much more complicated to specify the exact bacterial strain responsible for the fermentation of each carbohydrate substrate under investigation. In the present study, the measured factors providing information on the growth of the bifidobacteria were pH, pressure, VFA, and formation of H_2. The utilization of various carbohydrates was analyzed with the HPAEC-PAD method,

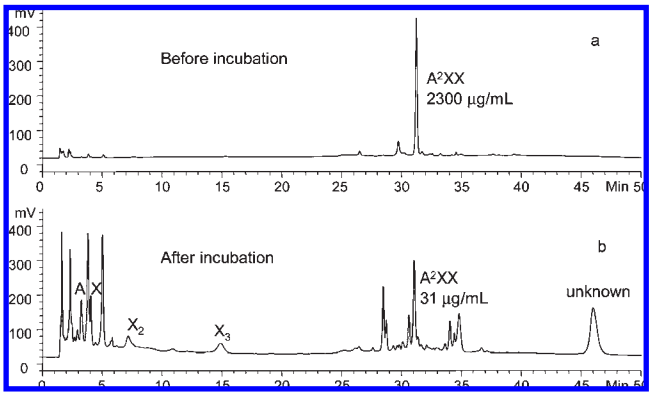

Figure 3. HPAEC-PAD analysis of the purified AXOS, A^2XX, (**a**) before and (**b**) after a 60 h growth experiment with the mixture of bifidobacteria (*B. adolescentis*, *B. breve*, and *B. longum*). Note that the sample in panel **a** is 40 times more diluted than in panel **b**. Abbreviations used for saccharides are as in **Figure 1**.

and the changes in bifidobacteria distribution were studied, using t-RFLP analysis. Polymeric WAX and RAX were also included as substrates, since fecal microbiota probably contain organisms producing endo-1,4-β-D-xylanases for their degradation.

After the incubation period, changes in pH were observed, indicating that the mixture of fecal microbiota fermented provided carbohydrates. In the samples with FOS, L-arabinose, D-xylose, XOS, and RAX and WAX hydrolysates, the end pH was 5. With polymeric RAX and WAX as carbon sources, the pH was somewhat higher (5.5) at the end of the fermentation period. With the purified oligosaccharides A^2XX and $A^{2+3}XX$, the pH was 6 and 4, respectively. The pressure increased in all of the samples (0.8−2.2 psi), and the least change was detected in the samples grown on $A^{2+3}XX$ and the greatest with RAX hydrolysate as a carbon source (**Table 2**). Hydrogen formation [0.2−3.8 c(v/v %)] was detected in the samples with all of the other carbon sources except $A^{2+3}XX$, where no hydrogen was found. Most of the hydrogen was formed in the samples grown on xylose and FOS. With fecal samples, the change in acetate concentration after the incubation period was typically about 15 mM, but in the samples with A^2XX and $A^{2+3}XX$, the change in acetate concentration was 22 and 31 mM, respectively. In most of the samples, some changes in propionate and butyrate concentrations were also detected (0.3−6.9 and −1.3−5.8 mM, respectively).

The L-arabinose, D-xylose, XOS, FOS, WAX and RAX hydrolysates, and polymeric wheat and rye flour AX were utilized almost completely, and only traces of these compounds (with polymers, traces of $X-X_4$) were seen in the HPAEC-PAD chromatograms. Purified monosubstituted AXOS (A^2XX) was also completely fermented, and only traces of xylose were found in the chromatograms. In the case of doubly substituted purified AXOS, $A^{2+3}XX$, the oligosaccharide was not consumed completely, as can be seen in **Figure 4**. Only 0.5 wt % of $A^{2+3}XX$ was left after incubation, but some L-arabinose (53 μg/mL), D-xylose (28 μg/mL), xylobiose (120 μg/mL), xylotriose (136 μg/mL), and A^2XX (25 μg/mL) were detected after a 48 h fermentation. The results clearly show that in these samples the A^2XX was formed during the incubation from $A^{2+3}XX$ by the bacteria in the fecal microbiota. However, the growth of intestinal bifidobacteria species at pH lower than 4.5 is very limited (*31*), suggesting clearly that bacteria other than bifidobacteria ferment disubstituted AXOS. This is supported by the altered fermentation pattern seen in the VFA distribution in **Table 2**.

