

# *In vitro fermentability of xylo-oligosaccharide and xylo-polysaccharide fractions with different molecular weights by human faecal bacteria*

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1 ***In vitro* fermentability of xylo-oligosaccharide and xylo-polysaccharide fractions with**  
2 **different molecular weights by human faecal bacteria**

3

4 Ai Ling Ho<sup>a,1</sup>, Ondrej Kosik<sup>b</sup>, Alison Lovegrove<sup>b</sup>, Dimitris Charalampopoulos<sup>a</sup>, Robert A.  
5 Rastall<sup>a\*</sup>

6

7 <sup>a</sup>Department of Food and Nutritional Sciences, University of Reading, Whiteknights, P.O.  
8 Box 226, Reading RG6 6AP, United Kingdom.

9 <sup>b</sup>Centre for Crop Genetic Improvement, Dept. of Plant Biology and Crop Science,  
10 Rothamsted Research, Harpenden, Herts AL5 2JQ, United Kingdom.

11

12 \*Corresponding author (Tel.: +44 (0)118 378 6726; Fax: +44 (0)118 931 0080)

13

14 E-mail addresses: alho@ums.edu.my (A.L. Ho), ondrej.kosik@rothamsted.ac.uk (O. Kosik),  
15 alison.lovegrove@rothamsted.ac.uk (A. Lovegrove), d.charalampopoulos@reading.ac.uk (D.  
16 Charalampopoulos), r.a.rastall@reading.ac.uk (R. A. Rastall)

17

18 <sup>1</sup>Present/permanent address: Faculty of Food Science and Nutrition, Universiti Malaysia  
19 Sabah, Jalan UMS, 88450 Kota Kinabalu, Sabah, Malaysia.

20

21

22 **Abstract**

23 Xylo-oligosaccharides and xylo-polysaccharides (XOS, XPS) produced by autohydrolysis  
24 of the fibre from oil palm empty fruit bunches (OPEFB) were purified using gel filtration  
25 chromatography to separate the XOS and XPS from the crude autohydrolysis liquor. Six  
26 mixed fractions of refined XOS and XPS with average degree of polymerisation (avDP) of  
27 4-64 were obtained. These were characterised in terms of their composition and size by  
28 HPLC, MALDI-ToF-MS (selected fractions) and carbohydrate gel electrophoresis (PACE).  
29 They were assessed in batch culture fermentations using faecal inocula to determine their  
30 ability to modulate the human faecal microbiota *in vitro* by measuring the bacterial growth,  
31 organic acid production and the XOS assimilation profile. The gut microbiota was able to  
32 utilise all the substrates and there was a link between the XOS/XPS degree of  
33 polymerisation with the fermentation properties. In general, XOS/XPS preparations of  
34 lower avDP promote better *Bifidobacterium* growth and organic acid production.

35

36 **Keywords**

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

39

40 **1. Introduction**

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

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

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

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

67 al., 2002; Okazaki, Fujikawa & Matsumoto, 1990; Palframan, Gibson & Rastall, 2003a)  
68 and in *in vivo* studies in humans and animals (Campbell, Fahey & Wolf, 1997; Childs et al.,  
69 2014; Chung, Hsu, Ko & Chan, 2007). Pure culture studies using XOS from corn cob and  
70 rice husk autohydrolysis with  $DP \leq 4$  also enhanced the growth of bifidobacteria despite  
71 having some acetyl groups and/or uronic acid substituents (Gullón et al., 2008; Moura et  
72 al., 2007). In Kabel, Kortenoeven, Schols & Voragen (2002a), a XOS preparation with  
73 wider range mixed DP (DP 2-11) was used and when fermented *in vitro* with human faecal  
74 inocula, the substrate was almost completely degraded in 20-40 h of fermentation. The  
75 fermentation rate and the SFCA profiles however varied depending on the substituents that  
76 were present, whereby the linear XOS and arabinose substituted XOS (AXOS) were  
77 fermented faster than acetylated XOS and methylglucuronylated XOS was the slowest. The  
78 bacteriology profile however was not the focus in that study, so the way the substituents  
79 modulate the gut microbiota is unknown. Increases in potentially health-positive bacterial  
80 groups such as *Bifidobacterium* spp. and *Lactobacillus/Enterococcus* spp. were seen with  
81 high average molecular weight arabinoxylans of 66,278 and 354 kDa (Hughes, Shewry, Li,  
82 Gibson, Sanz & Rastall, 2007). However, the arabinoxylans tested also significantly  
83 promoted clostridial growth. Van Craeyveld (2008) in a more systematic study on the  
84 influence of the average degree of polymerisation (avDP) and average degree of arabinose  
85 substitution (avDAS) of XOS preparation in the cecum of rats, showed that low molecular  
86 weight AXOS (avDP-avDAS of 5-0.27 and 3-0.26) increased *Bifidobacterium* spp.  
87 significantly more than high molecular weight AXOS (avDP –avDAS of 61-0.58). On the  
88 other hand, the measured branched SCFA was the lowest with avDP 61, so this could  
89 potentially suppress the metabolites from protein fermentation.

