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In Vitro Fermentability of Differently Substituted Xylo-oligosaccharides

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Xylo-oligosaccharides (XOS) with various substituents were fermented in vitro by fecal inocula (FI) from four human volunteers to study the influence of substitution on the ability and rate of fermentation and on the production of short-chain fatty acids (SCFA) and lactate. By all FI used nonsubstituted XOS (nXOS) and arabino-XOS (AXOS) were fermented more quickly than the more complex structures of acetylated XOS (AcXOS) and XOS containing a 4-*O*-methylglucuronic acid group ($GlcA_{me}XOS$). In the first stage (0−40 h) of the fermentations of nXOS and AXOS mainly acetate and lactate were formed. The fermentations of AcXOS and $GlcA_{me}XOS$ resulted in a lower lactate production, whereas the concentration of propionate and butyrate increased. These results put emphasis on the detailed elucidation of the structural features of nondigestible oligosaccharides in general to understand their fermentation mechanisms more precisely.

KEYWORDS: In vitro; fermentation; xylo-oligosaccharides; short-chain fatty acids; structural features

INTRODUCTION

From a nutritional point of view xylo-oligosaccharides (XOS) usually are considered to be nondigestible oligosaccharides (NDOs), which are not degradable by the low-pH gastric fluid or by human and animal digestive enzymes and will therefore reach the large bowel intact (*1*, *2*). In the large bowel NDOs can be fermented by the intestinal flora into mainly short-chain fatty acids (SCFA; acetate, propionate, and butyrate), lactate, CO_2, and H_2 (*3*, *4*). Furthermore, NDOs are frequently reported to have an effect on the composition of the colonic flora, which often is the result of an increase in bifidobacteria (*5*−*7*). Bifidobacteria are considered to exhibit a range of biological activities related to host health, for example, the inhibitory effect on the growth of pathogens (*8*). Additionally, the production of SCFA is related with a number of health effects, for example, bowel function, calcium absorption, lipid metabolism, and reduction of the risk of colon cancer (*9*−*11*).

XOS are reported to be preferentially fermented by bifidobacteria. This was shown in in vitro experiments as well as in vivo (*1*, *4*, *12*). Fermentability studies of XOS were mainly performed using linear and low molecular weight (MW) XOS [degree of polymerization (DP) < 4]. These studies do not answer the question about the influence of substituents to XOS on the mode of fermentation by the human intestinal flora. The importance of being able to distinguish between differently substituted (xylo-) oligosaccharides was recently indicated by Van Laere et al. (*2*), who included linear XOS and arabino-xylo-oligosaccharides (AXOS) in a fermentation study of a range of (complex) plant cell wall derived oligosaccharides. In this study it was found that linear XOS were fermented by more intestinal strains tested as compared to the branched AXOS.

A good source to obtain differently substituted XOS is agroindustrial xylan-rich byproducts, such as hard woods, brewery spent grain, corn cobs, and wheat bran. Through a mild hydrothermal treatment the xylan present in these byproducts is (partly) broken down into a variety of soluble XOS. The structures obtained depend on the structural features of the xylan originally present in the byproducts used (*13*, *14*). In a former publication we have described the purification and characterization of several series of XOS obtained from hydrothermally treated brewery spent grain and *Eucalyptus* wood. These included nonsubstituted XOS as well as XOS substituted with arabinose or acetyl groups and/or 4-*O*-methylglucuronic acid (*15*). In this paper, we study the fermentation by fecal inocula (FI) of the previously purified series of XOS. The focus of our study is the influence of various substituents of XOS on the ability and rate of fermentation and on the production of SCFA and lactate.

EXPERIMENTAL PROCEDURES

Xylo-oligosaccharides Mixtures. The four series of XOS used in this fermentation study were obtained and purified as described previously (*15*). The italic names in parentheses refer to the previous publication. Three of the series of XOS (DP 3−15) used were obtained from hydrothermally treated *Eucalyptus* wood: acetylated XOS (*Euc NI A* → AcXOS), linear XOS (*Euc NI A saponified* → nXOS), and nonacetylated XOS containing a 4-*O*-methylglucuronic acid or 4-*O*-methylglucuronic acid plus a hexose, most likely galactose (*Euc AII# plus AIII# saponified* → $GlcA_{me}XOS$). The fourth series of XOS used was obtained from hydrothermally treated brewery spent grain. To

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obtain sufficient amounts of the arabino-xylo-oligosaccharides (AXOS) in the range of DP 3−11, the pool *BSG IB* (containing higher MW arabinoxylan) was degraded by a purified and well-characterized endo-(1,4)-β-D-xylanase III (*16*). A solution of *BSG IB* in water (2.5 mg/mL) was adjusted to pH 5 with sodium hydroxide and incubated with endo-(1,4)-β-D-xylanase III (0.1 μg/mL) for 30 h at 40 °C. Finally, the enzyme was inactivated (5 min, 100 °C) before the digest was used in the fermentation experiment.