The bifidobacterial 16S rRNA was extracted from the samples taken before and after incubation of the fecal microbiota with two

Table 2. Formation of H_2, Pressure, and VFA (Acetate, Propionate, and Butyrate) during the Incubation Period (48 h) in Fecal Microbiota Cultivations[a]

carbohydrate	end pH	H_2 (v/v %)	pressure (Δ psi)	acetate (Δ mM)	propionate (Δ mM)	butyrate (Δ mM)
D-xylose	5	3.8	1.8	15.3	6.9	0.4
L-arabinose	5	2.0	1.9	15.4	2.6	2.3
XOS	5	2.1	2.0	16.7	4.3	4.0
RAX hydrolysate	5	1.4	2.2	13.9	2.2	4.9
WAX hydrolysate	5	2.0	2.0	13.4	1.9	2.9
RAX	5.5	0.2	1.1	14.4	2.3	3.5
WAX	5.5	1.0	1.6	16.0	2.9	5.8
A^2XX	6	2.1	1.6	22.1	4.6	5.4
$A^{2+3}XX$	4	0	0.8	31.2	0.3	−1.3
FOS	5	4.0	2.0	13.5	1.8	3.3

[a] The pH values of the samples were measured at the end of the experiment. Pressure (Δ psi) and VFA (Δ mM): pressure and VFA formation are compared with 0-sample at 48 h.

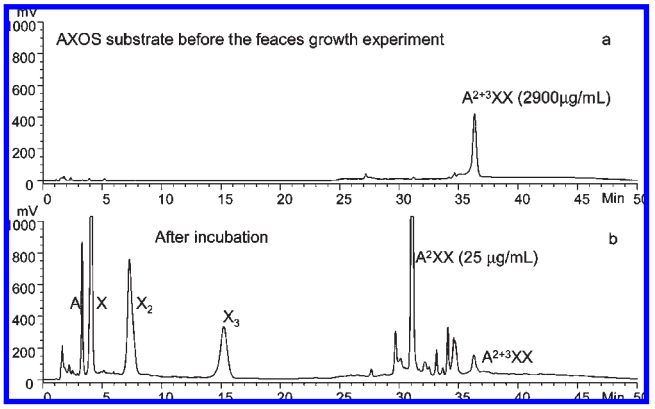

Figure 4. HPAEC-PAD analysis of the purified AXOS ($A^{2+3}XX$) (**a**) before and (**b**) after fermentation with feces slurry. Note that the sample in panel **a** is 40 times more diluted than in panel **b**. Abbreviations used for saccharides are as in **Figure 1**.

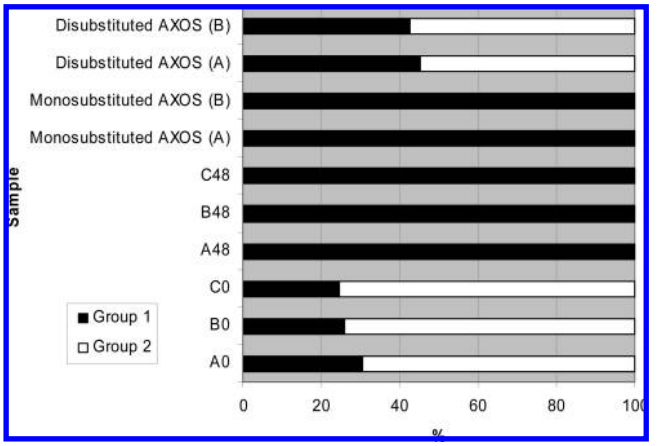

Figure 5. Distribution of bifidobacteria according to t-RFLP analysis, before incubation (A0, B0, and C0, three parallel samples) and after 48 h of incubation with no carbon source added (A48, B48, and C48) and with monosubstituted AXOS (A^2XX) or disubstituted AXOS ($A^{2+3}XX$) as a carbon source. Group 1, *B. pseudocatenulatum*, *B. catenulatum*, and *B. longum*; group 2, *B. breve*, *B. infantis*, *B. adolescentis*, *B. bifidum*, and *B. dentium*.

purified AXOS and with no added carbon source and analyzed, using t-RFLP to evaluate the effect of AXOS on the distribution of bifidobacteria. For analyzing the t-RFLP data, the relevant bifidobacteria were devided into two groups based on their known terminal fragment length sizes in base pairs (bp); group 1, *B. pseudocatenulatum* (299 bp), *B. catenulatum* (299 bp), and *B. longum* (299 bp); group 2, *B. breve* (470 bp), *B. infantis* (480 bp), *B. adolescentis* (469 bp), *B. bifidum* (467 bp), and *B. dentium* (468 bp). The distribution of these two bifidobacteria groups was measured before and after 48 h of incubation.