90           In a previous study, results have demonstrated that it is possible to produce purified  
91   XOS fractions of a variety of avDP from oil palm biomass autohydrolysis liquor (Ho et al.,  
92   2014). The aim of this work was to study the effect of XOS and XPS obtained from  
93   purification of autohydrolysed OPEFB at different avDP 4-64 upon the gut microbiota  
94   population. The rationale for inclusion of higher avDP XOS/XPS preparations in this study  
95   is they may have better persistence into the distal colon, with potential benefits to chronic  
96   gut diseases.

97

## 98   **2. Materials and methods**

### 99   2.1 Preparation of XOS/XPS fractions

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

109

### 110   2.2 Characterization of XOS/XPS fractions

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

113 The apparent molar mass of samples was determined by high performance liquid  
114 chromatography (HPLC) (Agilent 1100 series, Winnersh, UK). A size exclusion column  
115 BIOSEP-SEC S2000 (Phenomenex, Cheshire, UK) was used at 30 °C with 50 mM NaNO<sub>3</sub>  
116 as mobile phase at 0.7 mL/min. The eluate was detected using a refractive index (RI)  
117 detector. External standards with different molecular weights, i.e. xylose,  
118 maltooligosaccharides (DP 2-5) and dextrans (1-71 kDa, Sigma, Dorset, UK) were used for  
119 calibration.

120 The composition of the XOS/XPS samples was assayed by HPLC to quantify free  
121 monosaccharides (glucose, xylose and arabinose), aliphatic acids (acetic acid, formic acid  
122 and levulinic acid) and furan derivatives (furfural and 5-hydroxymethylfurfural, HMF)  
123 compounds. An Aminex HPX-87H column (BioRad, Hemel Hempstead, UK) was used at  
124 50 °C with 5 mM H<sub>2</sub>SO<sub>4</sub> as mobile phase. The monosaccharides and aliphatic acids were  
125 detected with a RI detector while furfural and HMF were detected using a diode array  
126 detector (DAD) at 280 nm.

127 The oligosaccharide content was determined by an indirect method using  
128 quantitative acid hydrolysis; this was done by mixing the XOS/XPS sample with H<sub>2</sub>SO<sub>4</sub>  
129 (72 % w/w) to obtain a final acid concentration of 4 % (w/w) and the sample was heated at  
130 121 °C for 60 min to induce hydrolysis. The post hydrolysed liquor was analysed with  
131 HPLC and the oligosaccharide concentration was expressed as the increase in sugar  
132 monomers (Sluiter et al., 2006).



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

135

### 136 2.3 MALDI-ToF-MS of XOS fractions

137           MALDI-ToF-MS was used to analyse the extracted OPEFB XOS fractions in native  
138 and permethylated form for XOS avDP 4, 7 and 14. Initial analysis of the native fractions  
139 did not produce strong signals (Figure S1) so analyses were also performed with  
140 permethylated fractions. Permethylation of XOS fractions was performed using the  
141 NaOH/DMSO slurry method using 0.5 mL of methyl iodide (Ciucanu & Kerek, 1984).  
142 Permethylated glycans were dried under a stream of nitrogen and re-dissolved in 100  $\mu$ L of  
143 methanol. Five  $\mu$ L of native or permethylated XOS fractions (10 mg/mL) were mixed with  
144 5  $\mu$ L of 2, 5-dihydroxybenzoic acid (DHB, 10 mg/mL dissolved in 50 % MeOH with 1%  
145 TFA, v/v) matrix. One  $\mu$ L of native or permethylated glycans mixed with DHB matrix was  
146 spotted onto a MALDI target plate and allowed to air-dry. MALDI-ToF-MS was carried  
147 out using a Micromass MALDI-LR mass spectrometer (Waters, Manchester, UK) using a  
148 mass acquisition between  $m/z$  450 and 3 000. The MALDI set-up was as described by  
149 Marsh et al. (2011). Glycan adduct ions  $[M + Na]^+$  were assigned. Experimentally  
150 determined masses were interpreted using GlycoMod (ExPaSy).