Fermentation of XOS. Fermentation of the nXOS, AcXOS, $GlcA_{me}$-XOS, and AXOS was performed using fecal inocula (FI) of four human volunteers. FI were prepared from fresh feces in buffered peptone water (Oxoid) with cysteine−HCl (0.5 g/L) in ~10-fold dilution. A medium consisting of 1 g/L of neutralized bacterial peptone (Oxoid), 8 g/L of sodium chloride (Merck), and 0.5 g/L of L-cysteine−HCL was adjusted to a pH of 6.7 using a 6 N NaOH solution (*17*). In an anaerobic chamber (atmosphere of 80% N_2, 10% CO_2, and 10% H_2) the 10-fold diluted feces was diluted further (10000×) with the medium described.

Sterile solutions (S1) were prepared of each of the XOS mixtures in water [6% (w/v)] and of pure water as blank. A sterile solution (S2) of bacto yeast nitrogen base in water [Difco; 13.4% (w/v)] was prepared. Additionally, a sterile solution (S3) in water was prepared containing a salt solution [40% (v/v); $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.2 g/L), K_2HPO_4 (1 g/L), KH_2PO_4 (1 g/L), $NaHCO_3$ (10 g/L), and NaCl (2 g/L)], casein enzymatic hydrolysate [N-Z-AmineA; bovine milk (Sigma; 5% (w/v)], and sodium thioglycolate [Sigma; 0.5% (w/v)]. The three solutions S1, S2, and S3 were combined (9.1 mL) in a ratio (v/v) of 17:1:2, respectively (pH 6−7). The latter solutions (four substrates and one blank) were each inoculated with 20% (v/v) of the 10000× diluted fecal inocula (four inocula) at 37 °C in an anaerobic chamber. From each tube (altogether 20) samples were taken at time 0, 0.5, 7, 19, 27, 43, 55, 67, 75, and 94 h and stored at −80 °C. Of each sample enzyme activity was inactivated (5 min, 100 °C) and centrifuged, and the supernatants were analyzed (*2*).

Cell Material from Bacterial Growth. The residue (cell material) of each sample, obtained after inactivation and centrifugation of the bacterial suspensions (94 h of fermentation), was mixed with 1 mL of pure water. The turbidity of the mixed solutions was measured by an UV-mini 1240 UV−vis spectrophotometer (Shimadzu) at 600 nm giving information about the density of cell material from bacterial growth in all samples.

Short-Chain Fatty Acids and Lactate. The contents of the SCFA (butyrate, propionate, and acetate) and lactate were determined on a Spectra Physics 8800 system HPLC, using an Aminex HPX-87H column and detection by refractive index (RI; Spectrasystem RI-150) (*18*).

Total Sugar Content. The total content of carbohydrates present in the fermentation experiments was determined by an automated orcinol sulfuric acid assay (*19*) using an autoanalyzer (Skalar Analytical BV, Breda, The Netherlands).

High-Performance Anion-Exchange Chromatography (HPAEC) (pH 12). To analyze the oligosaccharides present during the fermentations, HPAEC was performed on a Dionex system equipped with a CarboPac PA-1 column (4 mm i.d. × 250 mm) in combination with a CarboPac PA guard column (3 mm × 25 mm) and pulsed ampomeric detection (PAD) (*20*). Elution (1 mL/min) of the oligomers in the fermentation samples (diluted 12 times) was performed using the following gradient: 50−90 mM sodium acetate in 100 mM NaOH during 0−5 min, 90−130 mM sodium acetate in 100 mM NaOH during 10 min, and 130−520 mM sodium acetate in 100 mM NaOH in 15 min. Each elution was followed by a washing (30 min; isocratic 1 M sodium acetate in 100 mM NaOH) and equilibration step (20 min).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). For MALDI-TOF MS of oligosaccharides during fermentation, a Voyager-DE RP Biospectrometry workstation (PerSeptive Biosystems Inc., Framingham, MA) was used, operated as described by Daas et al. (*21*). The mass spectrometer was calibrated with a mixture of maltodextrins (mass range = 365−2309 Da as sodium adducts).

