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A combined metabolomic and phylogenetic study reveals putatively prebiotic effects of high molecular weight arabino-oligosaccharides when assessed by *in vitro* fermentation in bacterial communities derived from humans

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ARTICLE INFO

ABSTRACT

Prebiotic oligosaccharides are defined by their selective stimulation of growth and/or activity of bacteria in the digestive system in ways claimed to be beneficial for health. However, apart from the short chain fatty acids, little is known about bacterial metabolites created by fermentation of prebiotics, and the significance of the size of the oligosaccharides remains largely unstudied.

By *in vitro* fermentations in human fecal microbial communities (derived from six different individuals), we studied the effects of high-mass (HA, >1 kDa), low-mass (LA, <1 kDa) and mixed (BA) sugar beet arabino-oligosaccharides (AOS) as carbohydrate sources. Fructo-oligosaccharides (FOS) were included as reference. The changes in bacterial communities and the metabolites produced in response to incubation with the different carbohydrates were analyzed by quantitative PCR (qPCR) and Liquid Chromatography–Mass Spectrometry (LC–MS), respectively.

All tested carbohydrate sources resulted in a significant increase of *Bifidobacterium* spp. between 1.79 fold (HA) and 1.64 fold (FOS) in the microbial populations after fermentation, and LC–MS analysis suggested that the bifidobacteria contributed to decomposition of the arabino-oligosaccharide structures, most pronounced in the HA fraction, resulting in release of the essential amino acid phenylalanine. Abundance of *Lactobacillus* spp. correlated with the presence of a compound, most likely a flavonoid, indicating that lactobacilli contribute to release of such health-promoting substances from plant structures.

Additionally, the combination of qPCR and LC–MS revealed a number of other putative interactions between intestinal microbes and the oligosaccharides, which contributes to the understanding of the mechanisms behind prebiotic impact on human health.

1. Introduction

The human gastrointestinal system produces a large amount of enzymes capable of catalyzing the hydrolysis of various disaccharides and a few specific polysaccharides (starches). However, most complex oligo- and polysaccharides including e.g. arabinan, galactan, and fructo-oligosaccharides cannot be degraded by the human digestive enzymes. These substrates need to be metabolized by the very diverse ecosystem of bacteria inhabiting the human gut [1]. Some of these saccharides are prebiotics, defined as selectively fermented ingredients that result in specific beneficial changes in the composition and/or activity of the

gastrointestinal microbiota, thus conferring benefit(s) upon host health [2].

Inter-bacterial interactions, as well as interactions between bacteria and host are based on a variety of mechanisms. Biochemical messages can be sent e.g. by simple or complex abiotic molecules or by genetic sequences [3]. Previous studies of prebiotic degradation were mainly focused on bacterial production and epithelial absorption of short chain fatty acids (SCFA) [4–6]. A far more exhaustive approach is metabolomic footprinting of the bacterial exo-metabolome, that can be defined as the complete pool of molecules excreted by a bacterial community into the surroundings [7]. Compounds involved in signaling between bacteria or to the host will be present in the metabolomic footprint, and the metabolomics approach has previously been found useful in studies of the intestinal microbial ecosystem [8,9].

By *in vitro* fermentation in human fecal slurry, it was recently shown that long-chain arabino-oligosaccharides (AOS) derived from sugar beet pulp had a potentially better bifidogenic effect than shorter-chain AOS, and moreover that the bifidogenic effect of longer chain AOS was as good as the effect exerted by recognized prebiotic fructo-oligosaccharides that were used as control [10,11]. However, a more detailed evaluation of the microbial response is required to better interpret the overall mechanisms governing the changes in the microbial communities and their potential effects on the host. The present study was thus undertaken to provide a more detailed evaluation. We addressed whether *in vitro* fermentation of differently sized AOS molecules caused different changes in fecal bacterial communities isolated from six healthy humans. Additionally, the putatively prebiotic (health-promoting) effect of AOS was addressed using a metabolomics-based approach.

2. Materials and methods

2.1. Arabino-oligosaccharide (AOS) substrates

AOS substrates from sugar beet were obtained from Danisco A/S (Nakskov, Denmark). The AOS were derived from 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 [12]. The pulp was dried prior to extraction.

Separation of the base solution of arabino-oligosaccharides (BA) according to size was performed in a 200 mL stirred membrane reactor model 8200 (Millipore, Billerica, MA) equipped with a 1 kDa MWCO regenerated cellulose membrane (Millipore, Billerica, MA) connected to compressed nitrogen for flux regulation. Filtration was performed at room temperature. Filtration was performed at 3 bar until the retentate volume was 30% of the sample volume, and followed by diafiltration in one sample volume of deionized water. The permeates enriched in low molecular weight oligosaccharides (<1 kDa) were denoted LA and the retentate enriched in high molecular weight oligosaccharides (>1 kDa) was denoted HA. Free sugar content and monosaccharide composition was determined by acid hydrolysis and High Performance Anionic Exchange Chromatography (HPAEC) as described previously [11].

2.2. Size exclusion

High Performance 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 Corp., Sunnyvale, CA). Samples were separated on a Shodex SB-806HQGPCcolumn (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 used with a flow rate of 0.5 mL/min. Temperature was maintained at 40 °C. Data were collected and analyzed with the program Chromeleon 6.80 SP4 Build 2361 software (Dionex Corp., Sunnyvale, CA).

2.3. Removal of monosaccharides from the substrates

In order to reduce the amount of monosaccharides present in the AOS-based substrates, we carried out an initial bacterial fermentation using *Lactobacillus acidophilus* NCFM (ATCC 700396). This strain was kindly provided by Danisco A/S and chosen because we have previously mapped the metabolites consumed and produced by this strain [13], and because its genome sequence [14] does not contain the enzymes needed for AOS degradation.

L. acidophilus NCFM colonies were grown anaerobically at 37 °C overnight in MRS broth (Oxoid Ltd., Basingstoke, Hempshire, England) and diluted 10^7 fold into Semi Synthetic Medium (SSM) [15] containing 1% glucose. The cultures were incubated for 7 h, resulting in approximately $10^{5.8}$ CFU/ml of *L. acidophilus* NCFM, and subsequently further diluted 100 fold into SSM containing either 1% glucose (control), 20 g/L of BA, 20 g/L LA, or 20 g/L HA. After 24 h of anaerobic incubation with the bacteria removing non-arabinan monosaccharides from the media, the cultures were centrifuged at 3000g for 5 min at 4 °C, where after supernatants were sterile filtrated in order to remove remaining *L. acipdophilus* NCFM cells and kept in at 4 °C until further use. Final concentrations of the AOS were calculated to be approximately 10 g/L.