151

### 152 2.4 Analysis of XOS/XPS fractions by carbohydrate gel electrophoresis

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

155 digestion with xylanase 11. Briefly, for xylanase digested samples, 200 µg aliquots were  
156 digested with Xyn11 (4 µL ≈ 21.92 µg; Prozomix, UK) for 16 h at 40 °C in total volume of  
157 500 µL. Digestion was terminated by boiling the samples for 30 min and samples were  
158 dried *in vacuo*. Aliquots (200 µg) of undigested XOS/XPS fractions were also dried down.  
159 All samples together with standard xylo-oligosaccharides (Xyl<sub>1-6</sub>; Megazyme, Ireland) were  
160 labelled with ANTS and ran on acrylamide gel as described by Kosik, Bromley, Busse-  
161 Wicher, Zhang & Dupree (2012). Gels were visualized under UV light using a GelDoc-It  
162 TS2 imager (UVP, Germany) equipped with a GFP emission filter (513-557 nm).

163

#### 164 2.5 *In vitro* batch fermentation

165         The six different fractions of XOS/XPS along with commercial XOS (avDP2,  
166 Shandong Longlive Biotechnology Co. Ltd (SLBC), China) and birch wood xylan (Sigma,  
167 Dorset, UK) were evaluated for the ability to modulate the gut microbiota using an *in vitro*  
168 batch culture fermentation system inoculate with human faecal sample.  
169 Fructooligosaccharides (avDP 4, Raftilose®, Orafti, Tienan, Belgium) were used as the  
170 positive control. The *in vitro* fermentation was carried out a 50 mL working volume glass  
171 jacketed bioreactors, sterile of stirred batch culture fermentation system. The carbohydrates  
172 sources were added at 1 % (w/v). The basal medium (per litre) consisted of: 2 g peptone  
173 water, 2 g yeast extract, 0.1 g NaCl, 0.04 g K<sub>2</sub>HPO<sub>4</sub>, 0.04 g KH<sub>2</sub>PO<sub>4</sub>, 0.01 g MgSO<sub>4</sub>·7H<sub>2</sub>O,  
174 0.01 g CaCl<sub>2</sub>·6H<sub>2</sub>O, 2 g NaHCO<sub>3</sub>, 2 mL Tween 80, 0.05 g haemin, 0.01 mL vitamin K<sub>1</sub>, 0.5  
175 g L-cysteine-HCl, 0.5 g bile salt and 4 mL resazurin solution (0.25 g/L).

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

182           The fermentation was carried out at pH 6.7-6.9, controlled using an automated pH  
183 controller (Fermac 260; Electrolab, Tewkesbury, UK) and at 37 °C (using a  
184 thermocirculator) under anaerobic atmosphere, which was achieved through continuous  
185 sparging with nitrogen gas. Samples (5 mL) were taken from each fermentation vessel at 0,  
186 10, 24 and 36 h for organic acid analysis and bacterial enumeration using the fluorescent *in*  
187 *situ* hybridisation (FISH) technique.

188

## 189 2.6 Enumeration of bacteria

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

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

213 The probes used were Bif164 (Langendijk et al., 1995), Bac303 (Manz, Amann,  
214 Ludwig, Vancanneyt & Schleifer, 1996), Lab158 (Harmsen, Elfferich, Schut & Welling,  
215 1999), Ato291 (Harmsen, et al., 2000), Prop853 (Walker, Duncan, McWilliam Leitch,  
216 Child & Flint, 2005), Erec482 (Franks et al., 1998), Rrec584 (Walker et al., 2005),  
217 Fprau655 (Hold, Schwiertz, Aminov, Blaut & Flint, 2003), Chis150 (Franks et al., 1998),  
218 and mixed Eub338 I, II, III (Daims, Brühl, Amann, Schleifer & Wagner, 1999) for  
219 enumerating *Bifidobacterium* spp., *Bacteroides-Prevotella*, *Lactobacillus-Enterococcus*,

