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Abstract

Xylo-oligosaccharides and xylo-polysaccharides (XOS, XPS) produced by autohydrolysis of the fibre from oil palm empty fruit bunches (OPEFB) were purified using gel filtration chromatography to separate the XOS and XPS from the crude autohydrolysis liquor. Six mixed fractions of refined XOS and XPS with average degree of polymerisation (avDP) of 4-64 were obtained. These were characterised in terms of their composition and size by HPLC, MALDI-ToF-MS (selected fractions) and carbohydrate gel electrophoresis (PACE). They were assessed in batch culture fermentations using faecal inocula to determine their ability to modulate the human faecal microbiota *in vitro* by measuring the bacterial growth, organic acid production and the XOS assimilation profile. The gut microbiota was able to utilise all the substrates and there was a link between the XOS/XPS degree of polymerisation with the fermentation properties. In general, XOS/XPS preparations of lower avDP promote better *Bifidobacterium* growth and organic acid production.

Keywords

Autohydrolysis; *in vitro* fermentation; Oil palm empty fruit bunches; Xylo-oligo and xylo-polysaccharides

1. Introduction

The benefits of non-digestible oligosaccharides (NDOs) in modulating the intestinal and colonic microbiota that have an effect on human gut health have been well established and the study of NDOs derived from plant cell walls as emerging prebiotics has raised much interest. This is because plant cell walls, especially derived from cereal grains, are

part of our dietary fibre intake; the particular components of plant cell walls of interest in the context of prebiotics are the hemicelluloses. Hemicelluloses are the second most abundant class of polysaccharides available in the plant kingdom with xylan being the most common. Xylans have a backbone of β-(1→4) linked xylose units that are often substituted with arabinose, methylated or non-methylated glucuronic acid, acetic acid or ferulic acid (Ebringerová, Hromadkova & Heinze, 2005). Thus, depending on the origin of the plant cell wall and treatment process, various xylo-oligosaccharides (XOS, avDP ≤ 20) or xylo-polysaccharides (XPS, avDP > 20) with or without branching can be obtained.

In this regard, plant lignocellulosic biomass generated at the agricultural field and processing plant, which was once considered as waste for disposal, offers an enormous potential resource as a basic feedstock for XOS production (Moure, Gullón, Domínguez & Parajó, 2006). In the context of biorefining, hydrothermal treatments such as autohydrolysis have been investigated as an initial step of a possible multi-stage process for the utilisation of lignocellulosic materials, as it can produce soluble oligosaccharides, leaving cellulose and lignin in the solid phase for other usage (Parajó, Garrote, Cruz & Domínguez, 2004). The XOS obtained from autohydrolysis treatment also retain some substituents that are present in the native xylan such as acetyl groups, which could have an effect on their fermentability by the human intestinal microbiota (Kabel, Schols & Voragen, 2002b).

Earlier studies on the ability of XOS to modulate the intestinal microbiota investigated linear XOS of small molecular weight with a DP around 2-3. The low molecular weight XOS significantly promoted the growth of bifidobacteria and led to an increase in short chain fatty acid (SCFA) production in the bacterial cultures (Crittenden et

al., 2002; Okazaki, Fujikawa & Matsumoto, 1990; Palframan, Gibson & Rastall, 2003a) and in *in vivo* studies in humans and animals (Campbell, Fahey & Wolf, 1997; Childs et al., 2014; Chung, Hsu, Ko & Chan, 2007). Pure culture studies using XOS from corn cob and rice husk autohydrolysis with DP ≤ 4 also enhanced the growth of bifidobacteria despite having some acetyl groups and/or uronic acid substituents (Gullón et al., 2008; Moura et al., 2007). In Kabel, Kortenoeven, Schols & Voragen (2002a), a XOS preparation with wider range mixed DP (DP 2-11) was used and when fermented *in vitro* with human faecal inocula, the substrate was almost completely degraded in 20-40 h of fermentation. The fermentation rate and the SFCA profiles however varied depending on the substituents that were present, whereby the linear XOS and arabinose substituted XOS (AXOS) were fermented faster than acetylated XOS and methylglucuronylated XOS was the slowest. The bacteriology profile however was not the focus in that study, so the way the substituents modulate the gut microbiota is unknown. Increases in potentially health-positive bacterial groups such as *Bifidobacterium* spp. and *Lactobacillus/Enterococcus* spp. were seen with high average molecular weight arabinoxylans of 66,278 and 354 kDa (Hughes, Shewry, Li, Gibson, Sanz & Rastall, 2007). However, the arabinoxylans tested also significantly promoted clostridial growth. Van Craeyveld (2008) in a more systematic study on the influence of the average degree of polymerisation (avDP) and average degree of arabinose substitution (avDAS) of XOS preparation in the cecum of rats, showed that low molecular weight AXOS (avDP-avDAS of 5-0.27 and 3-0.26) increased *Bifidobacterium* spp. significantly more than high molecular weight AXOS (avDP –avDAS of 61-0.58). On the other hand, the measured branched SCFA was the lowest with avDP 61, so this could potentially suppress the metabolites from protein fermentation.

In a previous study, results have demonstrated that it is possible to produce purified XOS fractions of a variety of avDP from oil palm biomass autohydrolysis liquor (Ho et al., 2014). The aim of this work was to study the effect of XOS and XPS obtained from purification of autohydrolysed OPEFB at different avDP 4-64 upon the gut microbiota population. The rationale for inclusion of higher avDP XOS/XPS preparations in this study is they may have better persistence into the distal colon, with potential benefits to chronic gut diseases.

2. Materials and methods

2.1 Preparation of XOS/XPS fractions

The XOS/XPS preparation was according to Ho et al. (2014). Briefly, the fibre of dried oil palm empty fruit bunches (OPEFB) was subjected to non-isothermal autohydrolysis treatment in a two litre capacity stainless steel reactor (Parr Instruments Co., llinois, United States) with an operational temperature 210 ^{o}C and a liquid to solid ratio of 8 (w/w). The liquor obtained from autohydrolysis treatment was filtered and purified using preparative gel filtration chromatography (GFC) (Ho et al., 2014) with a BPG 100/950 column filled with Superdex 30^{TM} (Amersham Pharmacia Biotech, Uppsala, Sweden). The liquid fractions were freeze dried and then pooled together to obtained six mixed freeze-dried XOS/XPS with a range of avDP.

2.2 Characterization of XOS/XPS fractions

Prior to the determination of average molar mass and chemical compositions, the XOS/XPS samples were dissolved in deionised water to obtain a concentration of 10 g/L.

The apparent molar mass of samples was determined by high performance liquid chromotography (HPLC) (Agilent 1100 series, Winnersh, UK). A size exclusion column BIOSEP-SEC S2000 (Phenomenex, Cheshire, UK) was used at 30 ^{o}C with 50 mM $NaNO_3$ as mobile phase at 0.7 mL/min. The eluate was detected using a refractive index (RI) detector. External standards with different molecular weights, i.e. xylose, maltooligosaccharides (DP 2-5) and dextrans (1-71 kDa, Sigma, Dorset, UK) were used for calibration.

The composition of the XOS/XPS samples was assayed by HPLC to quantify free monosaccharides (glucose, xylose and arabinose), aliphatic acids (acetic acid, formic acid and levulinic acid) and furan derivatives (furfural and 5-hydroxymethylfurfural, HMF) compounds. An Aminex HPX-87H column (BioRad, Hemel Hempstead, UK) was used at 50 ^{o}C with 5 mM H_2SO_4 as mobile phase. The monosaccharides and aliphatic acids were detected with a RI detector while furfural and HMF were detected using a diode array detector (DAD) at 280 nm.