2.4. Subjects and fecal sample collection

Fecal samples were collected from six healthy volunteers (four women and two men). None of the participants had been treated with antibiotics for at least 3 months before enrolment and had no history of gastrointestinal disorder. The mean age of the participants was 41 ± 9 years. The samples were collected in airtight containers at home by the participants and stored at 4 °C (limited storage time was encouraged [16]) until delivery to the laboratory, where the samples were processed immediately. The fecal samples were homogenized in 50% glycerol (1:1 dilution) in an anaerobic cabinet (Macs Work Station, Don Whitley) containing 10% H_2, 10% CO_2, and 80% N_2, and stored at -80 °C until further analysis, as described below.

2.5. In vitro fermentation by human fecal bacterial communities

Fermentation studies were carried out to assess the effect of BA, LA and HA on the microbial composition and activity in human fecal samples, while parallel incubations with the established bifidogenic substrate fructo-oligosaccharide FOS (BENEO-Orafti, Tienen, Belgium) and no carbohydrates, respectively, were used as references. Fecal samples prepared as described above were defrosted in an anaerobic cabinet and 10% (w/v) fecal slurry was prepared by mixing the samples with anoxic PBS (Oxoid, Greve, Denmark) immediately before fermentation.

Sterile SSM supernatants prepared as described above were mixed 1:1 with sterile minimal basal medium containing 2 g/L of peptone water (Oxoid Ltd., Basingstoke, Hempshire, England), 1 g/L of yeast extract (Sigma Chemical co., St. Louis, Missouri, USA), 0.1 g/L of NaCl (Merck KGaA, Darmstadt, Germany), 0.04 g/L of K_2HPO_4 (Merck KGaA, Darmstadt, Germany), 0.04 g/L of KH_2PO_4 (Merck KGaA, Darmstadt, Germany), 0.01 g/L of $MgSO_4 7H_2O$ (Merck KGaA, Darmstadt, Germany), 0.01 g/L of $CaCl_2 2H_2O$ (Merck KGaA, Darmstadt, Germany), 2 g/L of $NaHCO_3$ (Merck KGaA, Darmstadt, Germany), 0.5 g/L of L-cysteine hydrochloride (Sigma Chemical co., St. Louis, Missouri, USA), 50 mg/L of hemin (Sigma Chemical co., St.

Louis, Missouri, USA), 10 μl/L of vitamin K1 (Sigma Chemical co., St. Louis, Missouri, USA), 0.05 g/L manganese sulfate monohydrate (Merck KGaA, Darmstadt, Germany) and 1 mL/L of Tween 80 (VWR, Darmstadt, Germany). The pH of the final solution was adjusted to 7. Estimated concentrations of AOS in the medium were approximately 5 g/L. Positive controls were made by adding 5 g/L of FOS to the SSM supernatant prepared by NCFM fermentation of glucose, and negative controls by adding nothing to the same SSM supernatant. All solutions were reduced over night in an anaerobic cabinet and inoculated with fecal slurry prepared as described above to a final concentration of 1% w/v feces. Tube caps were loosely placed on the vials to allow gas exchange but minimize evaporation. Each fermentation was carried out in triplicates for each fecal community, carbohydrate source and controls. The fermentation was non-pH controlled and non-stirred due to the low reaction volume (6–7 mL) and was carried out in an anaerobic cabinet at 37 °C. At the beginning of the fermentation (time 0), and after 24 hours (time 24), 1 ml samples were taken and centrifuged at 3000g for 15 min at 4 °C. The supernatants were used for metabolite profiling and the pellets were used for extraction of bacterial DNA as described below.

2.6. Extraction of bacterial DNA

DNA was extracted from each of the triplicate fermentation samples using the QIAamp DNA Stool mini kit (Qiagen, Hilden, Germany) with preceding bead beating as previously described [17]. The concentration of the purified DNA was measured by Qubit® 2.0 Fluorometer (Invitrogen) and the DNA was stored at −20 °C until use.

2.7. Real-time quantitative PCR (qPCR) assay

Amplification and detection of purified bacterial DNA by qPCR was performed with the ABI Prism 7900 HT from Applied Biosystems using optical grade 384-well plates. Each amplification reaction was done in duplicate for each of the triplicate fermentation samples in a final volume of 11 μl containing; 5.50 μl SYBR® Green Master Mix (Applied Biosystems, Denmark), 200 nM of each of the primers (Eurofins MWG Synthesis GmbH, Ebersberg, Germany), 2 μl template DNA (1 ng/μL), and Nuclease-free water

purified for PCR (Qiagen). The amplification program comprised one cycle at 50 °C for 2 min; one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s; 60 °C for 1 min, and finally one cycle of melting curve analysis for amplicon specificity at 95 °C for 15 s, 60 °C for 20 s and increasing ramp rate by 1.92 °C/min until 95 °C for 15 s. The qPCR data was baseline corrected and N_0-values, representing initial concentrations of the specified 16S rRNA genes, were calculated using the LinRegPCR software (version 11.1, based on Ruijter et al. [18]). All results were calculated as means of duplicate N_0 estimations, equal values required. The relative quantities of gene targets encoding 16S rRNA sequences of the bacterial taxa were calculated using N_0 (bacterial target)/N_0 (total bacterial population). The applied specific 16S rRNA-targeting primers are listed in Table S1 (supplementary data). Prior to quantification, all primers were tested to confirm sensitivity and specificity using DNA from pure bacterial species [19].

2.8. Metabolism quenching

Time 0 and time 24 h supernatants from the fermentations were quickly quenched by transferring into cold methanol (Fluka, Sigma-Aldrich, Steinheim, Germany, stored at −80 °C) in the ratio 1:1 to stop the metabolism. Samples were stored at −80 °C and centrifuged at 15,000g for 5 min at 4 °C just before LC−MS (Liquid Chromatography−Mass Spectrometry) analysis was carried out as described below.