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

224

## 225 2.7 Organic acid analysis

226 An aliquot (1 mL) of sample from each sampling time was centrifuged at 13 000 x g  
227 for 10 min and the supernatant was stored at -20 °C until analysis. Organic acids analysis  
228 was performed using an HPLC (1100 series; Agilent, Winnersh, UK) with refractive index  
229 detection. Prior to the analysis, the samples, after thawing, were centrifuged at 13 000 x g  
230 for 10 min and the supernatants were filtered through a 0.22 µm filter unit. An ion  
231 exclusion column, Rezex ROA-Organic Acid H+ (8%) (Phenomenex, Cheshire, UK) was  
232 used for the analysis, using 2.5 mM H<sub>2</sub>SO<sub>4</sub> as eluent. The column was heated at 84 °C and  
233 the eluent flow rate was set at 0.5 mL/min. The injection volume used was 20 µL with 40  
234 min run time. Organic acids were quantified using standard calibration curves for lactate,  
235 acetate, propionate, butyrate and valerate at concentrations of 12.5, 25, 50, 75 and 100 mM.  
236 Formate was determined using a formate dehydrogenase-based assay kit (Megazyme,  
237 Ireland).

238

## 239 2.8 Carbohydrate assimilation profile during fermentation

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

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

252

## 253 2.9 Statistical analysis

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

260

## 261 **3. Results and discussion**

### 262 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

266 **Table 1**  
 267 Composition of OPEFB fibre fractions (g/100 g freeze dried sample) obtained after GFC purification<sup>a</sup>  
 268

Sample no.	avDP <sup>b</sup>	Residues in linkage (g/100 g)				Ratio <sup>c</sup>		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

<sup>a</sup>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.

<sup>b</sup>avDP – Average degree of polymerization as determined by size exclusion chromatography