The oligosaccharide content was determined by an indirect method using quantitative acid hydrolysis; this was done by mixing the XOS/XPS sample with H_2SO_4 (72 % w/w) to obtain a final acid concentration of 4 % (w/w) and the sample was heated at 121 ^{o}C for 60 min to induce hydrolysis. The post hydrolysed liquor was analysed with HPLC and the oligosaccharide concentration was expressed as the increase in sugar monomers (Sluiter et al., 2006).

The total phenolic content was assayed spectrophometrically by the Folin Ciocalteu method using gallic acid as standard (Singleton & Rossi, 1965).

2.3 MALDI-Tof-MS of XOS fractions

MALDI-ToF-MS was used to analyse the extracted OPEFB XOS fractions in native and permethylated form for XOS avDP 4, 7 and 14. Initial analysis of the native fractions did not produce strong signals (Figure S1) so analyses were also performed with permethylated fractions. Permethylation of XOS fractions was performed using the NaOH/DMSO slurry method using 0.5 mL of methyl iodide (Ciucanu & Kerek, 1984). Permethylated glycans were dried under a stream of nitrogen and re-dissolved in 100 µL of methanol. Five µL of native or permethylated XOS fractions (10 mg/mL) were mixed with 5 µL of 2, 5-dihydroxybenzoic acid (DHB, 10 mg/mL dissolved in 50 % MeOH with 1% TFA, v/v) matrix. One µL of native or permethylated glycans mixed with DHB matrix was spotted onto a MALDI target plate and allowed to air-dry. MALDI-ToF-MS was carried out using a Micromass MALDI-LR mass spectrometer (Waters, Manchester, UK) using a mass acquisition between *m/z* 450 and 3 000. The MALDI set-up was as described by Marsh et al. (2011). Glycan adduct ions $[M + Na]^+$ were assigned. Experimentally determined masses were interpreted using GlycoMod (ExPaSy).

2.4 Analysis of XOS/XPS fractions by carbohydrate gel electrophoresis

Polysaccharide Analysis by Carbohydrate Gel Electrophoresis (PACE) was used to analyse the extracted OPEFB XOS/XPS fractions. Fractions were run with and without

digestion with xylanase 11. Briefly, for xylanase digested samples, 200 µg aliquots were digested with Xyn11 (4 µL ≈ 21.92 µg; Prozomix, UK) for 16 h at 40 °C in total volume of 500 µL. Digestion was terminated by boiling the samples for 30 min and samples were dried *in vacuo*. Aliquots (200 µg) of undigested XOS/XPS fractions were also dried down. All samples together with standard xylo-oligosaccharides (Xyl_{1-6}; Megazyme, Ireland) were labelled with ANTS and ran on acrylamide gel as described by Kosik, Bromley, Busse-Wicher, Zhang & Dupree (2012). Gels were visualized under UV light using a GelDoc-It TS2 imager (UVP, Germany) equipped with a GFP emission filter (513-557 nm).

2.5 *In vitro* batch fermentation

The six different fractions of XOS/XPS along with commercial XOS (avDP2, Shandong Longlive Biotechnology Co. Ltd (SLBC), China) and birch wood xylan (Sigma, Dorset, UK) were evaluated for the ability to modulate the gut microbiota using an *in vitro* batch culture fermentation system inoculate with human faecal sample. Fructooligosaccharides (avDP 4, Raftilose®, Orafti, Tienan, Belgium) were used as the positive control. The *in vitro* fermentation was carried out a 50 mL working volume glass jacketed bioreactors, sterile of stirred batch culture fermentation system. The carbohydrates sources were added at 1 % (w/v). The basal medium (per litre) consisted of: 2 g peptone water, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g $MgSO_4.7H_2O$, 0.01 g $CaCl.6H_2O$, 2 g $NaHCO_3$, 2 mL Tween 80, 0.05 g haemin, 0.01 mL vitamin K_1, 0.5 g L-cysteine-HCl, 0.5 g bile salt and 4 mL resazurin solution (0.25 g/L).

The fermentation of each substrate was carried out in triplicate with each of three healthy human faecal donors, who had not taken prebiotic or probiotic products for 3 months, or antibiotics for six months prior to the study. Each vessel containing fermentation medium was inoculated with 5 mL of faecal slurries, which was prior diluted at 10 % (w/w) with anaerobic phosphate-buffered saline (PBS, 0.1 M) and homogenised in a stomacher (Stomacher 400; Seward, West Sussex, UK) for 2 min at medium speed.

The fermentation was carried out at pH 6.7-6.9, controlled using an automated pH controller (Fermac 260; Electrolab, Tewkesbury, UK) and at 37 ^{o}C (using a thermocirculator) under anaerobic atmosphere, which was achieved through continuous sparging with nitrogen gas. Samples (5 mL) were taken from each fermentation vessel at 0, 10, 24 and 36 h for organic acid analysis and bacterial enumeration using the fluorescent *in situ* hybridisation (FISH) technique.

2.6 Enumeration of bacteria

The target faecal bacteria groups were enumerated by FISH using 16S rRNA targeted oligonucleotide probes labelled with the fluorescent Cy3 dye. An aliquot (375 µL) of sample from each sampling time was mixed with 3 volumes of 4 % (w/v) cold paraformaldehyde (PFA) solution. The duration of fixation was 5-10 h at 4 ^{o}C, followed by centrifugation at 13 000 x *g* for 5 min; the cell pellet was then washed twice with 1 mL cold filter sterilised PBS. The washed cells were then resuspended in 150 µL PBS and 150 µL of absolute ethanol and stored at -20 ^{o}C until analysis.

To further process the PFA-fixed sample, 10 µL of each sample was diluted with PBS/SDS (sodium dodecyl sulphate) diluent and the diluted samples (20 µL) were applied onto six-well of a polytetrafluoroethylene/poly-ι-lysine coated slide (Tekdon Inc., Myakka City, FL). The samples were dried at 48 ^{o}C for 15 min in a desktop plate incubator and then dehydrated using a series of ethanol solution at 50 %, 80 % and 96 % (v/v) for 3 min each. The excess ethanol was evaporated by drying the slides in a desktop plate incubator for 2 min followed by addition of 50 µL of mixed hybridisation solution (5 µL oligonucleotide probe solution and 45 µL hybridisation buffer) onto each well. The slide with samples were hybridised in a microarray hybridisation incubator (Grant-Boekel, Cambridge, UK) for 4 h, washed in 50 mL washing buffer for 15 min and dipped in cold distilled water for 2 s. Slides were dried with compressed air and a drop of PVA-DABCO antifade (polyvinyl alcohol mounting medium with 1, 4-diazabicyclo (2.2.2) octane) was added onto each well. The microscope cover slip was placed on each slide and the cell numbers of microorganisms were determined by direct counting under an epifluorescence microscope (Eclipse 400; Nikon, Surrey, UK) with Fluor 100 lens. A total of 15 fields of view were counted for each well.

The probes used were Bif164 (Langendijk et al., 1995), Bac303 (Manz, Amann, Ludwig, Vancanneyt & Schleifer, 1996), Lab158 (Harmsen, Elfferich, Schut & Welling, 1999), Ato291 (Harmsen, et al., 2000), Prop853 (Walker, Duncan, McWilliam Leitch, Child & Flint, 2005), Erec482 (Franks et al., 1998), Rrec584 (Walker et al., 2005), Fprau655 (Hold, Schwiertz, Aminov, Blaut & Flint, 2003), Chis150 (Franks et al., 1998), and mixed Eub338 I, II, III (Daims, Brühl, Amann, Schleifer & Wagner, 1999) for enumerating *Bifidobacterium* spp., *Bacteroides-Prevotella*, *Lactobacillus-Enterococcus*,

Atopobium cluster, propionate producing bacteria (Clostridium cluster IX), *Eubacterium rectale-Clostridium cocoides* group (Clostridium cluster XIVa and XIVb), *Roseburia* spp., *Faecalibacterium prausnitzii* cluster *Clostridium histolyticum* group (Clostridium cluster I and II) and total bacteria, respectively.