2.9. Metabolite detection by LC−MS

Metabolite profiling was done on a Dionex Ultimate 3000 RS liquid chromatograph (Dionex, Germering, Germany) coupled to a Bruker maXis time of flight mass spectrometer equipped with an electrospray ionsource (ESI-Qtof-MS) (Bruker Daltonics, Bremen, Germany). Analytes were separated on a Kinetex pentafluorophenyl column 50 × 2.10 mm, 2.6 μm, 100Å (Phenomenex, USA), using a binary solvent system: 10 mM ammonium formate at pH 3.5 in water (A), and acetonitrile (B). This column was chosen over the typical C-18 columns due to its ability to provide better separation of polar compounds. Solvent programming was: isocratic 0% B at 0 min followed by a linear gradient up to 100% B in 7 min retaining 100% B for 8 min. Flow rate was 0.25 mL/min

Table 1
Relative fold change of bacteria target from samples incubated with either BA, LA, HA or FOS compared to the NC samples (set to 1).

ID	Bacterial taxa	Substrates			
		BA	LA	HA	FOS
F	Firmicutes	0.80 (±0.07)	0.78 (±0.07)	0.80 (±0.08)	0.96 (±0.07)
Cc	• C. coccoides group	0.59 (±0.11)	0.54 (±0.16)*	0.68 (±0.09)	0.85 (±0.11)
Rs	○ Roseburia spp.	1.18 (±0.09)	1.18 (±0.10)	1.07 (±0.12)	1.16 (±0.17)
Cl	• C. leptum subgroup	0.97 (±0.15)	0.86 (±0.17)	0.85 (±0.11)	0.94 (±0.11)
Ls	• Lactobacillus spp.	1.17 (±0.12)	1.10 (±0.13)	1.11 (±0.09)	1.14 (±0.11)
B	Bacteroidetes	0.90 (±0.13)	0.90 (±0.10)	0.80 (±0.13)	0.80 (±0.09)
Bs	• Bacteroides spp.	0.82 (±0.15)	0.80 (±0.12)	0.74 (±0.13)	0.70 (±0.14)
Bf	○ Bac. fragilis group	0.92 (±0.21)	0.91 (±0.10)	0.85 (±0.21)	0.78 (±0.14)
Ps	• Prevotella spp.	0.96 (±0.13)	0.93 (±0.10)	0.97 (±0.08)	0.99 (±0.10)
As	• Alistipes spp.	0.80 (±0.07)	0.74 (±0.06)*	0.77 (±0.06)	0.71 (±0.04)*
	Actinobacteria				
Bis	• Bifidobacterium spp.	1.72 (±0.28)**	1.72 (±0.26)***	1.79 (±0.24)***	1.64 (±0.22)**
	Verrucomicrobia				
Am	• Akk. muciniphila	1.26 (±0.09)	1.28 (±0.09)	0.80 (±0.13)	0.96 (±0.04)
	Proteobacteria				
E	• Enterobacteriaceae	0.90 (±0.09)	1.14 (±0.09)	1.10 (±0.10)	0.94 (±0.25)
Ds	• Desulfovibrio spp.	0.81 (±0.05)	0.80 (±0.04)	0.66 (±0.05)**	0.74 (±0.07)*

All calculated data are means ± SEM of the six fecal communities. Asterisks designate a significant difference from samples taken after 24 h of incubation without any added carbon-source (NC samples) ($P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***)). No significant difference in the relative density of bacterial taxa after fermentation was obtained when comparing the four substrates.

Table 2
Annotated metabolites from LC–MS analysis in positive mode found to separate fermentation samples according to carbohydrate source (Fig. 1).

Number	RT [min]	M_{LCMS} [Da]	Adduct	M_{REF} [Da]	Error [mDa]	M_{MSMS}	Metabolite candidate
1	1.7	166.086493	M+H	166.086255	0.2	120.081723	Phenylalanine
2	0.8	188.175739	M+H	188.175738	0.0	171.150205	N′-Acetylspermidine
						128.071353	
						117.103928	
						100.077105	
						64.024444	
3	0.8	104.106928	M+NH₄	104.106988	0.1	NF	Iso-Valeraldehyde
4	1.5	121.062386	M+NH₄	121.060766	1.6	103.054712	3-Oxoalanine
						93.069976	
						57.993688	
5	1.1	138.091331	M+H	138.091340	0.0	121.064816	Tyramine
						93.070169	
6	0.9	139.050163	M+NH₄	139.050200	0.0	121.039480	Cysteine
7	0.7	140.068238	M+Na	140.068197	0.1	96.078471	Betaine
	0.7	156.042329	M+K	156.042137	0.2	NF	5-Aminopentanoic acid
	0.7	257.147484	2M+Na	257.147175	0.3	140.069251	N-Methyl-a-aminoisobutyric acid
							Valine
							Norvaline
8	1.3	155.081504	M+NH₄	155.0815	0.0	NF	Aminobenzoic acid
9	1.0	137.045787	M+H	137.045787	0.0	NF	Hypoxanthine
10	6.6	282.279062	M+H	282.279141	0.0	265.253039	Oleamide
						247.245066	
						149.024021	
						142.122957	
						114.091440	
						111.117935	
						70.272614	
11	6.3	321.240053	M+Na	321.240013	0.0	NF	3-Oxooctadecanoic acid
12	6.1	323.255570	M+Na	323.255663	0.1	NF	(R)-3-Hydroxy-octadecanoic acid

RT – retention time on the chromatogram; MLCMS – *m/z* (mass to charge) ratio measured; MREF – *m/z* ratio of metabolites given in the referral data bases; Error – difference between MLCMS and MREF; MMSMS – *m/z* ratio of the fragment ions after MSMS; NF – no fragmentation or fragments not visible in the given MS settings.

at 0 min, and increased to 0.4 mL/min at 7 min. Solvent composition and flow were returned to initial conditions at 8.2 min. The total runtime was 10 min, the oven temperature was 40 °C, and injection volumes were 1 μL. The following key mass spec settings were used: Nebulizer pressure 2 bar, drying gas 10 L/min, 200 °C, capillary voltage 4000 V. Data collection range was from 50 to 800 Da/e at 1 Hz. Samples were analyzed in both positive and negative mode. External and internal calibration was done using sodium formate clusters (Sigma–Aldrich, Steinheim, Germany). Lock-mass calibration using hexakis(1H,1H,2H-perfluoroetoxy)-phosphazene, (Apollo Scientific, Stockport, UK) was applied in order to increase mass accuracy. MSMS fragmentation of the selected masses (Tables 2 and 3) was done using nitrogen as collision gas with collision energy at 14 eV at

Table 3
Annotated metabolites from LC–MS analysis in negative mode found to separate fermentation samples according to carbohydrate source (Fig. 2).