The group 2 bacteria predominated before the incubation, but in the control samples with no added carbon source, only group 1 bifidobacteria were found after 48 h (**Figure 5**). With monosubstituted AXOS (A^2XX) as a carbon source, the distribution of bifidobacteria groups was the same as in the control samples after 48 h, whereas with disubstituted AXOS ($A^{2+3}XX$), group 2 also predominated after the incubation period. In this experiment, the monosubstituted AXOS (A^2XX), which were almost completely utilized by the bacteria in the fecal slurry, induced the growth or survival of group 1 bifidobacteria, as was also the case in the samples with no added carbon source. With the less efficiently fermented disubstituted AXOS, the proportion of group 1 bifidobacteria increased about 20% during the incubation, as compared with the control samples before incubation. The quantities of bifidobacteria were not achieved with the used method.

On the basis of the t-RFLP analysis, fermentation pattern, pH, and HPAEC-PAD analyses, the disubstituted AXOS were fermented during rapid acidification, leading to high acetate concentrations and low pH. As a result, the bifidobacteria populations were similar to those in the control samples, and unknown fecal bacteria were presumably responsible for the partial fermentation of $A^{2+3}XX$. All of the other carbohydrates tested were probably fermented over acetate into butyrate.

DISCUSSION

The focus here was to examine the fermentation patterns of AX-derived carbohydrates by pure bifidobacteria and fecal microbiota and to analyze the utilization of carbohydrates, especially that of XOS and AXOS in detail by HPAEC-PAD. The substrates studied were pure or mixed mono- and oligosaccharides. The AXOS were prepared, using GH10 endo-1,4-β-D-xylanase to produce oligosaccharides with nonreducing end-terminal Ara*f* branches to restrict the action of exo-1,4-β-D-xylosidases and thus prevent the liberation of free xylose for bacterial growth. Fundamental studies with different (A)XOS are important not only for the development of prebiotic oligosaccharide products but also to better understand the in vivo consumption and effects of AX in the colon, which is the result of a cooperative cross-feeding process between different bacteria.

Our results indicate clear substrate preferences among the various bifidobacteria strains. L-Arabinose was consumed by *B. longum* ATCC 15707 but not by *B. adolescentis* ATCC 15703. When D-xylose was present as the only carbon source, it was fermented slowly by *B. adolescentis*, whereas xylose was not

efficiently consumed when *B. adolescentis* was cultivated on AX hydrolysates. In contrast, *B. longum* was able to utilize xylose only from the mixture of several carbohydrates. *B. adolescentis* effectively fermented xylobiose and xylotriose, whereas *B. longum* used neither. *B. breve* ATCC 15700 may be able to slowly utilize some xylobiose and AXOS from a mixture, since some reduction in their quantities was detected after incubation, as compared with the starting situation. *B. adolescentis* and *B. longum* were able to grow about equally on AX hydrolysates, but the growth observed was clearly less than that on FOS, indicating more restricted fermentability of a mixture rich in AXOS. The consumption of AXOS from the hydrolysates was detected by both species; however, both of these bacteria utilized only parts of the AXOS, that is, *B. adolescentis* used the backbone XOS and *B. longum* the Ara*f* substituents. Thus, new detailed information was obtained here on the use of more complex oligosaccharides, which does not always mean complete utilization.