2.7 Organic acid analysis

An aliquot (1 mL) of sample from each sampling time was centrifuged at 13 000 x *g* for 10 min and the supernatant was stored at -20 ^{o}C until analysis. Organic acids analysis was performed using an HPLC (1100 series; Agilent, Winnersh, UK) with refractive index detection. Prior to the analysis, the samples, after thawing, were centrifuged at 13 000 x *g* for 10 min and the supernatants were filtered through a 0.22 μm filter unit. An ion exclusion column, Rezex ROA-Organic Acid H+ (8%) (Phenomenex, Cheshire, UK) was used for the analysis, using 2.5 mM H_2SO_4 as eluent. The column was heated at 84 ^{o}C and the eluent flow rate was set at 0.5 mL/min. The injection volume used was 20 μL with 40 min run time. Organic acids were quantified using standard calibration curves for lactate, acetate, propionate, butyrate and valerate at concentrations of 12.5, 25, 50, 75 and 100 mM. Formate was determined using a formate dehydrogenase-based assay kit (Megazyme, Ireland).

2.8 Carbohydrate assimilation profile during fermentation

The assimilation profile for the nine different carbohydrates substrates used for the batch culture fermentations was determined by High Performance Anion-Exchange

Chromatography (HPAEC, Dionex, Camberley, UK) using a CarboPac PA-1 column and Pulsed Amperometric Detection (PAD). Samples were filtered and diluted with deionised water at a dilution factor of 30. The injection volume was 25 μL and the flow rate 1 mL/min with the following linear gradient: 8.75 mM NaOH and 2.4 mM sodium acetate from 0-19 min; 30 mM NaOH and 25 mM sodium acetate from 20-44 min; and 96.875 mM NaOH and 150 mM sodium acetate from 45-49 min. After 50 minutes, a washing step was performed with 125 mM NaOH and 500 mM sodium acetate for 10 min and the column was then equilibrated for 20 min with 8.75 mM NaOH and 2.4 mM sodium acetate. Standard glucose, arabinose, xylose and xylose oligomers (DP 2-6, Megazyme, Ireland) were used for identification.

2.9 Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 17.0. One-way analysis of variance (ANOVA) and Tukey's posthoc test was used to determine significant differences among the bacterial group populations and organic acid concentrations among the different substrates. A paired independent t-test was also used to determine significant changes for each bacterial group concentration at inoculation and subsequent sampling point. Differences were considered to be significant when $p<0.05$.

3. Results and discussion

3.1 Characterization of the XOS/XPS fractions

263 The chemical analysis of the six fractions of purified and freeze dried OPEFB
264 autohydrolysis liquor is shown in Table 1. In all cases, XOS/XPS were the dominant
265

Table 1

Composition of OPEFB fibre fractions (g/100 g freeze dried sample) obtained after GFC purification[a]

Sample no.	avDP[b]	Residues in linkage (g/100 g)				Ratio[c]		Free Monomers (g/100 g)		Total phenolics (g/100 g)
		Glc	Xyl	Ara	AcO	Ara/Xyl	AcO/Xyl	Xylose	Acetic acid	
1	4	1.75	62.25	1.49	9.16	0.02	0.37	1.23	1.73	0.46
2	7	1.62	65.38	1.16	10.30	0.02	0.39	0.86	0.80	0.37
3	14	1.56	67.32	1.22	11.23	0.02	0.42	0.65	0.48	0.33
4	28	1.61	67.68	1.18	12.43	0.02	0.46	0.57	0.46	0.31
5	44	2.31	64.00	1.21	12.75	0.02	0.50	n.d	0.48	0.43
6	64	2.83	59.28	1.16	12.95	0.02	0.55	n.d	0.46	0.43

[a]In freeze dried form and reconstitute with deionised water to give final concentration of 10 g/L. Calculations were made by assuming the freeze dried samples have 5% moisture content.

[b]avDP – Average degree of polymerization as determined by size exclusion chromatography

[c]Ratio in mol/mol

AcO - acetyl groups linked to oligosaccharides; n.d. – not detected

oligosaccharides, accounting for 78-83 % of the total oligosaccharides. The highest XOS/XPS yield was found in the middle fractions (avDP 14 and 28); free monomeric compounds (xylose and acetic acid) were present at slightly higher concentrations in XOS fractions with lower DP (avDP 4 and 7) than in the other fractions with free xylose absent in XPS fractions with higher DP (avDP 44 and 64). The oligosaccharides of the higher DP fractions (XPS, avDP 44 and 64) were more acetylated. The acetyl groups contribute to the oligosaccharides solubility in water (Nabarlatz, Ebringerová & Montané, 2007) and this may be the reason that high molecular weight XOS fractions were present in OPEFB autohydrolysis liquor. The arabinose content was rather low for all fractions, with an arabinose to xylose ratio of approximating 0.02. The gluco-oligosaccharides (GlcOS) were presumably derived from cellulose and were present at 2-3% w/w. There was also a small amount of total phenolic compounds (<0.5 % w/w) found in all samples.

OPEFB fractions (avDP 4, 7 and 14) were analysed by MALDI-ToF-MS (larger avDP fractions were too large for MALDI-ToF-MS analysis). XOS/XPS fractions were all analysed in both their native and permethylated forms by MALDI-ToF-MS. All XOS/XPS fractions analysed in their native form showed acetylated pentose oligosaccharide ions (labelled $Pent_nAc_n$, the $_n$ denoting the number of pentose (Pent) or acetyl (Ac) groups respectively). In avDP 4 the most dominant ion is *m/z* 917.27 ($Pent_6Ac_2$) (Fig. 1) with acetylated oligosaccharides ranging from $Pent_4Ac_2$ (*m/z* 653.19) to $Pent_9Ac_4$ (*m/z* 1397.42). Also present are pentose oligosaccharides with no acetylation or other modifications with DP 6 to 9 (*m/z* 833.25 to 1229.38) and hexose oligosaccharides of DP 4-8 (*m/z* 689.21 to 1337.42). There could also be small pentose oligosaccharides with methylated-glucuronic acid substitutions (ions at *m/z* 637.18 and 685.18) found in the native avDP 4 fraction. The

293 permethylated version of avDP 4 fraction (data not shown); although the acetylated
294 residues of the pentose oligosaccharides are lost, we were able to see a pentose ladder
295 starting from $Pent_3$ (*m/z* 549.25) up to $Pent_9$ (*m/z* 1509.69) and ladder of pentose
296 oligosaccharide substituted with single glucuronic acid up to DP 8 (Pen_1HexA_1, *m/z* 447.18
297 to Pen_7HexA_1, *m/z* 1407.63) that could not be observed in native form of the sample.
298 Similarly to the native version of avDP 4 XOS fraction hexose oligosaccharide ladder was
299 observed (Hex_3 *m/z* 681.33- Hex_8 *m/z* 1701.83) These data confirm the data in Table 1
300 which showed gluco-oligosaccharides (hexose oligosaccharides), xylo- and arabino-
301 oligosaccharides (pentose oligosaccharides) and acetylated oligosaccharides. Mass
302 spectrometry of OPEFB fractions of avDP 7 and avDP 14 also confirmed the data in Table
303 1. The predominant ions were the acetylated pentoses e.g. *m/z* 785.18 ($Pent_5Ac_2$) up to
304 $Pent_9Ac_5$ ion (*m/z* 1439.43) and methylated glucuronic acid substituted oligosaccharides
305 were also present (*m/z* 637.18 and *m/z* 685.18) (Supplementary Fig. 1a). The permethylated
306 avDP7 fraction (Supplementary Fig. 1b) also contained hexose oligosaccharides (Hex_4, *m/z*
307 885.43 to Hex_{10} *m/z* 2110.03) as well as pentose oligosaccharide substituted with
308 glucuronic acid ($Pent_2HexA_1$, *m/z* 607.26 to $Pent_9HexA_1$, *m/z* 1727.77). In OPEFB the
309 avDP 14 fraction (Supplementary Fig. 1c) acetylated pentose oligosaccharides range from
310 $Pent_5Ac_2$, *m/z* 785.23 to $Pent_{18}Ac_6$, *m/z* 2669.82. Also, observed in the permethylated
311 avDP14 (Supplementary Fig.1d) are glucuronic acid substituted pentoses, $Pent_3HexA_1$ (*m/z*
312 767.33) to $Pent_{13}HexA_1$ (*m/z* 2368.07).