Number	RT [min]	M_{LCMS} [Da]	Adduct	M_{REF} [Da]	Error [mDa]	M_{MSMS}	Metabolite candidate
13	2.9	195.066074	M−H	195.066283	0.2	151.094347	Homoveratric acid
						136.065370	
						121.041572	
14	0.8	133.070545				NF	Fatty acid derivative
15	3.3	457.135071				397.114107	Arabionofuranosyl structure
						367.102362	
						325.094322	
						265.075597	
						235.062259	
						193.052703	
16	3.2	263.073298	M−H	263.071400	1.9	191.074171	Flavonoid
						165.073565	
17	6.3	297.243324	M−H	297.243530	0.2	NF	3-Oxooctadecanoic acid
18	1.0	151.026420	M−H	151.026149	0.2	108.020397	Xanthine
19	3.2	165.055711	M−H	165.055718	0.0	147.048091	Phenyllactic acid
						119.052135	
						103.059652	
20	0.6	131.071452	M-H	131.071368	0.0	NF	Fatty acid derivative
21	6.6	279.233124	M−H	279.232954	0.1	NF	Linoleic acid
							Derivative of linoleic acid
22	0.9	175.049132	M−H	175.047272	1.9	157.036324	Allantoic acid
						131.052535	
						113.039588	

RT – retention time on the chromatogram; MLCMS – *m/z* (mass to charge) ratio measured; MREF – *m/z* ratio of metabolites given in the referral data bases; Error – difference between MLCMS and MREF; MMSMS – *m/z* ratio of the fragment ions after MSMS; NF – no fragmentation or fragments not visible in the given MS settings.
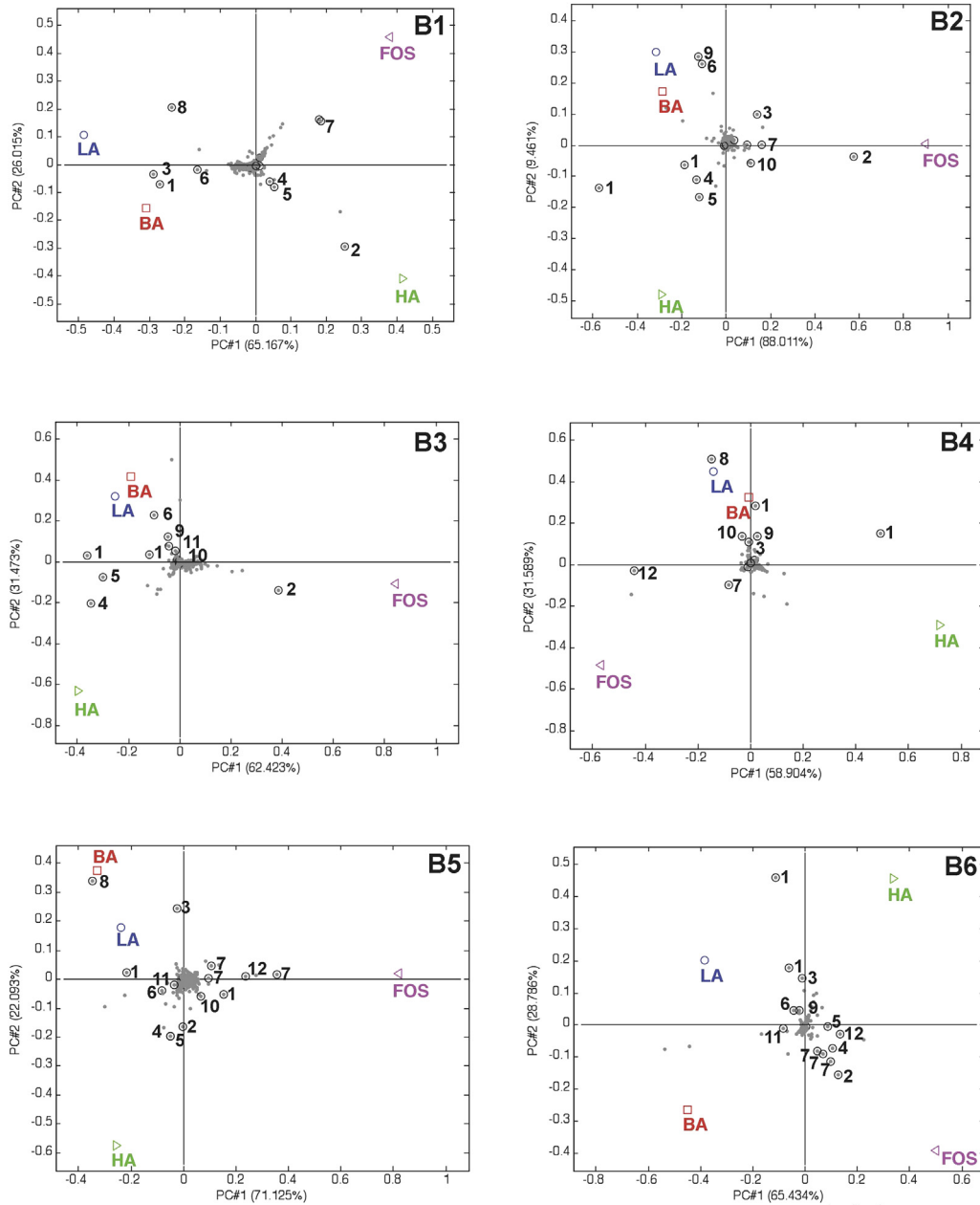
Fig. 1. PCA bi-plots (scores and loadings present on the same plot) of the fermentation samples with different arabino-oligosaccharides BA, LA and HA and the established prebiotic FOS. Data from LC−MS analyses in positive mode for tested bacterial floras separately (B1−B6). Numbers refer to metabolite candidates as listed in Table 2. Phenylalanine (1) was observed on the border of the 1 min and 2 min bucket, which is why PCA plots are showing metabolite no. 1 twice. LC−MS chromatogram studies (data not shown) confirmed that it was indeed the same metabolite.

m/z 100 ramped linearly to 20 eV at m/z 500 and to 30 eV at m/z 1000.

2.10. Metabolite identification

A putative metabolite identification was based on the exact mass to charge ratio (m/z) with a very low measurement error, and MSMS fragments (Tables 2 and 3) using metabolite data from the Human Metabolome Database (HMDB [20]) and the Metabolite Mass Spectral Database (METLIN [21]) where MSMS spectra of selected metabolites are presented, and references to the bacterial metabolism presented in the discussion part of this paper. Metabolites that could not be identified reliably but showed some

significance were included in the further data analyses as unknown metabolites given a number.