The growth of *B. adolescentis* ATCC 15703 (DSM 20083), *B. longum* ATCC 15707 (DSM 20213), and *B. breve* ATCC 15700 (DSM 20219) on D-xylose, L-arabinose, XOS, and AXOS was previously reported by several groups, and the results obtained in the present study are generally in agreement with these. *B. adolescentis* ATCC 15703 earlier showed modest growth on xylose (*30*, *32*), although Crittenden et al. (*19*) and Gullón et al. (*33*) speculated on the inability of *B. adolescentis* to utilize xylose. In the present study, clear growth on pure xylose was detected after a prolonged incubation time (140 h). Furthermore, HPAEC-PAD analysis showed that xylose was consumed by *B. adolescentis* ATCC 15703; however, no reduction in xylose content was detected in the AX hydrolysates. This difference may actually explain the diverging conclusions on the ability of *B. adolescentis* ATCC 15703 to ferment xylose. In previous studies, the growth of *B. longum* ATCC 15707 was detected with pure xylose (*32*), whereas in the present study xylose was utilized by *B. longum* ATCC 15707 only when present in a mixture with xylobiose and AXOS. The result that *B. longum* ATCC 15707 can, but *B. adolescentis* ATCC 15703 cannot, utilize L-arabinose is well in agreement with published data (*18*, *30*, *34*). Previous reports also indicated the inability of *B. breve* ATCC 15700 to grow on pure xylose and L-arabinose (*32*, *34*, *35*).

The strong growth of *B. adolescentis* ATCC 15703 on commercial XOS (X_2, X_3, and X_4) is in line with several previous reports (*18*, *30*, *33*, *36*). The main diverging findings between this study and previous results are found in the ability of XOS to promote growth of *B. longum* ATCC 15707. In the study of Crittenden et al., *B. longum* ATCC 15707 (VTT E-96664) was able to grow effectively on a mixture of XOS (Xylo-oligo 70, Suntory Ltd., Osaka, Japan), which, however, contained over 40% free monosaccharides, including xylose and some glucose. Thus, the growth of *B. longum* could have occurred due to the utilization of the monosaccharides instead of XOS (*19*). Gullón et al. (*33*) reported modest growth of *B. longum* ATCC 15707 on XOS prepared from rice husks. In addition to XOS, the product contained some free arabinose and xylose, and because of the raw material used for XOS production, it could also have contained some AXOS, all of which could have promoted the growth of *B. longum* ATCC 15707 (*33*). However, Van Laere et al. (*36*) reported the ability of *B. longum* ATCC 15707 to grow on a xylose-free XOS mixture (X_2, X_3, and X_4). In the present study, XOS as the only carbon source was too challenging for *B. breve* ATCC 15700, which was also observed in the study by Palframan et al. (*32*), while Gullón et al. (*33*) reported on the ability of the same strain to grow on XOS. Studies with AXOS are more rare than with XOS. Van Laere et al. (*36*) reported that AXOS (mainly L-Ara*f* doubly substituted XOS) were fermented partially by *B.adolescentis* ATCC 15703 and completely by *B. longum* ATCC 15707. This supports our data that both strains are able to consume AXOS, but we were only able to demonstrate partial consumption of AXOS by *B. longum* ATCC 15707, that is, utilization of the L-Ara*f* released but not the residual XOS.

The differences between the results obtained in this study and previously could have occurred partially because the experiments were executed in a small volume, using an extremely nutrient-poor medium. The downscaling of the cultivation volume of the pure cultures had to be performed in test tubes where normally 10 mL of medium was added to secure the preservation of anaerobic conditions and the reliable measurement of OD. The small (3 mL) volume changed the proportions of the medium and the airspace and possibly made it more challenging to obtain completely anaerobic conditions. Furthermore, this small volume hindered the usage of the reducing agent (Na_2S), which may also have changed the growth environment. However, use of this small-scale method was a necessity for testing carbohydrates that are difficult to obtain in large amounts. All of the experiments were performed on a small scale to obtain comparable results. Long cultivation times, up to 140 h, were used in the present study to ensure growth in the somewhat atypical conditions applied. Other reported studies have used much shorter fermentation times, which may also explain some of the differences observed. There are also divergences in the nutrient basal medium used in various studies. Moreover, the purity and composition of the XOS and AXOS used have varied, which may have affected the induction of uptake systems and enzyme production.