<sup>c</sup>Ratio in mol/mol

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

269

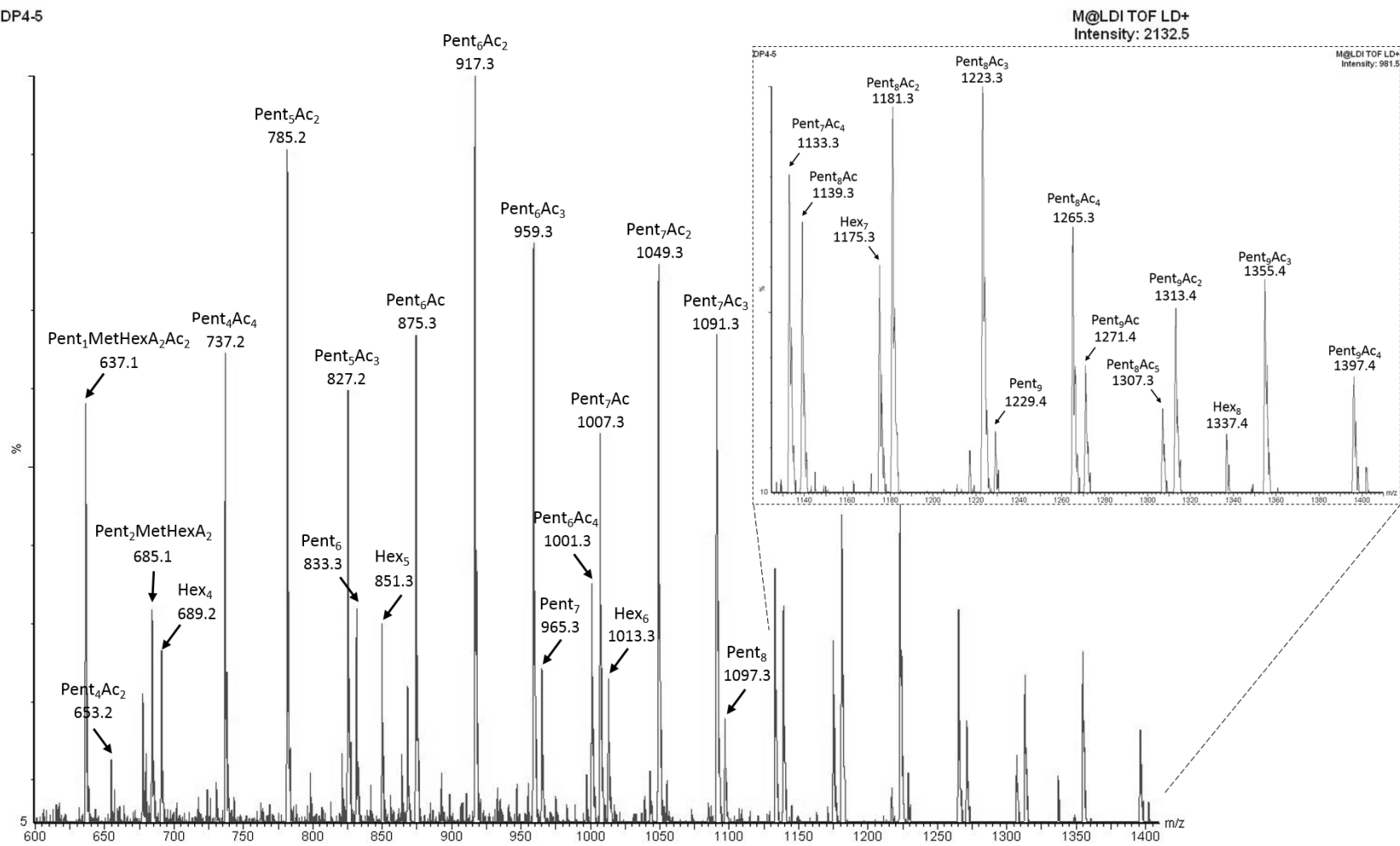


270 oligosaccharides, accounting for 78-83 % of the total oligosaccharides. The highest  
271 XOS/XPS yield was found in the middle fractions (avDP 14 and 28); free monomeric  
272 compounds (xylose and acetic acid) were present at slightly higher concentrations in XOS  
273 fractions with lower DP (avDP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

282 OPEFB fractions (avDP 4, 7 and 14) were analysed by MALDI-ToF-MS (larger  
283 avDP fractions were too large for MALDI-ToF-MS analysis). XOS/XPS fractions were all  
284 analysed in both their native and permethylated forms by MALDI-ToF-MS. All XOS/XPS  
285 fractions analysed in their native form showed acetylated pentose oligosaccharide ions  
286 (labelled  $\text{Pent}_n\text{Ac}_n$ , the  $n$  denoting the number of pentose (Pent) or acetyl (Ac) groups  
287 respectively). In avDP 4 the most dominant ion is  $m/z$  917.27 ( $\text{Pent}_6\text{Ac}_2$ ) (Fig. 1) with  
288 acetylated oligosaccharides ranging from  $\text{Pent}_4\text{Ac}_2$  ( $m/z$  653.19) to  $\text{Pent}_9\text{Ac}_4$  ( $m/z$  1397.42).  
289 Also present are pentose oligosaccharides with no acetylation or other modifications with  
290 DP 6 to 9 ( $m/z$  833.25 to 1229.38) and hexose oligosaccharides of DP 4-8 ( $m/z$  689.21 to  
291 1337.42). There could also be small pentose oligosaccharides with methylated-glucuronic  
292 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<sub>3</sub> (*m/z* 549.25) up to Pent<sub>9</sub> (*m/z* 1509.69) and ladder of pentose  
296 oligosaccharide substituted with single glucuronic acid up to DP 8 (Pen<sub>1</sub>HexA<sub>1</sub>, *m/z* 447.18  
297 to Pen<sub>7</sub>HexA<sub>1</sub>, *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<sub>3</sub> *m/z* 681.33- Hex<sub>8</sub> *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<sub>5</sub>Ac<sub>2</sub>) up to  
304 Pent<sub>9</sub>Ac<sub>5</sub> 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<sub>4</sub>, *m/z*  
307 885.43 to Hex<sub>10</sub> *m/z* 2110.03) as well as pentose oligosaccharide substituted with  
308 glucuronic acid (Pent<sub>2</sub>HexA<sub>1</sub>, *m/z* 607.26 to Pent<sub>9</sub>HexA<sub>1</sub>, *m/z* 1727.77). In OPEFB the  
309 avDP 14 fraction (Supplementary Fig. 1c) acetylated pentose oligosaccharides range from  
310 Pent<sub>5</sub>Ac<sub>2</sub>, *m/z* 785.23 to Pent<sub>18</sub>Ac<sub>6</sub>, *m/z* 2669.82. Also, observed in the permethylated  
311 avDP14 (Supplementary Fig.1d) are glucuronic acid substituted pentoses, Pent<sub>3</sub>HexA<sub>1</sub> (*m/z*  
312 767.33) to Pent<sub>13</sub>HexA<sub>1</sub> (*m/z* 2368.07).