2.11. Statistical analysis of the PCR data

Statistical analysis of the qPCR data was performed with the GraphPad Prism software (version 5.03; GraphPad Software Inc., La Jolla, CA). One-way analysis of variance and Tukey's multiple comparison tests were used to determine significant differences in the density of selected bacterial taxa in the different fermentations (NC, FOS, BA, LA and HA). Homogeneity of variance was assessed using Bartlett's test for equal variances. Log-transformations were performed before statistical analysis of qPCR measurements that did not meet this criterion. The nonparametric Kruskal−Wallis test
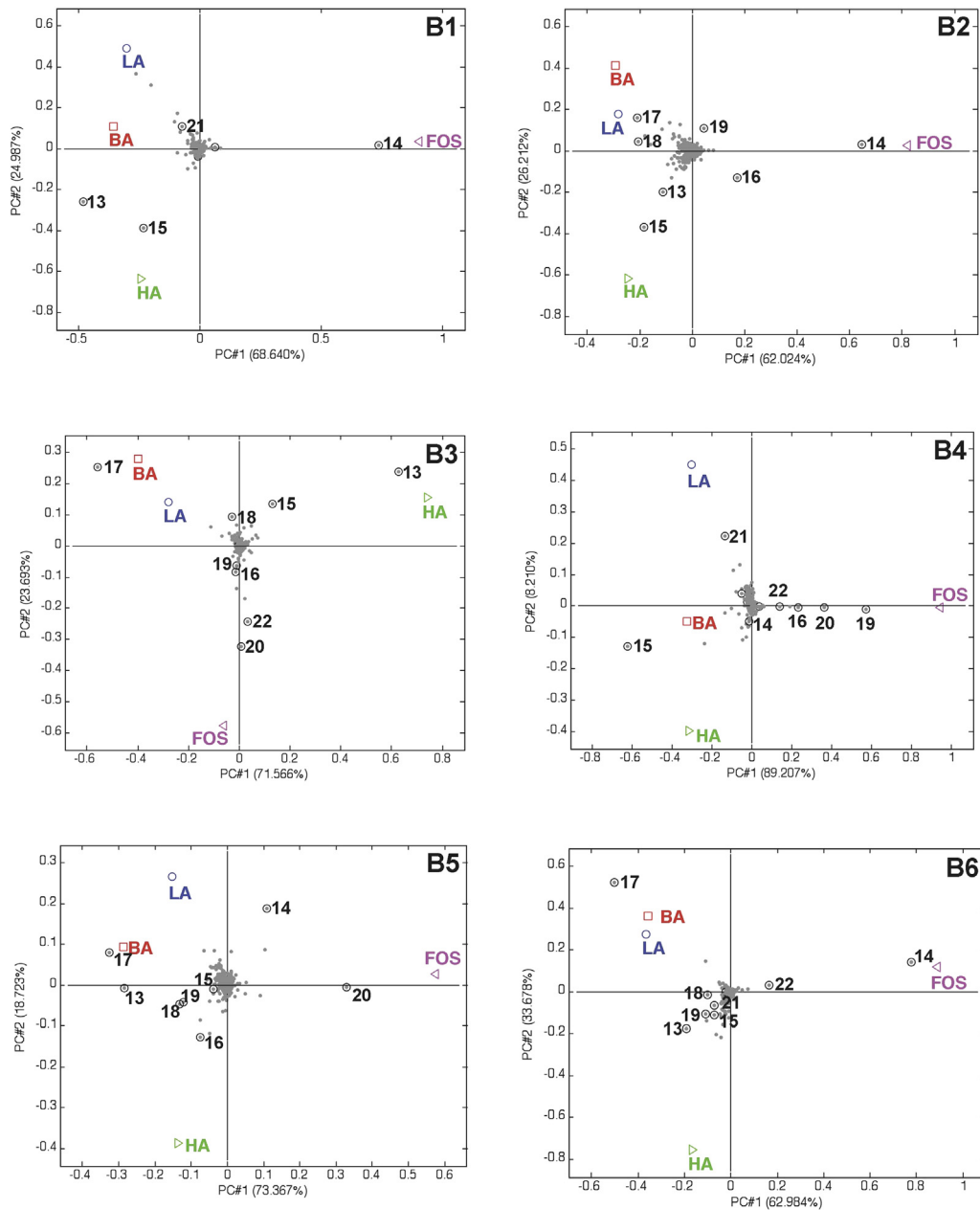
Fig. 2. PCA bi-plots (scores and loadings present on the same plot) of the fermentation samples with different arabino-oligosaccharides BA, LA and HA and the established prebiotic FOS. Data from LC–MS analyses in negative mode for tested bacterial floras separately (B1–B6). Numbers refer to metabolite candidates as listed in Table 3.

and Dunn's multiple comparison tests were used for datasets, which did not have homogeneity of variance even after log-transformation. Tests were considered statistically significant when P-values lower than 0.05 were obtained.

2.12. Principal component analysis (PCA)

The basic data analysis of the LC–MS data were based on the bucketing approach: the centroid data were grouped into buckets of 1 min and 1 m/z differences in the range from 0.5 to 9 min and 50–800 m/z and normalized by the sum of buckets in the analysis by use of Profile Analysis 2.0 (Brucker Daltonics, Bremen, Germany). The typical number of buckets was 6750 per sample. Each bucket was considered as a variable independently of whether the content

of the bucket could be attributed to a specific metabolite or not. All variables were exported to Excel for further data analyses.

The next step of the data analysis was done in Excel, using a set of criteria, presented below, to select metabolites present in the medium after 24 h and produced exclusively due to the addition of the given carbohydrate:

First, the metabolites which were present already before the fermentation (CH_0), were subtracted from the metabolites present after 24 h of fermentation (CH_{24}), and the remaining metabolites were represented as an average value CH(A) of intensities of the given bucket in three independent fermentations (I, II and III):

$$CH_{24}(I) - CH_0(I) = CH(I); \text{ if } CH(I) \leq 0, \text{ then } CH(I) = 0;$$
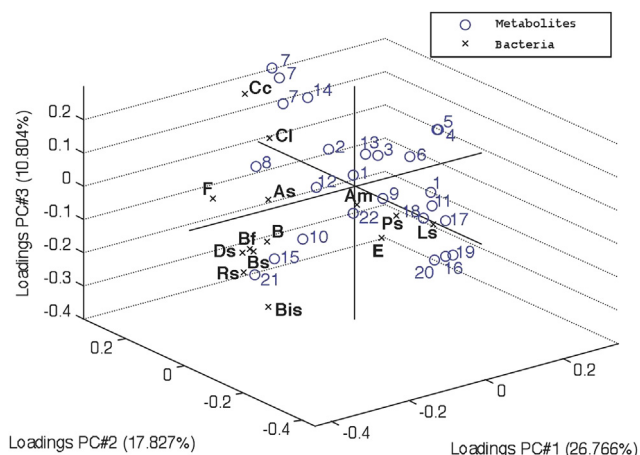$$(CH(I) + CH(II) + CH(III))/3 = CH(A)$$

Fig. 3. PCA loading plot of selected metabolites (numbers corresponding to Tabels 2 and 3) combined with microbial abundance data (Abbreviations corresponding to Table 1).