The samples were desalted with anion-exchange material (AG 50W-X8 resin; Bio-Rad) and mixed with a matrix solution (1 μL of sample in 9 μL of matrix). The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid and 3 mg of 1-hydroxyisoquinoline in a 1-mL mixture of acetonitrile/water (300 μL/700 μL). Of the prepared (sample plus matrix) solutions 1 μL was put on a gold plate and allowed to dry at room temperature before analysis by MALDI-TOF MS.

RESULTS

Four series of differently substituted XOS and a fermentation blank (without carbohydrate source added) were fermented by fecal inocula from four human volunteers. All XOS studied consisted of a β-D-(1,4)-linked xylopyranosyl backbone and varied in DP and type of substituent, as reported previously (*14*, *15*). In summary, nXOS are linear (DP 2−11), AcXOS are highly acetylated (DP 2−11), $GlcA_{me}$XOS each contain a 4-*O*-methylglucuronic acid group or in addition a hexose, most likely galactose (DP 3−10), AXOS are singly and doubly substituted with arabinose (DP 2−10), and arabinoxylan material having a higher MW is present (MW > 1500 Da).

The XOS in the samples taken during the fermentation experiment (up to 94 h) were monitored using an automated orcinol sulfuric acid assay, HPAEC, and MALDI-TOF MS to determine both the total carbohydrates present and individual components. From the results obtained with HPAEC the amount of nXOS, AcXOS, and $GlcA_{me}$XOS present during fermentation compared to the amount at the start of the fermentation was calculated (total area percent). The relative amount of the AXOS was calculated in a similar way but from the total carbohydrate content (orcinol sulfuric acid assay). The AXOS contained some higher MW material as well, which could not be separated and quantified by HPAEC. The relative amount of the XOS in the four XOS mixtures present during the fermentation by four human FI is shown in **Figure 1**.

During the first 20 h of the experiment the pH of all XOS samples was adequate to allow bacteria to grow (pH 5−7). However, during the first 20 h a difference in degradation between the four XOS mixtures was observed. The susceptibility of the various XOS to fermentation by FI 1−3 decreased in the following order: AXOS > nXOS > AcXOS > $GlcA_{me}$XOS. In contrast, for fermentation by FI 4 the order was nXOS > AXOS > AcXOS > $GlcA_{me}$XOS. However, as can be seen from **Figure 1**, the $GlcA_{me}$XOS were fermented quite well after an adaptation time of ~20 h (FI 1−3). FI 4 fermented this substrate again divergently; it needed almost no adaptation time but fermented the $GlcA_{me}$XOS gradually. Analysis of the fermentation blanks did not show any carbohydrates to be present or formed during fermentation (0−94 h).

Figure 2 shows the amounts of the different XOS present in the nXOS and AcXOS mixtures during the fermentation related to the amount present at the start (HPAEC; peak area percent) by FI 1 and 2. The results of the fermentations of the different DPs of nXOS and AcXOS by FI 3 and 4 are reported in the text. Analysis of the XOS composition of the $GlcA_{me}$XOS and AXOS mixtures by HPAEC was not accurate enough to estimate the relative amount of these XOS. From a comparison of the XOS profiles of the fermentations of the different DPs of nXOS by FI 1 and 2 during the first 30 h, it was obvious that DP 3 and 4 were fermented more readily, whereas DP 8−10 were less rapidly fermented. The fermentation pattern of nXOS (DP 3−10) by FI 3 resembled that of FI 1, whereas FI 4 fermented even the higher DPs of the nXOS in a much shorter time. During the first 30 h of the fermentation of the AcXOS by FI 1−3 mainly DP 3 was fermented, similarly to the nonsubstituted DP 3. The AcXOS fermentation by FI 4 resembled that of FI 2, with the difference that FI 4 was able to ferment DP 4 of the AcXOS at the same rate as DP 3. AcXOS of a higher DP (5−