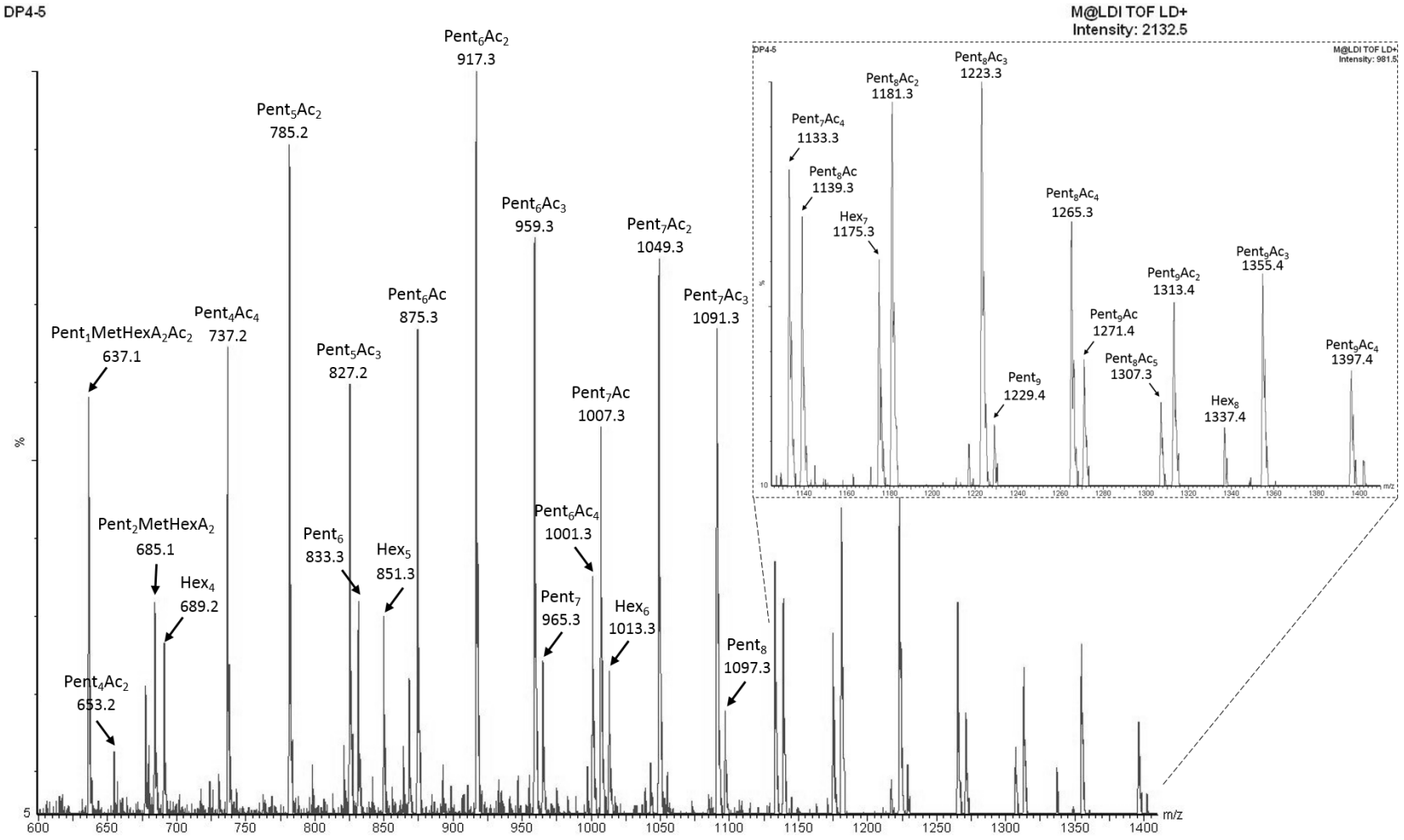


Fig. 1. MALDI-ToF-MS spectrum of native glycans isolated from OPEFB XOS fraction avDP 4. The glycan adduct ions $[M+Na]^+$ are indicated for acetylated pentose oligosaccharides ($Pent_nAc_n$), for pentose oligosaccharides ($Pent_n$), for pentoses with methylated-glucuronic acid substitution ($Pent_nMetHexA_nAc_n$) and for hexose oligosaccharides (Hex_n).

The DP ranges of the OPEFB fractions obtained from MALDI-ToF-MS analysis were as follows: avDP 4 (DP 2-9), avDP 7 (DP 3-12), avDP 14 (DP 3-18). The OPEFB fractions were also xylanase cleaved and visualised by polysaccharide analysis using carbohydrate gel electrophoresis (PACE) (Fig.2) which confirms the predominant oligosaccharides were xylo-oligosaccharides and that the gel filtration fractionation of the avDP 4 to avDP 64 contained similar oligosaccharides but with increasing xylose chain length.

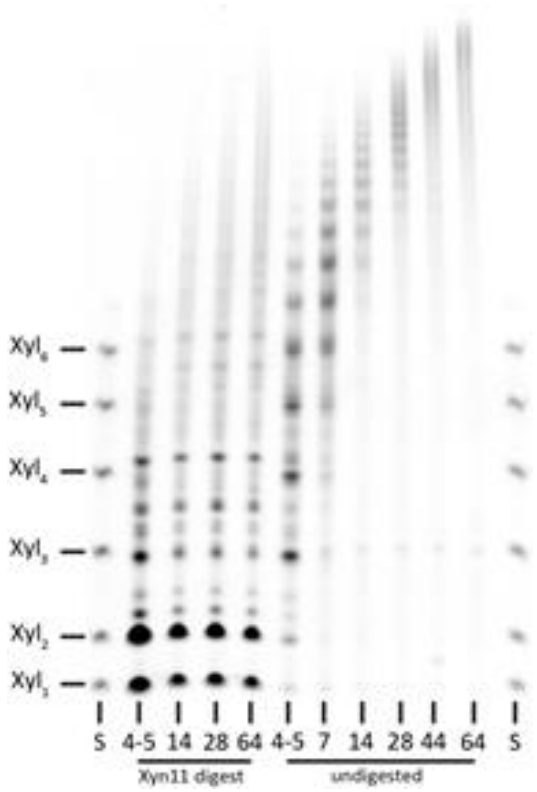


Fig. 2. PACE gel showing separation of extracted OPEFB XOS fractions digested with Xyn11 and undigested. S - Standard xylose$_{1\text{-}6}$ ladder; 4-5 = avDP 4; 14 = avDP 14; 28 = avDP 28 and 64 = avDP 64, digested with Xyn11. 4-5 = avDP 4; 7 = avDP 7; 14 = avDP 14; 28 = avDP 28; 44 = avDP 44 and 64 = avDP 64, undigested OPEFB XOS fractions.