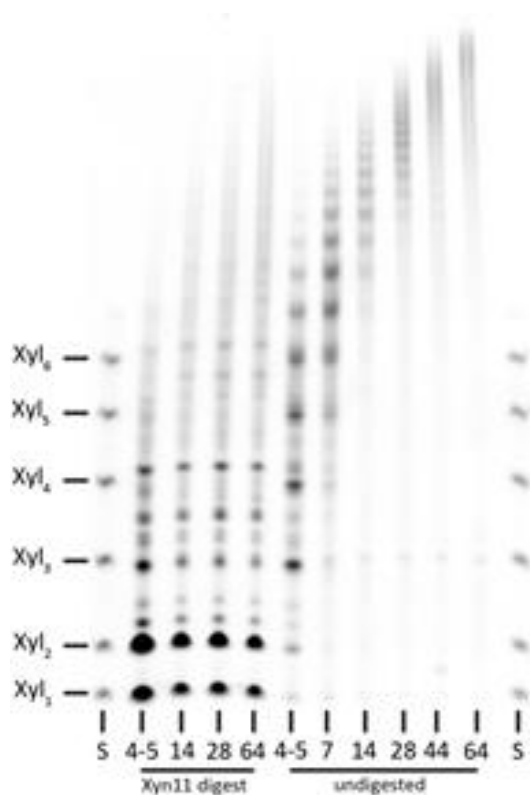
DP4-5



313

314 **Fig. 1.** MALDI-ToF-MS spectrum of native glycans isolated from OPEFB XOS fraction avDP 4. The glycan adduct ions  
 315  $[M+Na]^+$  are indicated for acetylated pentose oligosaccharides (Pent<sub>n</sub>Ac<sub>n</sub>), for pentose oligosaccharides (Pent<sub>n</sub>), for pentoses with  
 316 methylated-glucuronic acid substitution (Pent<sub>n</sub>MetHexA<sub>n</sub>Ac<sub>n</sub>) and for hexose oligosaccharides (Hex<sub>n</sub>).

317 The DP ranges of the OPEFB fractions obtained from MALDI-ToF-MS analysis  
 318 were as follows: avDP 4 (DP 2-9), avDP 7 (DP 3-12), avDP 14 (DP 3-18). The OPEFB  
 319 fractions were also xylanase cleaved and visualised by polysaccharide analysis using  
 320 carbohydrate gel electrophoresis (PACE) (Fig.2) which confirms the predominant  
 321 oligosaccharides were xylo-oligosaccharides and that the gel filtration fractionation of the  
 322 avDP 4 to avDP 64 contained similar oligosaccharides but with increasing xylose chain  
 323 length.  
 324



325  
 326

327 **Fig. 2.** PACE gel showing separation of extracted OPEFB XOS fractions digested with  
 328 Xyn11 and undigested. S - Standard xylose<sub>1-6</sub> ladder; 4-5 = avDP 4; 14 = avDP 14; 28 =  
 329 avDP 28 and 64 = avDP 64, digested with Xyn11. 4-5 = avDP 4; 7 = avDP 7; 14 = avDP  
 330 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 ( $p \geq 0.05$ ). 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<sup>a</sup>

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

<sup>a</sup>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&lt;0.05; \*\*Significant differences from value at 0 h, p&lt;0.01 (Value at 0 h is shown in the far left under ‘Probe’ column)

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

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

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



**Table 3**Mean organic acid concentrations in pH-controlled batch cultures at 0, 10, 24 and 36 h<sup>a</sup>

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

<sup>a</sup>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&lt;0.05; \*\*Increased significant differences from value at 0 h, p&lt;0.01