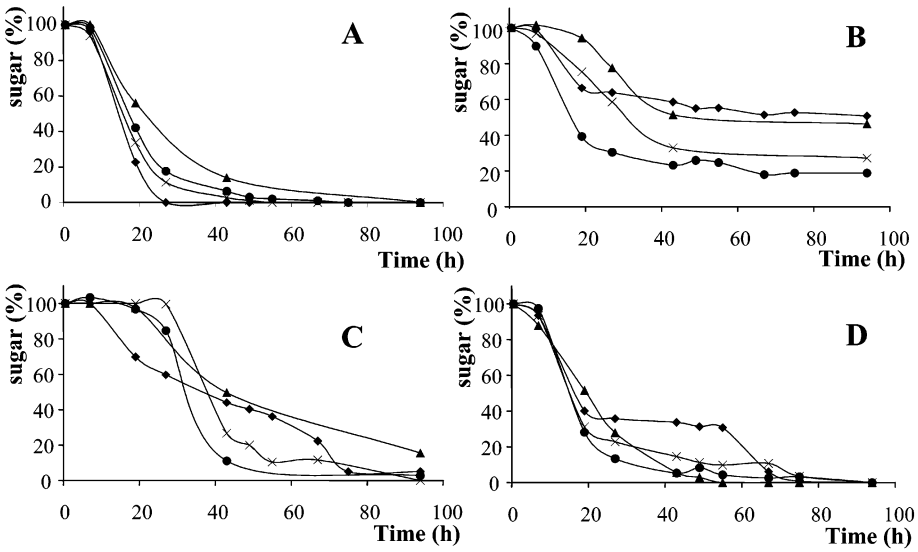

Figure 1. Degradation patterns of nXOS (A), AcXOS (B), $GlcA_{me}XOS$ (C), and AXOS (D) during 94 h of fermentation by fecal inocula from four persons (●, FI 1; ▲, FI 2; ×, FI 3; ◆, FI 4).

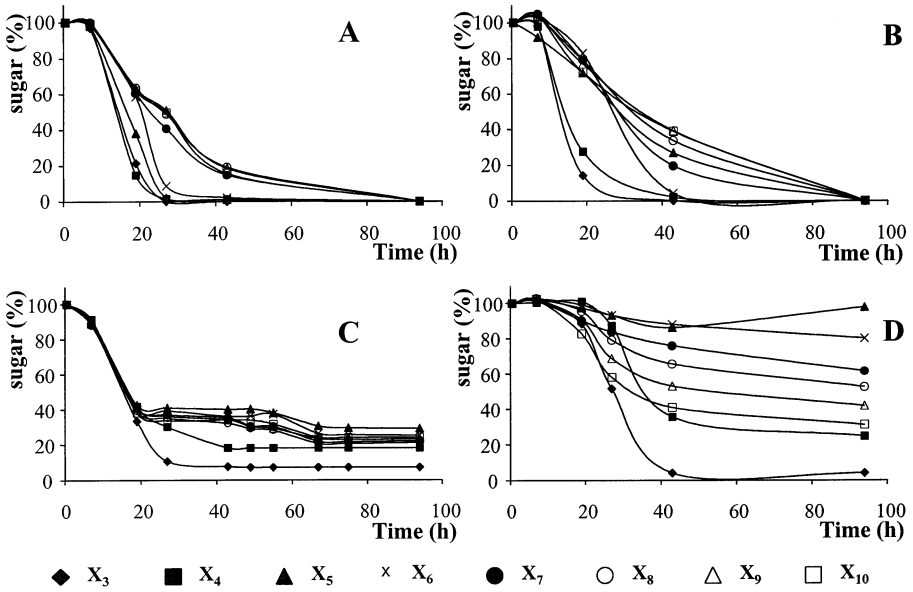

Figure 2. Degradation patterns of different DPs during 94 h of fermentation of nXOS [FI 1 (A) and FI 2 (B)] and AcXOS [FI 1 (C) and FI 2 (D)].

10) were fermented more slowly (FI 1, 2, and 4). Nevertheless, at the end of these fermentations the amount of DP 5 and 6 was higher than of DPs 9 and 10. This "accumulation" of DP 5 and 6 was also observed in the fermentation of AcXOS by FI 3. However, in the AcXOS fermentation by FI 3 DPs 8−10 were consumed more rapidly than DPs 3 and 4.