331 3.2 Bacterial enumeration

332 Changes in the bacterial populations during the *in vitro* fermentations with the
333 different XOS fractions are shown in Table 2. A significant increase ($p<0.05$) of
334 *Bifidobacterium* population, ranging between 0.5-0.8 log cells/mL for all time points
335 compared to time 0 h was observed for the XOS fractions with avDP of 4, 7 and 14,
336 commercial XOS and FOS. In the case of the XOS fractions with avDP of 28 and 44,
337 significant increases ($p<0.05$) were observed for the 10 h sample, whereas for the XOS
338 fraction with avDP of 64, although an increase was observed for the 10 h sample, this was
339 not statistically significant ($p\geq0.05$). For all these higher avDP (28, 44, 64) fractions, the
340 concentrations were sustained for the 24 h and 36 h samples and were not statistically
341 different to 0 h. Taking into account the above and the fact that the effect of the XOS
342 fractions with low avDP (avDP 4-14) on the *Bifidobacterium* population was similar to that
343 of commercial XOS, which mainly consists of DP 2-3, it can be inferred that bifidobacteria
344 preferred the lower molecular weight XOS fractions. This is also supported by the fact that
345 birch wood xylan did not have a significant effect on the *Bifidobacterium* population. In the
346 pure culture study, there were few strains of *Bifidobacterium* capable of fermenting high
347 molecular weight XOS or xylan (Palframan, Gibson & Rastall, 2003b). The reason for the
348 increase in the *Bifidobacterium* population at 10 h for the XOS fractions of avDP 14, 28, 44
349 could be that the bifidobacteria utilise the low molecular weight XOS, which were present
350 in the fractions as demonstrated by the MALDI-ToF-MS. Another possibility is that higher
351 molecular weight XOS was hydrolysed to smaller XOS molecules by other microorganisms
352 such as *Bacteroides* (Chassard, Goumy, Leclerc, Del'homme & Bernalier-Donadille, 2007;

Table 2

Mean bacterial populations in pH-controlled batch cultures at 0, 10, 24 and 36 h[a]

Probe	Time (h)	Bacterial population (log_{10} cells/ml batch culture fluid) in substrate								
		OPEFB XOS (avDP 4)	OPEFB XOS (avDP 7)	OPEFB XOS (avDP 14)	OPEFB XPS (avDP 28)	OPEFB XPS (avDP 44)	OPEFB XPS (avDP 64)	Birch wood xylan	XOS (SLBC)	FOS (Raftilose)
Bif164	10	8.38 (0.19)^{ab*}	8.37 (0.18)^{ab*}	8.41 (0.27)^{ab*}	8.31 (0.16)^{ab*}	8.26 (0.16)^{ab*}	8.22 (0.10)^{ab}	8.15 (0.10)^{a}	8.65 (0.13)^{b**}	8.64 (0.08)^{b**}
	24	8.56 (0.14)^{a*}	8.50 (0.19)^{a*}	8.59 (0.16)^{a*}	8.40 (0.29)^{a}	8.36 (0.28)^{a}	8.29 (0.28)^{a}	8.25 (0.29)^{a}	8.53 (0.06)^{a**}	8.48 (0.12)^{a*}
7.85(0.09)	36	8.41 (0.15)^{a*}	8.46 (0.13)^{a*}	8.54 (0.10)^{a**}	8.30 (0.24)^{a}	8.24 (0.21)^{a}	8.10 (0.21)^{a}	8.01 (0.23)^{a}	8.38 (0.19)^{a*}	8.31 (0.35)^{a}
Bac303	10	8.58 (0.08)^{a*}	8.62 (0.17)^{a}	8.64 (0.27)^{a}	8.62 (0.13)^{a*}	8.46 (0.26)^{a}	8.43 (0.14)^{a*}	8.48 (0.34)^{a}	8.54 (0.11)^{a}	8.63 (0.20)^{a}
	24	8.50 (0.14)^{a**}	8.50 (0.06)^{a**}	8.71 (0.04)^{a*}	8.59(0.25)^{a*}	8.50 (0.44)^{a}	8.41 (0.50)^{a}	8.59 (0.35)^{a}	8.42 (0.13)^{a*}	8.46 (0.21)^{a}
8.10(0.09)	36	8.30 (0.17)^{a}	8.31 (0.12)^{a}	8.46 (0.04)^{a**}	8.33 (0.32)^{a}	8.43 (0.29)^{a}	8.27 (0.46)^{a}	8.32 (0.14)^{a*}	8.29 (0.20)^{a}	8.15 (0.08)^{a}
Lab158	10	8.30 (0.19) ^{a}	8.42 (0.25) ^{a}	8.45 (0.23) ^{a*}	8.45 (0.20) ^{a*}	8.38 (0.26) ^{a}	8.29 (0.05) ^{a*}	8.27 (0.14) ^{a}	8.51 (0.13) ^{a*}	8.45 (0.22) ^{a}
	24	8.36 (0.17) ^{a}	8.50 (0.19) ^{a*}	8.57 (0.20) ^{a*}	8.46 (0.07) ^{a**}	8.29 (0.35) ^{a}	8.24 (0.24) ^{a}	8.42 (0.19) ^{a*}	8.35 (0.14) ^{a*}	8.30 (0.15) ^{a*}
7.97(0.04)	36	8.31 (0.17) ^{a}	8.45 (0.12) ^{a*}	8.46 (0.14) ^{a*}	8.27 (0.11) ^{a}	8.10 (0.31) ^{a}	8.04 (0.32) ^{a}	8.13 (0.19) ^{a}	8.28 (0.24) ^{a}	8.32 (0.35) ^{a}
Ato291	10	8.22 (0.03)^{ab*}	8.19 (0.05)^{ab*}	8.12(0.05)^{ab**}	8.07 (0.18)^{a}	8.00 (0.07)^{a}	8.05 (0.14)^{a}	7.97 (0.32)^{a}	8.42 (0.17)^{ab**}	8.56 (0.20)^{b**}
	24	8.14 (0.09)^{bcd*}	8.08 (0.10)^{abc**}	7.93 (0.11)^{ab}	7.99 (0.03)^{abc*}	7.87 (0.04)^{ab}	7.72 (0.05)^{a}	8.00 (0.20)^{abc}	8.35 (0.20)^{cd*}	8.51 (0.23)^{d**}
7.78(0.10)	36	7.88 (0.23)^{abc}	7.80 (0.17)^{ab}	7.81 (0.16)^{ab}	7.69 (0.10)^{a}	7.60 (0.15)^{a}	7.57 (0.15)^{a}	7.66 (0.21)^{a}	8.22 (0.24)^{bc}	8.37 (0.05)^{c**}
Prop853	10	7.90 (0.04)^{a*}	8.07 (0.12)^{a}	8.08 (0.08)^{a*}	8.11 (0.05)^{a**}	8.05 (0.03)^{a**}	8.01 (0.23)^{a}	7.92 (0.32)^{a}	7.99 (0.23)^{a}	7.97 (0.25)^{a}
	24	8.03 (0.26)^{a}	8.12 (0.09)^{a*}	8.17 (0.08)^{a*}	8.13 (0.14)^{a*}	8.04 (0.30)^{a}	7.87 (0.37)^{a}	7.98 (0.23)^{a}	8.02(0.32)^{a}	7.97 (0.37)^{a}
7.71(0.05)	36	7.87 (0.33)^{a}	7.92 (0.16)^{a}	7.86 (0.19)^{a}	7.78 (0.13)^{a}	7.74 (0.41)^{a}	7.68 (0.41)^{a}	7.76 (0.20)^{a}	7.61 (0.12)^{a}	7.86 (0.24)^{a}
Erec482	10	8.09 (0.20) ^{a}	8.18 (0.47) ^{a}	8.28 (0.48) ^{a}	8.29 (0.37)^{a}	8.15 (0.18) ^{a}	8.20 (0.14) ^{a}	8.20 (0.18) ^{a}	8.28 (0.30) ^{a}	8.31 (0.24) ^{a}
	24	8.26 (0.12) ^{a*}	8.44 (0.34) ^{a}	8.35 (0.51) ^{a}	8.43(0.27) ^{a}	8.08 (0.52) ^{a}	8.22 (0.27) ^{a}	8.24 (0.14) ^{a*}	8.36 (0.15) ^{a*}	8.33 (0.08) ^{a**}
7.99(0.04)	36	8.43 (0.10) ^{a*}	8.41 (0.33) ^{a}	8.27 (0.47) ^{a}	8.28 (0.09) ^{a*}	8.19 (0.32) ^{a}	8.13 (0.41) ^{a}	8.20 (0.33) ^{a}	8.28 (0.14) ^{a*}	8.14 (0.12) ^{a}
Rrec584	10	7.48 (0.16) ^{a}	7.48 (0.11) ^{a}	7.49 (0.18) ^{a}	7.45 (0.02) ^{a*}	7.38 (0.02) ^{a*}	7.35(0.06) ^{a*}	7.38 (0.12) ^{a}	7.52(0.22) ^{a}	7.41 (0.17) ^{a}
	24	7.61 (0.06)^{ab}	7.58 (0.11) ^{ab}	7.46 (0.19)^{a}	7.54 (0.10) ^{ab}	7.51 (0.17)^{ab}	7.50(0.06) ^{a}	7.50(0.15) ^{a}	7.85 (0.05) ^{b*}	7.76 (0.11) ^{ab*}
7.38(0.05)	36	7.70 (0.22)^{a*}	7.65 (0.15) ^{a}	7.65 (0.21) ^{a}	7.53 (0.07) ^{a*}	7.59 (0.20) ^{a}	7.60 (0.12) ^{a}	7.40 (0.20) ^{a}	7.87 (0.20) ^{a*}	7.75 (0.15) ^{a}
Fprau655	10	7.58 (0.26) ^{a}	7.67 (0.29) ^{a}	7.66 (0.30) ^{a}	7.72 (0.13) ^{a}	7.61 (0.19) ^{a}	7.62 (0.13) ^{a}	7.65 (0.30) ^{a}	7.53 (0.26) ^{a}	7.67 (0.34) ^{a}
	24	7.36 (0.08) ^{a}	7.45 (0.11) ^{a}	7.57 (0.24) ^{a}	7.84 (0.10) ^{a*}	7.51 (0.27) ^{a}	7.58 (0.22) ^{a}	7.74 (0.27) ^{a}	7.49 (0.20) ^{a}	7.60 (0.22) ^{a}
7.54(0.10)	36	7.44 (0.24) ^{a}	7.46 (0.02) ^{a}	7.47 (0.21) ^{a}	7.56 (0.19) ^{a}	7.40 (0.11) ^{a*}	7.55 (0.27) ^{a}	7.48 (0.28) ^{a}	7.34 (0.12) ^{a}	7.43 (0.25) ^{a}
Chis150	10	7.41 (0.21)^{a}	7.48 (0.09)^{a}	7.44 (0.13)^{a}	7.49 (0.10)^{a}	7.38 (0.07)^{a}	7.38 (0.15)^{a}	7.44 (0.09)^{a}	7.36 (0.11)^{a}	7.56 (0.27)^{a}
	24	7.34 (0.04)^{a}	7.34 (0.15)^{a}	7.23 (0.06)^{a}	7.31 (0.10)^{a}	7.27 (0.05)^{a}	7.24 (0.07)^{a}	7.36 (0.09)^{a}	7.28 (0.03)^{a}	7.34 (0.12)^{a}
7.33(0.05)	36	6.93 (0.12)^{a}	6.97 (0.08)^{a*}	6.95 (0.15)^{a}	6.91 (0.06)^{a*}	6.90 (0.16)^{a}	6.71 (0.07)^{a*}	6.95 (0.15)^{a*}	6.88 (0.08)^{a*}	6.80 (0.06)^{a*}
Eub338	10	9.17 (0.11) ^{a}	9.21 (0.12) ^{a*}	9.22 (0.10) ^{a*}	9.19 (0.06) ^{a*}	9.16 (0.15) ^{a}	9.10 (0.10) ^{a*}	9.06 (0.14) ^{a}	9.33 (0.18) ^{a}	9.30 (0.11) ^{a*}
	24	9.25(0.17) ^{a}	9.25 (0.10) ^{a*}	9.30 (0.14) ^{a*}	9.25 (0.13) ^{a*}	9.24 (0.18) ^{a}	9.12 (0.13) ^{a}	9.12 (0.16) ^{a}	9.27 (0.16) ^{a}	9.18 (0.11) ^{a}
8.79(0.06)	36	9.16(0.19) ^{a}	9.15(0.15) ^{a}	9.22 (0.13) ^{a}	9.08 (0.05) ^{a*}	9.08 (0.34) ^{a}	8.93 (0.33) ^{a}	8.91 (0.19) ^{a}	9.09 (0.17) ^{a}	9.06 (0.13) ^{a}