Secondly, the average amount of specific metabolites NC(A) produced only due to metabolism of the basal medium were identified by analysis of the control incubations (NC) carried out without addition of a carbohydrate source:

$NC_{24}(I)-NC_0(I) = NC(I)$; if $NC(I) \leq 0$, then $NC(I) = 0$;
$(NC(I) + NC(II) + NC(III))/3 = NC(A)$

Finally, the metabolite values M(A) to be included in the Principal Component Analysis (PCA) were calculated as: $CH(A)-NC(A) = M(A)$; If $M(A) \leq 0$, then $M(A) = 0$.

P-values describing differences between the experiments carried out with different substrates were calculated by *t*-test. PCA plots made in LatentiX 2.10 (Latent 5, Copenhagen, Denmark) were based on the metabolite buckets showing significant differences between the tested carbohydrate types (*P*-value < 0.05; data not shown). Data were only mean centered (to allow the higher intensity peaks/buckets to have a greater influence on the model) and normalized (2-norm). From the positive mode, due to the interference from TWEEN in the medium (which was necessary for growth of lactobacilli), all *m/z* buckets at 3 min, 4 min and 7 min were removed. From the negative mode buckets with mass 555.5, 665.5, 666.5 (lock mass), 187.5, 188.5 and 189.5 at all time buckets were disregarded due to the noise they were creating.

Selected metabolites from previous PCA plots were used together with the PCR data (before log-transformation) to create a PCA plot (Fig. 3) in LatentiX. Data was autoscaled and normalized (2-norm).

2.13. Statistical analysis of the LC–MS data

Heat maps were created to illustrate the *P*-values of differences between levels of selected metabolites present before and after fermentation, taking into consideration only metabolites which increased during fermentation. *P*-values were calculated using *t*-test in Excel. Data used in this analysis were normalized by the sum of buckets by use of Profile Analysis 2.0.

3. Results

3.1. Oligosaccharide composition

Size exclusion chromatography (Fig. S1) showed that the base solution of arabino-oligosaccharides (BA) had a dual distribution

with one peak at 24.7 min corresponding to monomers and a larger broader peak around 1.0 kDa corresponding to the oligosaccharides. The low molecular weight fraction (LA) showed a similar profile, but with a tendency towards a lower content of oligosaccharides below 1.0 kDa. The high molecular weight fraction (HA) showed one homogeneous peak around 1.0 kDa with only a minor peak at 24.7 min indicating that the content of monosaccharides was significantly reduced, but not removed completely. The monosaccharides in the BA fraction constituted about 30% of the dry matter and were mainly glucose (13.3% w/w), arabinose (10.1% w/w) and fructose (8.0% w/w). Other pectin derived free sugars like rhamnose, galacturonic acid, galactose, and fructose were found in small amounts (2.3% w/w in total). Acid hydrolysis revealed that 66.3% of the substrate was comprised of arabino-oligosaccharide moieties. The relatively high content of monosaccharides compared to similar substrates [11] might be due to the drying of the pulp prior to the acidic extraction. LC–MS analysis of the BA fraction revealed several peaks besides those identified by comparison to linear AOS, which indicated that, in accordance with previous data obtained for sugar beet derived AOS [11,22] the AOS applied in this study contained a mixture of linear and branched AOS (data not shown). Initial fermentation with *L acidophilus* NCFM removed basically all of the non-arabinan monosaccharides present in the AOS fractions, as detected by LC–MS (data not shown).

3.2. Quantitative PCR studies of bacterial community composition

After fermentation in fecal slurries obtained from six different healthy subjects, quantitative PCR was applied to measure the density of gene targets encoding 16S rRNA of selected bacterial taxonomic units. The ability of the substrates to selectively stimulate the growth of a given bacterial taxon was compared to that of the NC (No added Carbohydrate) fermentations (Table 1 and Figure S2). The fecal communities fermented on BA, LA and HA selectively increased the relative abundance of *Bifidobacterium* spp. significantly (*P* < 0.01, *P* < 0.001, and *P* < 0.001, respectively) as compared to the NC fermentations. The densities of bifidobacteria after fermentation of BA, LA and HA were not significantly different from the densities obtained by fermentation of FOS, which has a well established prebiotic effect [23]. Also the densities of *Lactobacillus* spp. appeared to be higher in the BA, LA, HA and FOS fermentations than in the NC samples, although this was not statistically significant (*P* = 0.18, *P* = 0.21, *P* = 0.16 and *P* = 0.23, respectively). The relative abundances of the *C. coccoides* group and *Alistipes* spp. were significantly lower in fecal communities fermented on LA than in the NC fermentations (*P* < 0.05 and *P* < 0.05, respectively). FOS fermentation resulted in a significantly lower relative abundance of *Alistipes* spp. and *Desulfovibrio* spp. than the NC fermentations (*P* < 0.05 and *P* < 0.05, respectively). A significantly lower density of *Desulfovibrio* spp. was additionally observed in the fecal communities fermented on HA (*P* < 0.01). No statistical difference in bacterial density after fermentation of the four different substrates was seen for the remaining investigated bacterial taxa.