Study of the patterns of degradation of the AcXOS by HPAEC provided only information regarding the size of the various oligomers, because at the high pH of the HPAEC eluents used acetyl groups are removed. In contrast, MALDI-TOF MS allows the monitoring of acetylated XOS, enabling us to follow the fermentation of these oligomers. MALDI-TOF MS also provided data about the degradation of the substituents of the $GlcA_{me}XOS$. As an example, the MALDI-TOF mass spectra of the fermented AcXOS and $GlcA_{me}XOS$ by FI 1 after 0.5, 19, and 43 h are presented in **Figures 3** and **4**, respectively. From these figures it can be seen that no masses were observed representing nonsubstituted XOS. The series of XOS each containing a 4-*O*-methylglucuronic acid plus a hexose (most likely galactose) were accumulated after 43 h of fermentation. The same observations were noticed for the fermentations of AcXOS and $GlcA_{me}XOS$ by the other three FI (not shown).

The production of acetate, propionate, butyrate, and lactate during the fermentation was measured, and for the fermentations by FI 1 and 2 these results are presented in **Figure 5**. The results obtained during the fermentation by FI 3 and 4 are given in the text.

Analysis of all fermentation blanks showed only after 40 h some production of SCFA, not exceeding 20 μmol/mL.

During the first 20 h of the fermentations of nXOS (FI 1, 2, and 4) and of AXOS (FI 1−3) mainly acetate and lactate were detected in a molar ratio of 1:1, respectively. However, during the nXOS fermentation by FI 3 and AXOS fermentation by FI 4 some propionate was formed as well, at the expense of lactate (0−19 h). From 19 until 94 h of fermentation of nXOS (FI 2−4) and AXOS (FI 1−4) also propionate and/or butyrate were detected, whereas the amount of lactate and, to a lesser extent, acetate decreased. Only during fermentation of nXOS by FI 1 (19−94 h) was no propionate or butyrate detected, and the amounts of lactate and acetate remained constant.

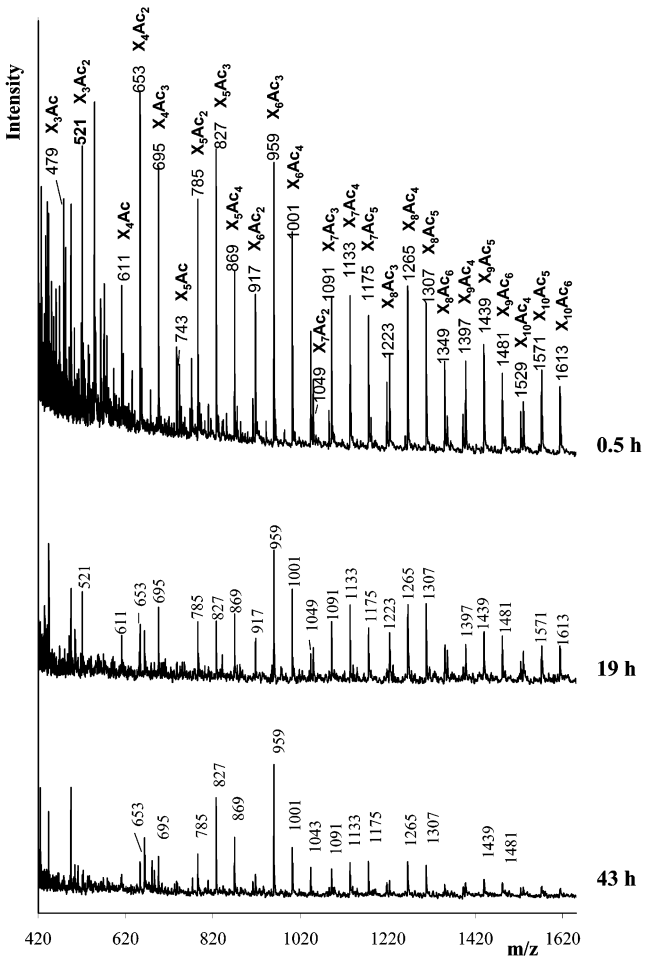

Figure 3. MALDI-TOF mass spectra obtained after 0.5, 19, and 43 h of fermentation of AcXOS (FI 1); masses of the sodium-adducted AcXOS are inserted (X = xylose; Ac = acetyl group).