[a]Standard deviation is shown in parentheses (n=3). Significant differences ($p<0.05$) between substrates are indicated with different letters in a same row.

[*]Significant differences from value at 0 h, $p<0.05$; [**]Significant differences from value at 0 h, $p<0.01$ (Value at 0 h is shown in the far left under 'Probe' column)

353

354 Falony, Calmeyn, Leroy & De Vuyst, 2009). This was also observed in studies carried out
355 by Mäkeläinen and co-workers (2010a; 2010b), a high molecular weight xylan (DP 35-40)
356 was not efficiently metabolised by a range of *Bifidobacterium* strains in pure culture studies
357 but when they tested the same xylan in a semi continuous colon simulator system using
358 faecal inoculum, they observed a significant increase in the *Bifidobacterium* sp. population.
359 Another bacterial group which had significant difference between substrates is the
360 *Atopobium* cluster. *Atopobium* has the highest count on FOS, significantly higher ($p<0.05$)
361 than OPEFB XOS of avDP 28, 44 and 64. These results are consistent with Hughes et al.
362 (2007) whereby the large molecular weight AXOS (278 kDa and 354 kDa) generally did
363 not induce growth of *Atopobium*.
364

365 3.3 Organic acid analysis

366 Table 3 shows the organic acid concentrations in the fermentations; acetate was the
367 leading SCFA produced, followed by propionate, formate, lactate and butyrate. Across all
368 substrates, formate and lactate were transient metabolites reaching maximum at 10 h.
369 Acetate and propionate concentration on the other hand continued to rise up to 24 h and/or
370 36 h, whereas butyrate, though present at low concentration initially, increased steadily up
371 to 36 h.
372 All OPEFB XOS produced significantly lower ($p<0.05$) amount of lactate than
373 commercial XOS and FOS. The wider DP distribution and possibility the presence of
374 substituents on OPEFB XOS may affect the accessibility for bifidobacterial fermentation.
375 Kabel et al. (2002a) also observed a higher amount of lactate in non-substituted XOS than

substituted XOS. According to Falony et al. (2009), metabolism in bifidobacteria produces more formate, acetate and ethanol at the expense of lactate when there is limited access to substrate. Different carbohydrates are known to promote the growth of different species of bifidobacteria, resulting in varying amount of lactate (Palframan et al., 2003b).

The initial acetate level in OPEFB XOS avDP 4 was high, possibly as a result of free acetic acid present in the low molecular weight substrate. XOS in all OPEFB fractions and the commercial XOS resulted in higher acetate and less propionate and butyrate than FOS. This typical profile corresponds with previous studies conducted on XOS and xylan fermentation (Englyst, Hay & Macfarlane, 1987; Kabel et al., 2002a; Rycroft, Jones, Gibson & Rastall, 2001).