In line with previous studies [11,24], major individual differences between intestinal bacterial ecosystems derived from different subjects were observed. The density of 16S rRNA genes of four different bacterial taxonomic units, showing alteration after fermentation (*Bifidobacterium* spp., *C. coccoides* group, *Alistipes* spp. and *Desulfovibrio* spp.), were thus determined for each of the six individual fecal communities (Table S2). Substantial individual differences were observed depending on substrate and bacterial target. However, fermentations in all six intestinal communities resulted in significantly higher increases of bifidobacteria than measured in the NC samples. When comparing the increase caused

by the three substrates (BA, LA and HA), it varied significantly among the six fecal communities. FOS fermentation resulted in significantly lower fold changes of bifidobacteria in three out of the six fecal communities than seen for the AOS based substrates. Only one fecal community (derived from individual 2) showed no significant difference in bifidobacterial increase caused by the tested substrates. The densities of the *C. coccoides* group were either unaltered or significantly lower after fermentation of the four substrates. However, fermentation of FOS generally caused a smaller decrease of *C. coccoides* than observed for BA, LA and HA. In all six bacterial communities, densities of *Alistipes* spp. and *Desulfovibrio* spp. were either unaltered or significantly lower after fermentation of the four substrates, however the extent of the reduction varied between communities.

3.3. Metabolomic studies

After quenching the metabolism, LC–MS analysis of samples taken before and after fermentation was performed using electrospray ionization in both positive and negative mode. After grouping of the LC–MS data in time-mass buckets, only buckets with significant differences between at least 2 substrates were included in the further analysis, which significantly reduced the number of variables (metabolites). PCA analysis of the reduced metabolite (bucket) profile data (Figs. S3 and S4) from positive mode did not show a clear clustering, indicating that none of the selected profile data explain a differentiation caused by the different fermentation substrates. However, in the negative mode, the selected profile data from FOS-fermentation samples clustered separately from all three types of AOS (Fig. S4). For each of the bacterial communities, we observed that BA and LA metabolite profiles were typically very similar to each other, but different from the profiles measured after fermentation of HA or FOS.

In order to identify effects of the fermentation substrates on the metabolite profiles and avoid masking caused by the differences between the individual bacterial communities, PCA analysis was carried out for each community separately and presented as bi-plots (Figs. 1 and 2). Based on this, metabolites that may contribute to the differences observed between samples fermented on the four substrates were selected as shown in Tables 2 and 3, and Fig. S5. As described above, the profiles obtained after BA and LA fermentation were typically quite similar to each other, but different from those from HA and FOS fermentation. This was also seen in the PCA plots based on the metabolite profiles from the individual bacterial communities. Based on the accurate mass data, phenylalanine (Fig. 1; No. 1; all individuals), xanthine (Fig. 2; No. 18; B2, B3, B5 and B6) and linoleic acid or its derivative (Fig. 2; No. 21; B1, B4 and B6) are likely candidates that contribute significantly to differentiate between AOS and FOS-fermented samples. In all six microbial communities, the metabolite corresponding to phenylalanine (1) was present in higher amounts in one or more of the AOS-fermented samples than in FOS-fermented samples (Fig. S5).

A metabolite corresponding to N′-acetylspermidine (Fig. 1; No. 2; B1, B2, B3, B5 and B6) was present in high levels in FOS and HA fermentation samples, but separated these from BA and LA fermentations, and although a significant increase of N′-acetylspermidine was observed in all bacterial communities after fermentation of one or more of the 4 oligosaccharides (Fig. S5), the highest increase of this metabolite candidate was observed in HA and FOS.

Oppositely, BA and LA fractions typically had a higher content of the metabolite candidates: cysteine (Fig. 1; No. 6; B1, B3, B5 and B6), aminobenzoic acid (Fig. 1; No. 8; B1, B4 and B5), hypoxanthine (Fig. 1; No. 9; B2, B3, B4 and B6) and 3-oxooctadecanoic acid (Fig. 1, No. 11; B3, B5, B6 and Fig. 2; No. 17; B2, B3, B5, B6).

HA fermentation fractions (but not FOS) were positively correlated to the presence of a metabolite corresponding to: 3-oxoalanine (Fig. 1; No. 4; B1, B2, B3, B5, B6), tyramine (Fig. 1; No. 5; B1, B2; B3; B5, B6), homoveratic acid (Fig. 2; No. 13; B1, B2; B3; B5, B6); and arabionofuranosyl structures (Fig. 2; No. 15; B1, B2, B3, B4 and B5).

Presence of the remaining metabolite candidates listed in Tables 2 and 3 depended highly on the bacterial community.

3.4. Combined data analysis of qPCR and LC–MS data

A PCA analysis was conducted for combined LC–MS and qPCR data. A loading plot combining selected metabolites (Tables 2 and 3) with all targeted bacteria taxa (Table 1) was created (Fig. 3) in order to reveal correlations between the presence of specific bacteria and specific metabolites.

The analysis indicated that abundance of the clostridial taxa (Cl and Cc), and a fatty acid derivative (14) was likely to co-occur. Additionally, a positive correlation was seen between the bacterial taxons *Bifidobacterium* spp. (Bis), *Roseburia* spp. (Rs), *Bacteroides* spp. (Bs) and the two metabolites corresponding to flavonoid C25H24O7 (15) and linoleic acid (21). *Lactobacillus* spp. (Ls) was seen to correlate with what could be 3-oxooctadecanoic acid (11 and 17), xanthine (18), flavonoid C15H14O3 (16), phenyllactic acid (19) and a fatty acid derivative (20). Interestingly, the outcomes also included a negative correlation between the possible metabolites: 3-oxooctadecanoic acid (11, 17) and (R)-3-hydroxy-octadecanoic acid (12) as well as between two different fatty acid derivatives (20 and14).

4. Discussion

Previous *in vitro* studies of sugar beet arabino-oligosaccharides (AOS) have shown their bifidogenic effect and influence on the gastrointestinal microbiota, and have indicated that the prebiotic potential of high molecular weight AOS may be slightly better than that of the shorter ones [10,11,24]. Our present results confirm that AOS, whether it was high molecular weight (HA, > 1 kDa), low molecular weight (LA < 1 kDa), or a mix of these (BA) selectively stimulated the growth of bifidobacterial species (Table 1 and S2), which are associated with positive effects on the host health [25–27]. One of the health promoting properties of bifidobacteria is known to be the production of conjugated linoleic acid [28], which is produced from linoleic acid available in the GIT, originating (among other sources) from consumed plant tissues. In line with this, a positive correlation between the linoleic acid (No. 21) and several bacterial taxa including bifidobacteria (Bis), was found in the present study (Fig. 3).