Two different strategies for utilization of oligosaccharides have been speculated for bifidobacteria: (i) small oligosaccharides are transported into the cell where intracellular enzymes further hydrolyze them to monosaccharides or (ii) extracellular glycosidases are produced to hydrolyze the oligosaccharides and the monosaccharides formed are transported into the cell (*37*). Further metabolism of the carbohydrates in bifidobacteria differs from that in other genera in the colon, and this metabolism is not yet fully understood (*32*).

Stronger growth of *B. adolescentis* ATCC 15703 on XOS than on xylose indicated that short XOS (X_2 and X_3) are transported into the cell rather than hydrolyzed extracellularly to xylose. The preference of *B. adolescentis* ATCC 15703 for di- and oligosaccharides as compared with monosaccharides was also noted in previous studies (*19*, *32*, *37*). Even though there are no reports yet on isolation and characterization of intra- or extracellular β-D-xylosidases from *B. adolescentis* ATCC 15703, the genome sequence has revealed several potential genes (*14*). Furthermore, Palframan et al. (*32*) showed that *B. adolescentis* ATCC 15703 produces extracellular β-D-xylosidase activity when cultivated on XOS. The presence of extracellular β-D-xylosidase was also indicated in the present study, since the xylose formed was detected after cultivation of *B. adolescentis* ATCC 15703 on XOS and AX hydrolysates. An increase in XOS chain length to degree of polymerization (DP) 4−6 reduces the efficacy of *B. adolescentis* ATCC 15703 in utilizing these XOS (*30*, *33*), most probably because longer XOS need to be first hydrolyzed by an extracellular enzyme to shorter XOS. Palframan et al. (*32*) reported that in contrast to *B. adolescentis* ATCC 15703, *B. longum* ATCC 15707 showed no extracellular β-D-xylosidase activity, which agrees with the present study in which no consumption of xylobiose was detected.

From the data obtained, it seems evident that AXOS are hydrolyzed extracellularly by α-L-arabinofuranosidases. Both *B. adolescentis* ATCC 15703 and *B. longum* ATCC 15707 were able to cleave the linkage between L-arabinose and xylose, since no short-chain AXOS were detected after cultivations with AX hydrolysates.

B. adolescentis ATCC 15703 is known to express two distinct extracellular α-L-arabinofuranosidases (*18*, *38*). *B. longum* ATCC 15707 undoubtedly also produces α-arabinofuranosidase(s) analogously to other *B. longum* strains (*19*, *20*), although the presence of α-L-arabinofuranosidase-encoding genes has not yet been shown.

B. breve K-110, isolated from human intestine, also produces *β*-D-xylosidase and α-L-arabinofuranosidase (*39*, *40*). On the other hand, preliminary results from the genome sequence of *B. breve* UCC2003 show the absence of AX-degrading enzymes (*38*). In the present and previous studies, *B. breve* ATCC 15700 was unable to grow on xylose or L-arabinose (*32*, *34*, *35*). Degnan and Macfarlane (*41*) showed that to transport L-arabinose, *B. breve* NCFB 2257 requires glucose in the cultivation medium, indicating cotransportation. The lack of cotransporation could be, in addition to lack of enzymes for the hydrolysis of (A)XOS, another potential explanation for the poor growth of *B. breve* ATCC 15700 when xylose, L-arabinose, and (A)XOS were the only carbohydrates present in the cultivation medium.

After the incubation of *B. adolescentis* with the WAX and RAX hydrolysates, twice as much free L-arabinose was found in the samples than could be formed from the short, quantified AXOS in the hydrolysates. This indicates that L-arabinose is also liberated from the longer, unidentified AXOS present in the samples. Furthermore, *B. longum* was not able to utilize xylobiose and xylotriose from the WAX and RAX hydrolysates, and the amounts of these short XOS detected in the samples after fermentation were, surprisingly, 7−8 times more than could be released from the quantified AXOS. Additional xylobiose and xylotriose may also originate in this case from longer AXOS. After releasing the L-Ara*f* substituents, *B. longum* may be able to hydrolyze the resulting XOS further, for example, using specific (exo)xylanase as is produced by *Aeromonas caviae* ME-1 (*42*). The formation of more xylobiose and xylotriose during growth of *B. longum* is rather interesting, because it does not consume these carbohydrates.