386

Table 3

Mean organic acid concentrations in pH-controlled batch cultures at 0, 10, 24 and 36 h[a]

Organic acid	Time (h)	Concentration (mM)								
		OPEFB XOS (avDP 4)	OPEFB XOS (avDP 7)	OPEFB XOS (avDP 14)	OPEFB XPS (avDP 28)	OPEFB XPS (avDP 44)	OPEFB XPS (avDP 64)	Birch wood xylan	XOS (Suntory)	FOS (Raftilose)
Lactate	0	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.78 (0.68)^{a}	0.79 (0.68)^{a}
	10	4.88 (2.92)^{a}	1.85 (2.32)^{a}	2.46 (2.38)^{a}	0.81 (1.40)^{a}	2.34 (1.44)^{a}	0.46 (0.79)^{a}	0.79 (0.72)^{a}	16.11 (5.89)^{b*}	19.29 (6.34)^{b*}
	24	0.56 (0.98)^{a}	0.32 (0.56)^{a}	1.02 (1.76)^{a}	0.00 (0.00)^{a}	0.50 (0.87)^{a}	0.00 (0.00)^{a}	0.32 (0.56)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}
	36	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.45 (0.78)^{a}	0.00 (0.00)^{a}	0.47 (0.81)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}
Formate	0	0.58 (0.04)^{d}	0.16 (0.07)^{bc}	0.11 (0.07)^{abc}	0.11 (0.06)^{abc}	0.13 (0.06)^{abc}	0.36 (0.04)^{a}	0.16 (0.03)^{c}	0.03 (0.04)^{ab}	0.01 (0.01)^{a}
	10	8.42 (8.28)^{a}	7.61 (7.02)^{a}	4.26 (5.39)^{a}	8.37 (6.06)^{a}	4.11 (5.44)^{a}	5.80 (7.78)^{a}	2.64 (1.64)^{a}	14.06 (3.49)^{a*}	14.96(5.90)^{a*}
	24	5.33(3.65)^{a}	5.66 (8.32)^{a}	3.54(6.12)^{a}	4.26 (7.38)^{a}	6.44 (5.58)^{a}	2.19 (3.56)^{a}	0.05 (0.08)^{a}	6.56 (5.94)^{a}	1.69 (2.86)^{a}
	36	0.00 (0.00)^{a}	1.93 (3.34)^{a}	0.55 (0.95)^{a}	0.00 (0.00)^{a}	2.34 (2.54)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	1.24 (2.15)^{a}	0.00 (0.00)^{a}
Acetate (A)	0	10.08 (2.41)^{c}	6.70 (1.79)^{bc}	6.00 (1.56)^{b}	5.62 (1.72)^{ab}	5.20 (0.50)^{ab}	5.18 (0.38)^{ab}	5.84 (0.06)^{ab}	2.18 (0.03)^{a}	2.31 (0.30)^{a}
	10	48.44 (21.23)^{a}	47.45 (24.27)^{a}	47.84 (23.12)^{a}	51.72 (24.48)^{a}	33.12 (22.07)^{a}	37.19 (27.31)^{a}	25.53 (6.71)^{a*}	54.82 (8.47)^{a**}	47.55 (11.02)^{a*}
	24	77.39 (21.26)^{b*}	71.61 (7.48)^{ab**}	78.37 (6.57)^{b**}	62.35 (11.82)^{ab*}	57.30 (28.36)^{ab}	43.50 (26.36)^{ab}	28.98 (7.96)^{a*}	60.19 (2.00)^{ab**}	43.10 (6.47)^{ab**}
	36	79.80 (22.19)^{b*}	68.68 (10.00)^{ab**}	78.70 (6.86)^{b**}	54.60 (10.09)^{ab*}	59.49 (27.07)^{ab}	41.44 (30.12)^{ab}	21.32 (7.03)^{a}	60.53 (3.77)^{ab**}	39.61 (7.66)^{ab*}
Propionate (P)	0	3.08 (0.52)^{a}	2.75 (0.19)^{a}	2.69 (0.18)^{a}	2.67 (0.19)^{a}	2.70 (0.10)^{a}	2.69 (0.22)^{a}	2.76 (0.18)^{a}	2.61 (0.11)^{a}	2.67 (0.42)^{a}
	10	9.23 (4.64)^{a}	13.84 (10.69)^{a}	15.60 (11.96)^{a}	12.77 (1.82)^{a*}	7.96 (1.94)^{a*}	11.37 (6.05)^{a}	10.28 (2.78)^{a*}	13.28 (8.04)^{a}	15.55 (14.71)^{a}
	24	16.57 (4.75)^{a*}	20.10 (7.20)^{a}	25.10 (8.72)^{a*}	18.43 (2.87)^{a*}	18.15 (10.37)^{a}	11.46 (10.51)^{a}	13.22 (4.72)^{a}	18.07 (8.97)^{a}	18.58 (16.11)^{a}
	36	17.93 (5.55)^{a*}	18.82 (6.41)^{a*}	25.70 (7.51)^{a*}	16.35 (2.54)^{a*}	19.27 (11.63)^{a}	11.28 (11.08)^{a}	9.91 (3.20)^{a}	17.96 (9.82)^{a}	18.22 (16.91)^{a}
Butyrate (B)	0	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}
	10	1.11 (1.72)^{a}	1.85 (1.84)^{a}	1.87 (2.33)^{a}	2.08 (1.47)^{a}	1.11 (1.44)^{a}	1.89 (1.06)^{a}	1.76 (0.86)^{a}	1.89 (1.65)^{a}	2.68 (1.08)^{a*}
	24	2.99 (1.81)^{a}	3.08 (2.13)^{a}	3.32 (3.40)^{ab}	3.67 (1.74)^{ab}	1.66 (1.46)^{a}	2.66 (2.48)^{a}	3.39 (1.92)^{ab}	11.41 (5.31)^{bc}	13.16 (3.29)^{c*}
	36	4.07 (1.75)^{ab}	3.52 (2.31)^{a}	4.49 (4.11)^{ab}	4.09 (1.03)^{ab*}	2.40 (2.02)^{a}	3.24 (3.60)^{a}	3.09 (2.79)^{a}	12.30 (4.64)^{bc*}	13.23 (2.49)^{c*}
Total	0	13.73 (2.70)^{b}	9.61 (1.85)^{ab}	8.80 (1.65)^{a}	8.40 (1.87)^{a}	8.04 (0.42)^{a}	8.23 (0.56)^{a}	8.77 (0.22)^{a}	5.59 (0.67)^{a}	5.77 (1.38)^{a}
	10	72.09(29.09)^{a}	72.60 (33.75)^{a}	72.03 (32.92)^{a}	75.74 (32.56)^{a}	48.64 (27.61)^{a}	56.71 (41.11)^{a}	41.00(11.33)^{a*}	100.15 (7.69)^{a**}	100.03 (1.46)^{a**}
	24	102.84 (27.40)^{a*}	100.77 (7.09)^{a**}	111.35 (6.55)^{a**}	88.71 (21.28)^{a*}	84.05 (44.15)^{a}	59.80 (42.27)^{a}	45.97(13.62)^{a*}	96.24 (4.10)^{a**}	76.52 (10.92)^{a**}
	36	101.80(26.14)^{ab*}	92.95 (6.64)^{ab**}	109.44 (2.12)^{b**}	75.04 (12.69)^{ab*}	83.96 (41.10)^{ab}	55.95 (44.40)^{ab}	34.79 (12.76)^{a}	92.03 (7.57)^{ab**}	71.05 (17.60)^{ab*}
A:P:B	0	1:0.3:0	1:0.4:0	1:0.5:0	1:0.5:0	1:0.5:0	1:0.5:0	1:0.5:0	1:1.2:0	1:1.2:0
	10	1:0.2:0.03	1:0.3:0.03	1:0.3:0.03	1:0.3:0.04	1:0.3:0.04	1:0.3:0.06	1:0.4:0.07	1:0.3:0.03	1:0.4:0.06
	24	1:0.2:0.04	1:0.3:0.04	1:0.3:0.04	1:0.3:0.06	1:0.3:0.04	1:0.3:0.06	1:0.5:0.1	1:0.3:0.2	1:0.5:0.3
	36	1:0.2:0.05	1:0.3:0.05	1:0.3:0.06	1:0.3:0.08	1:0.3:0.05	1:0.3:0.06	1:0.5:0.1	1:0.3:0.2	1:0.5:0.4

[a]Standard deviation is shown in parentheses with n=3. Significant differences ($p<0.05$) between substrates are indicated with different letters in a same row.