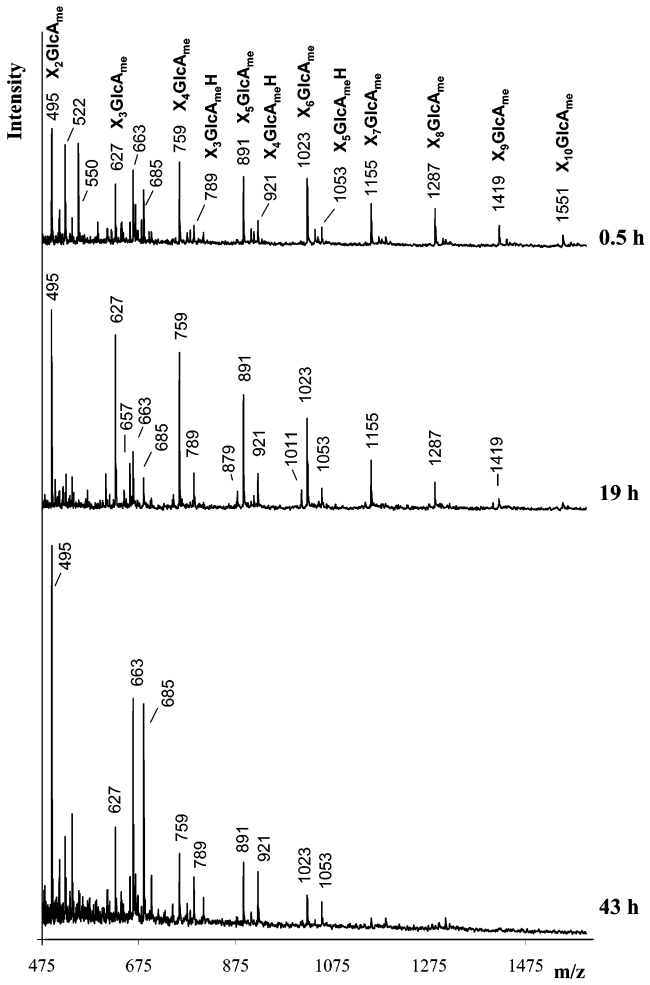

Figure 4. MALDI-TOF mass spectra obtained after 0.5, 19, and 43 h of fermentation of $GlcA_{me}$XOS (FI 1); masses of the sodium-adducted $GlcA_{me}$-XOS are inserted (X = xylose; $GlcA_{me}$ = 4-*O*-methylglucuronic acid).

During the first 27 h of the fermentations of AcXOS (pH 5−7) mainly acetate, lactate, and propionate were detected in a molar ratio of ∼1:0.8:0.2, respectively (FI 1, 2, and 4), but for FI 3 a divergent molar ratio was determined (1:0.5:0.3). The amount of SCFA and lactate remained quite constant from 27 until 94 h of the fermentations of AcXOS. However, by FI 2 some butyrate was formed, and a decrease in lactate was detected. During all $GlcA_{me}$XOS fermentations (pH 5−7), first mainly acetate and lactate were detected, followed by propionate and small amounts of butyrate (0−43 h). The molar ratios of acetate, lactate, propionate, and butyrate analyzed at 43 h were 1:0.2:0.5:0.09 (FI 1), 1:1.3:0.45:0.06 (FI 2), 1:0.05:0.6:0.03 (FI 3), and 1:0.2:0.2:0.01 (FI 4). The production of butyrate increased during fermentation of $GlcA_{me}$XOS from 43 until 94 h (FI 1−4), whereas the amount of lactate decreased substantially.

DISCUSSION

Fermentation of the XOS resulted in a decrease of oligomers present and in the production of SCFA and lactate. In all fermentations the observed increase in cell material from bacterial growth corresponded well with the decrease in total amount of XOS present (not shown). The type of bacteria grown were not analyzed, because the aim of our study was to compare the fermentation of differently substituted XOS mainly on the basis of degradation patterns and changes in structural features of the XOS tested rather than to perform a complete microbiological study.

During the first 20 h of the fermentations a different degradation pattern for the four XOS mixtures was observed. The nXOS and AXOS were fermented more rapidly than the $GlcA_{me}$XOS and AcXOS. However, after an adaptation time, the $GlcA_{me}$XOS were fermented at the same rate as the nXOS and AXOS.

The fact that the fermentation was not pH controlled may have influenced bacterial action. However, the pH after fermentation times of 20, 20, 27, and 43 h of the nXOS, AXOS, AcXOS, and $GlcA_{me}$XOS, respectively (pH 5−7), was still adequate to allow bacteria to grow. To look at the changes in structure of substituted XOS during fermentation, only results obtained of XOS samples having a pH >5 were used.