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

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

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

402

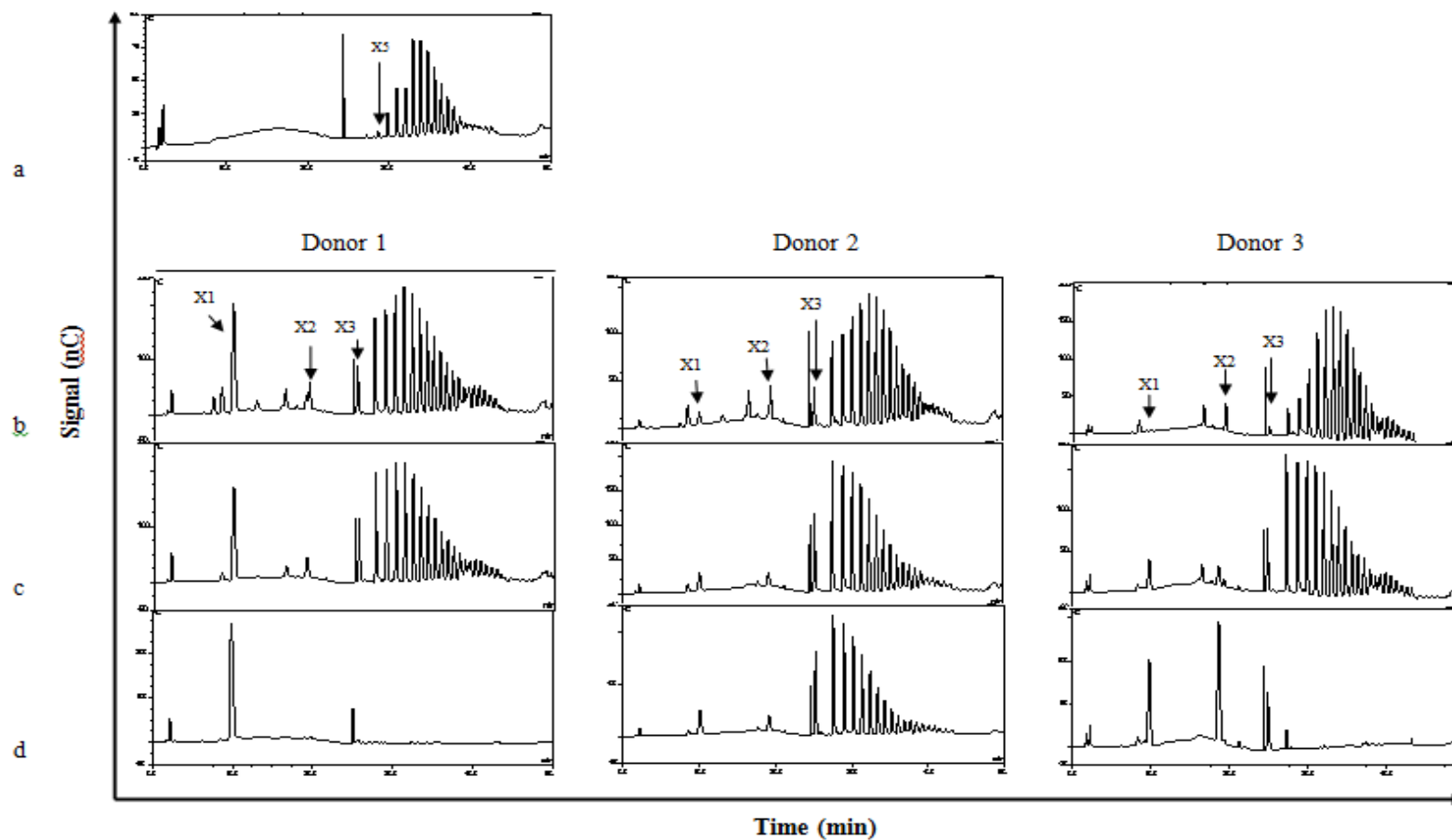
### 403 3.4 Carbohydrate assimilation profile during fermentation

404           The carbohydrate was profiled in the samples during the course of fermentation using  
405 HPAEC-PAD to observe the changes in DP. The assimilation profile of OPEFB XOS of  
406 avDP14 from each faecal donor is illustrated in Fig. 3. The three donors showed slight  
407 variation in magnitudes and trends that coincides with rather high standard deviation  
408 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.

425 **4. Conclusion**

426           The solubility of high avDP XOS/XPS preparation from OPEFB through  
427 autohydrolysis process is rather interesting as it could be incorporated into many food  
428 processes. The acetyl group may aid XOS/XPS solubility, however the impact of this on  
429 fermentation in the gut was not conclusive from the present results. Nevertheless, the  
430 degree of polymerisation has significant influence on OPEFB XOS/XPS fermentability by  
431 the gut microflora. The *in vitro* study conducted in this work shows the low avDP XOS (4,  
432 7, 14) were more selective to beneficial bacteria than the higher avDP XPS (22, 44, 64).  
433 OPEFB XOS fractions of avDP 14 appeared to be the most bifidogenic.

434

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441

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