Metabolites corresponding to phenylalanine (1), xanthine (18), linoleic acid or its derivatives (21) (based on accurate mass data) were all produced by AOS fermentation to a larger extent than seen for FOS (Fig. S5). A possible source of phenylalanine is bacterial degradation of arabionofuranosyl structures, present in the AOS [11]. The HA fermentation fraction, which was enriched for high-mass carbohydrates, was also enriched in feruloylated AOS, and in line with this, arabionofuranosyl structures (15) were partly explaining the observed difference between HA and LA or BA, respectively (Fig. 2). Higher amounts of feruloylated AOS in the HA fraction may lead to a higher probability of the non-digestible carbohydrate to reach the distal colon, potentially preventing the accumulation of toxic by-products of proteolysis and amino acid fermentation, which typically takes place in the absence of carbohydrates [29,30]. Phenylalanine produced by decomposition of the feruloylated AOS by the intestinal microbiota is an essential amino acid, and acts as precursor for tyrosine, signaling molecules such as

dopamine, noradrenaline and adrenaline as well as skin pigment — melanin [20,31]. In addition to a higher content of arabinofuranosyl structures, the HA fermentation fraction had a higher content of metabolites corresponding to 3-oxoalanine (4), tyramine (5) and homoveratric acid (13) than seen in the other fractions. 3-oxoalanine is found as an oxidation product of cysteine or serine containing substrates in anaerobic conditions [32,33], while the metabolite candidate cysteine (6) was typically correlated to fermentations with LA and BA (Fig. 1), indicating a different metabolism (pathway or rate of turnover) of sulfate containing amino acids in the high-mass fraction. Tyramine is produced mainly by LAB, and is reported to cause allergies, migraine and heart failure when consumed in very high concentrations [34,35], however, the impact of production of this essential monoamine by intestinal bacteria is unstudied. Homoveratric acid may have an effect on eukaryotic endocrine metabolic pathways and is found in urine samples [36,37], plant cells [38] and microbial cells [39]. Considering the phenolic structure of homoveratric acid and tyramine, the increase may be attributed to bacterial degradation of ferulic structures present in the AOS fractions.

A metabolite corresponding to N′-acetylspermidine (as determined by accurate mass) was typically increased in HA as well as FOS fermentations. N′-acetylspermidine has significant roles in many biological systems found in mammals, plants and microbes [20]. Whether bacterially-produced N′-acetylspermidines affect epithelial cell growth and proliferation remains to be addressed.

Generally, we observed that BA and LA metabolite profiles were similar to each other, but different from the profiles measured after fermentation of HA or FOS. In fact, no major differences in the metabolic profiles were observed between the BA and LA fractions (Figs. 1 and 2 and S5). We speculate that intestinal bacteria were more prone to metabolize the carbohydrates with lower mass, which were present in rather high amounts in BA (Fig. S1). Metabolites typically correlated with the BA and LA fractions corresponded to cysteine (6) and aminobenzoic acid (8). Cysteine, which may arise from bacterial metabolism of plant structures [31], and contributes to the maintenance of anaerobic conditions by binding free oxygen [40], is known to increase the pH during fermentation by buffering the environment [41]. This may explain the higher number of *Desulfovibrio* spp. observed in the BA and LA fermentations (Table S2), as previous reports show that cysteine supports the growth of *Desulfovibrio* [42]. Aminobenzoic acid may originate from degradation of phytochemicals or ferulic structures by the microbial communities [31]

A significant increase of a putative flavonoid (16) was observed in five out of six subjects for all of the tested carbohydrates (Fig. S5). Flavonoids may be released by microbial fermentation of plant structures present in the fecal matter, and are shown to have a number of biological effects on the human body, including antioxidative ramification of polyphenolic compounds active against cancer, atherosclerosis and chronic inflammation [43,44]. They are known to inhibit and induce a large number of mammalian enzymes [43] involved in cell division, proliferation and detoxification [45]. Absorption of flavonoids from the diet was long considered to be negligible, as they are present in foods bound to sugars as β-glycosides [46]. However, it has now been shown that the final biological activity of flavonoids depends on the intestinal bacterial metabolism, which breaks the β-glycosidic bonds and leads to biotransformation of flavonoic compounds, thereby changing their bioactivity [47]. Many lactic acid bacteria (LAB) are able to break the β-glycosidic bonds, and the observed increase in the number of bifidobacteria observed for all fermentation substrates may have caused a higher amount of free flavonoids to be released. A correlation

between the abundance of *Lactobacillus* spp (Ls) and the candidate flavonoid compound (16) was found (Fig. 3), indicating that also these species contributed to release of flavonoids.

Xanthine (18), and allantoic (22) acid are products of bacterial purine metabolism [31], and fermentations with at least three of the four tested oligosaccharides showed a significant increase in the abundance of these metabolites in all six microbial communities (Fig. S5), indicating an activation in this pathway compared to the incubations carried out without oligosaccharides.

3-Oxooctadecanoic acid (11, 17) and (R)-3-hydroxy-octadecanoic acid (12) are building blocks of the unsaturated fatty acids [31]. 3-Oxooctadecanoic acid (17) was seen to increase in all six microbiotas after fermentation of at least two of the AOS fractions, and also increased in four out of six microbiotas after FOS fermentation (Fig. S5). A negative correlation between Oxooctadecanoic acid and (R)-3-hydroxy-octadecanoic acid (11/17 and 12, Fig. 3) suggests that they are present in the same bacterial metabolic pathway — an increase in (R)-3-hydroxy-octadecanoic acid is possibly related to a decrease of 3-oxooctadecanoic [31]. Together with an observed negative correlation between the fatty acid derivatives no. 14 and 20, this suggests that AOS and FOS might have an impact on the unsaturated fatty acid metabolism carried out by the intestinal microbiota, but that different turnover rates and different pathways are used [31], depending on the microbiota composition as well as on the type of oligosaccharide. The co-occurrence of the clostridial taxa (Cl and Cc) and fatty acid derivative no.14 (Fig. 3), indicates involvement of clostridial butyrate producers in the (microbial interaction leading to) production of this particular derivative.

5. Conclusion

In conclusion, we find that the present investigation shows that combining PCR techniques with metabolic profiling provides a strong tool to study complex microbiotas and their interactions with given substrates. We have shown that the response to high-mass arabino-oligosaccharides (HA) resembled that of FOS more than seen for mixed and low-mass arabino-oligosaccharides (BA and LA), supporting that the high-mass arabinans were the most promising prebiotic candidates. The proposed list of metabolite candidates enhanced by the arabino-oligosaccharide substrates constitutes a new and substantial contribution to the understanding of the mechanisms behind prebiotic interaction with the host.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anaerobe.2014.05.007.

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