Studying the fermentation of AcXOS and $GlcA_{me}$XOS in more detail by MALDI-TOF MS, we concluded that the substituents were not easily degradable, because no masses were observed representing nonsubstituted XOS. Furthermore, it could be concluded that relatively low substituted AcXOS (DP 3 and DP >7) and $GlcA_{me}$XOS (DP >7) were fermented preferentially, resulting in an accumulation of relatively high substituted XOS (DP 5−7). This accumulation, caused by degradation of higher DPs into DP 5−7 and a lack of consumption of DP 5 and 6, most likely points to the conclusion that the substituents present delayed or completely hindered fermentation. The 4-*O*-methylglucuronic acid plus hexose containing XOS were fermented very poorly. During fermentation of all substituted XOS (partial) release of the substituents was immediately

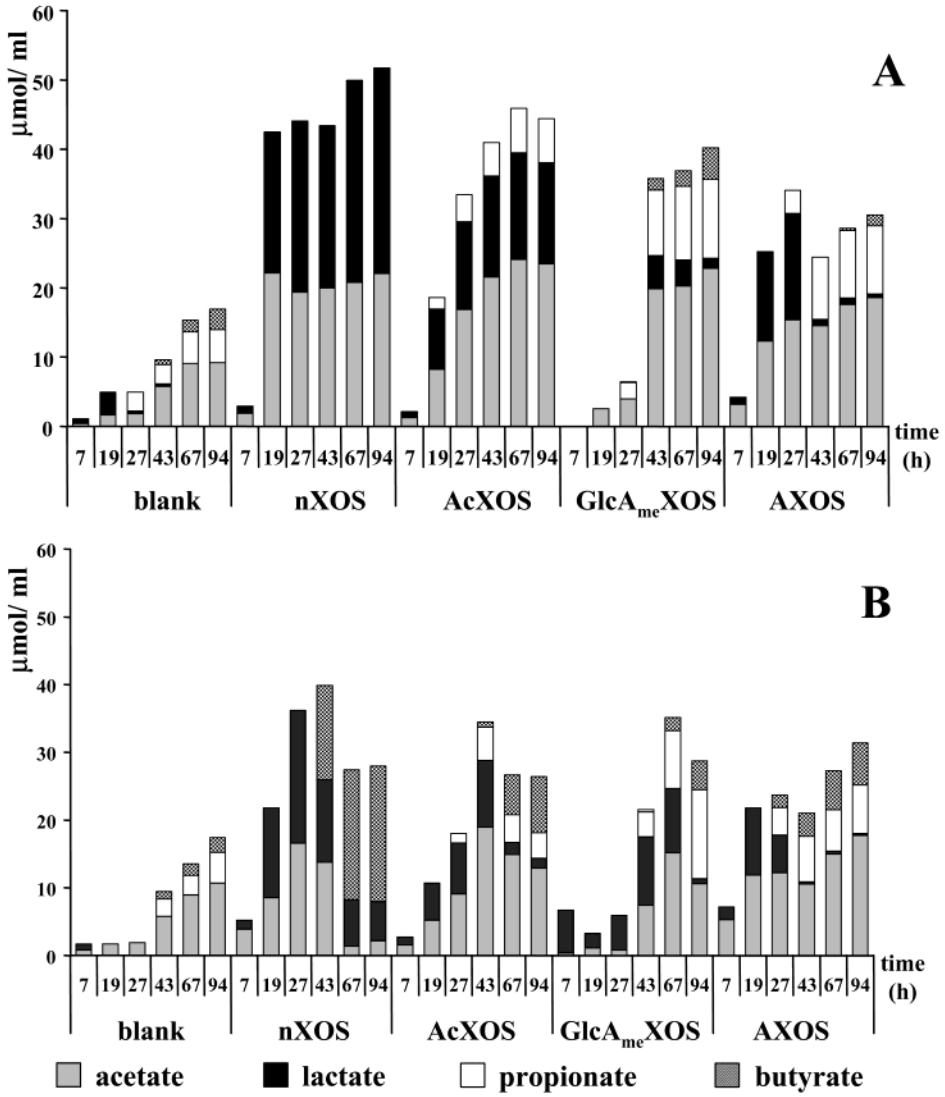

Figure 5. Production of SCFA and lactate during 94 h of fermentation of a blank, nXOS, AcXOS, GlcA$_{me}$XOS, and AXOS [FI 1 (A) and FI 2(B)].

followed by rapid fermentation of the remaining xyloses. Similar observations were described by Englyst et al. (*22*) for the fermentation of arabinose side chains of pectin, xylan, and arabinogalactan by mixed populations of human fecal bacteria. These results might point to the suggestion that the kind and amount of substituents per oligomer present influence the rate of fermentation.