The fermentation experiments with pure AXOS revealed that monosubstituted A^2XX is an easier substrate than the doubly substituted $A^{2+3}XX$, since the former was completely fermented both by a mixture of pure bifidobacteria and by fecal microbiota, whereas the latter were utilized only by the fecal bacteria mixture. However, even after the growth of the fecal microbiota, some mono- and oligosaccharides were detected, including A^2XX formed after cleavage of a single L-Ara*f* unit from the doubly substituted D-Xyl*p* residue in $A^{2+3}XX$. Removal of the first L-Ara*f* unit appears to be challenging, but after this, complete fermentation of the singly substituted AXOS formed can be achieved. Consumption of the doubly substituted AXOS was, however, slow since carbohydrates still detected after a 48 h of incubation of $A^{2+3}XX$ were readily utilized in other fermentations. Thus, it can be assumed that few bacteria species in the human fecal microbiota were able to liberate α-1→3-linked L-Ara*f* units from the doubly substituted D-Xyl*p* residue. The t-RFLP analysis of fecal samples indicated that various AXOS may result in different distributions of bifidobacteria, since not all AXOS support growth of the same bifidobacteria species. Because the doubly substituted AXOS were consumed by pure *B. adolescentis* ATCC 15703 and *B. longum* ATCC 15707 from the WAX hydrolysate, the pure $A^{2+3}XX$ may not have been able to induce the production of essential α-L-arabinofuranosidases. Gueimonde et al. (*20*) reported that the GH 51 α-L-arabinofuranosidase of *B. longum* NIZO B667 was induced by xylose and to a lesser extent by L-arabinose.

Interestingly, *B. adolescentis* ATCC 15703 did not ferment L-arabinose, although it has α-L-arabinofuranosidases. The need for this organism to produce these enzymes may be to degrade AXOS to obtain xylose, not L-arabinose. It is also tempting to hypothesize that the liberation of L-arabinose as a result of the action of *B. adolescentis* α-L-arabinofuranosidases may provide a carbon source for other bifidobacteria, for example, *B. longum*, indicating the complex synergisms of probiotic bacteria. This was indeed observed in the present study when pure A^2XX was completely consumed by the mixture of *B. adolescentis*, *B. breve*, and *B. longum*. The other indication of a possible versatile cross-synergy between *B. adolescentis* and *B. longum* was the buildup of xylobiose and xylotriose by *B. longum* when grown on AX hydrolysates, because the short XOS are the preferred substrates of *B. adolescentis*.

Detailed chromatographic analysis of mono- and oligosaccharides after the fermentations revealed new insight into carbohydrate, particularly AXOS, consumption by the bifidobacteria studied. Particularly, the fate of individual AXOS during fermentation has not previously been published. To our knowledge, this is also the first report showing that *B. adolescentis* ATCC 15703 did not consume the L-arabinose that it cleaved from various AXOS. On the basis of our results and combined with previous studies by other groups, short (A)XOS support growth of *B. adolescentis* ATCC 15703, whereas highly substituted AXOS, and even polymeric AX, support the growth of *B. longum* ATCC 15707, utilizing primarily the L-Ara*f* substituents. AXOS are clearly potential candidates for selective prebiotics, but the fermentation efficiency and the prebiotic effect appear to be dependent on the type of L-Ara*f* substitution. AXOS with doubly substituted D-Xyl*p* were fermented less efficiently than AXOS with singly substituted D-Xyl*p*. Although isolated $A^{2+3}XX$ was not fermented by the mixture of pure bifidobacteria strains, it was consumed by the versatile fecal microbiota. Doubly substituted AXOS were also utilized by *B. adolescentis* ATCC 15703 and *B. longum* ATCC 15707 when present in the hydrolysate together with xylose, XOS, and monosubstituted AXOS. Thus, a mixture of different singly and doubly substituted AXOS could function as a suitable, slowly fermenting, prebiotic substance. The results also reflected a complex cooperation between bifidobacteria in the consumption of AXOS. In the present study, pure AX-derived saccharides were used as the only carbon source, but in the colon, there are also other carbohydrates available. This may contribute to fermentation of the challenging AXOS through the activation of hydrolytic enzymes, transport systems, or activation of the bifidus pathway. Thus, further research is needed on the interactions of different putative prebiotics in relation to the growth of probiotic bacteria.

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