[*]Increased significantly from value at 0 h, $p<0.05$; [**]Increased significant differences from value at 0 h, $p<0.01$

The significant increase in acetate at 24 h and 36 h for XOS of avDP 4, 7, 14 and 28 can be linked to the two major acetate producers; *Bifidobacterium* spp. and the *Bacteroides-Prevotella* group.

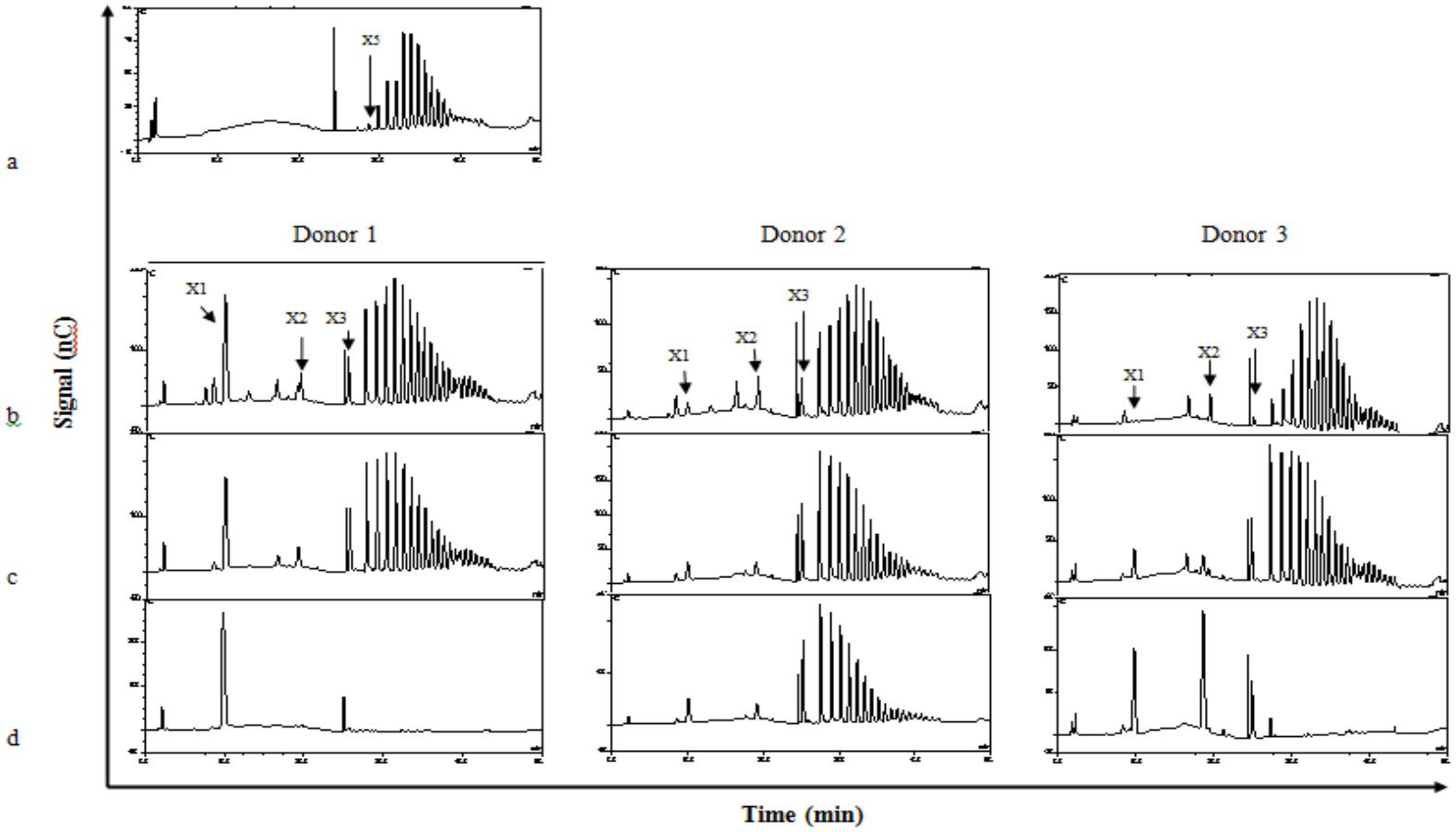
There was no significant increase in butyrate on any OPEFB XOS while commercial XOS resulted in similar butyrate level to FOS ($p \geq 0.05$). Nevertheless, the XOS preparation of lower avDP (4, 14, 28) were not different to commercial XOS ($p \geq 0.05$). Although the human gut microbiota has also been known to be able to further metabolise acetate to butyrate (Duncan, Barcenilla, Stewart, Pryde & Flint, 2002; Duncan et al., 2004), the conversion of acetate from OPEFB XOS to butyrate was generally lower.

The type and molecular weight of the substrates influenced rate and amount of organic acid produced. Based on total organic acid, it is noticeable that commercial XOS and FOS were the fastest fermentable substrates, reaching at least 100 mM at 10 h. As for OPEFB XOS, the three lowest avDP (4, 7, 14) reached 100 mM at 24 h while other fractions of higher avDP (28, 44, 64) had less than 100 mM and birch wood xylan, the least fermentable substrate had the lowest organic acid of all with 46 mM at 24 h.

3.4 Carbohydrate assimilation profile during fermentation

The carbohydrate was profiled in the samples during the course of fermentation using HPAEC-PAD to observe the changes in DP. The assimilation profile of OPEFB XOS of avDP14 from each faecal donor is illustrated in Fig. 3. The three donors showed slight variation in magnitudes and trends that coincides with rather high standard deviation observed in the organic acid data. At 10 h, donor 1 XOS were utilised faster, leaving behind

409 xylose. For donor 2, since the rate of fermentation is slower, some oligosaccharides were
410 still present at 10 h and without much increase of xylose. Donor 3 had a trend between
411 donor 1 and 2 whereby the XOS were also quickly fermented and broken down into xylose,
412 xylobiose and xylotriose. At 24 h there was no detectable sugar remaining in all the culture
413 samples. While the xylose and low DP XOS were being consumed by the bacteria,
414 accumulation could arise from continual digestion of XOS/XPS from the higher DP. This
415 similar degradation characteristic was also observed in XOS (DP 2-6) derived from rice
416 husk when fermented with a single bifidobacteria culture (Gullón et al., 2008).
417 Analysis with HPAEC-PAD however does not provide information on acetyl groups
418 as deacetylation occurs in the high pH eluent used in HPAEC (Kabel et al., 2002a). As such,
419 the chromatogram could not show the susceptibility of acetylated XOS during fermentation.
420



421

422 **Fig. 3.** Degradation profile of OPEFB XOS avDP 14 at different time by faecal culture from three donors using HPAEC-PAD:
423 (a) Substrate before fermentation, (b) Immediately after substrate addition into fermenter, (c) After 5 h, (d) After 10 h. X1, X2,
424 X3 on the chromatogram indicate the position of xylose, xylobiose and xylotriose, respectively.

4. Conclusion

The solubility of high avDP XOS/XPS preparation from OPEFB through autohydrolysis process is rather interesting as it could be incorporated into many food processes. The acetyl group may aid XOS/XPS solubility, however the impact of this on fermentation in the gut was not conclusive from the present results. Nevertheless, the degree of polymerisation has significant influence on OPEFB XOS/XPS fermentability by the gut microflora. The *in vitro* study conducted in this work shows the low avDP XOS (4, 7, 14) were more selective to beneficial bacteria than the higher avDP XPS (22, 44, 64). OPEFB XOS fractions of avDP 14 appeared to be the most bifidogenic.

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