SCFA and lactate were formed during the fermentations performed, and the total amount of these fermentation products increased with the consumption of total XOS. The results presented in **Figure 5** showed that the total concentration of acids produced in the medium without carbohydrate source (fermentation blank) was <20 μmol/mL, indicating that most acids were produced from the XOS as substrates. A very low production of acids in the blanks by a mix of human FI was also indicated by Hartemink et al. (*23*) as well.

For all fermentations described a distinction could be made between the first stage of the fermentation (0−40 h) and a second stage (>40 h). In the first stage of the fermentations the pH decreased, whereas in the second stage the pH remained constant or even increased slightly. In the first stage of the nXOS and AXOS fermentations, mainly acetate and lactate were formed. Lactic acid bacteria (e.g., *Lactobacillus* and *Enterococcus* species) and *Bifidobacterium* spp. may play an important role in this part of the fermentation, as they do not produce butyrate or propionate but they do produce acetate and lactate (*24*). A high concentration of acids formed might be desirable because, by a decrease in pH, the growth of potentially pathogenic microorganisms and the growth of putrefactive bacteria will be inhibited (*7*, *24*, *25*). The preference for bifidobacteria to ferment low-substituted XOS, both in vitro and in vivo, has been described previously (*1*, *4*). Contrarily, oat XOS were not selective for bifidobacteria exclusively, because *Bacteroides* spp., *Clostridium* spp., *Lactobacillus acidophilus*, and *Klebsiella pneumoniae* also showed moderate growth on these substrates (*2*, *26*). Also, the more branched wheat arabinoxylan hydrolysates (singly and doubly substituted arabinoxylo-oligosaccharides) could only be (partly) fermented by the *Bifidobacterium* spp. and *Bacteroides* spp. tested (*2*). The latter observations corresponded well with our results that in the first stage of the fermentation of AcXOS and GlcAmeXOS in addition to acetate and lactate also propionate and some butyrate were formed. This is most likely due to the growth of several intestinal bacteria and not specifically of lactic acid bacteria.

In the second stage of all fermentations, in which less carbohydrate degradation was observed, also propionate and butyrate was produced. Butyrate is reported to be related to antitumor effects in vitro and in animal studies of colon cancer (*27*−*30*). Butyrate was detected in the fermentations of nXOS, AXOS, and GlcA$_{me}$XOS by almost all FI but was hardly

detected during AcXOS fermentation. However, butyrate was observed mainly when all XOS were already degraded. Butyrate can be produced directly from carbohydrates by many different intestinal species, especially clostridia. Furthermore, some *Clostridium* species can metabolize lactate to butyrate, carbon dioxide, and water (*24*). Therefore, because in most of the XOS fermentations lactate decreased in favor of butyrate and all XOS had already been degraded, substantial secondary fermentation through lactate is expected to have occurred.

In conclusion, the nXOS, AXOS, AcXOS, and $GlcA_{me}XOS$, obtained from hydrothermally treated xylan-rich byproducts, were fermented by human FI. Both the oligosaccharide degradation patterns of the XOS and the patterns in SCFA and lactate formed depended on the particular structure of the XOS studied. Although this study aimed to reveal differences in structure in relation to fermentation, it was also demonstrated that interperson variation occurred. Our results suggest that it is useful to correlate detailed structural features of NDOs with their behavior in fermentation studies to be able to better understand and control the mechanisms involved.

ABBREVIATIONS USED

AcXOS, acetylated xylo-oligosaccharides; AX, arabinoxylan; AXOS, arabino-xylo-oligosaccharides; DP, degree of polymerization; $GlcA_{me}XOS$, 4-*O*-methylglucurono-XOS; HPAEC, high-performance anion-exchange chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MW, molecular weight; NDO, nondigestible oligosaccharides; nXOS, linear xylo-oligosaccharides; RI, refractive index; SCFA, short-chain fatty acids; XOS, xylo-oligosaccharides.

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