

*Oat bran, but not its isolated bioactive  $\beta$ -glucans or polyphenols, have a bifidogenic effect in an in vitro fermentation model of the gut microbiota*

Article

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1 Oat bran, but not its isolated bioactive  $\beta$ -glucans or polyphenols, have a bifidogenic  
2 effect in an *in vitro* fermentation model of the gut microbiota<sup>1-4</sup>

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12 short chain fatty acids, prebiotic, bifidogenic

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19

## 20 Abstract

21 Wholegrain oats are known to modulate the human gut microbiota and have prebiotic  
22 properties (increase the growth of some health promoting bacterial genera within the colon).  
23 Research to date mainly attributes these effects to the fibre content; however, oat is also a rich  
24 dietary source of polyphenols, which may contribute to the positive modulation of gut  
25 microbiota. *In vitro* anaerobic batch-culture experiments were performed over 24 h to evaluate  
26 the impact of two different doses (1 and 3 % w/v) of oat bran, matched concentrations of  $\beta$ -  
27 glucan extract or polyphenol mix, on the human faecal microbiota composition using 16S RNA  
28 gene sequencing and short chain fatty acid analysis (SCFA). Supplementation with oats  
29 increased the abundance of Proteobacteria ( $p < 0.01$ ) at 10 h, Bacteroidetes ( $p < 0.05$ ) at 24h  
30 and concentrations of acetic and propionic acid increased at 10 and 24 h compared to negative  
31 control. Fermentation of the 1 % w/v oat bran resulted in significant increase in SCFA  
32 production at 24 h ( $86 \pm 27$  mM vs.  $28 \pm 5$  mM;  $p < 0.05$ ) and a bifidogenic effect, increasing  
33 the relative abundance of *Bifidobacterium unassigned* at 10h and *Bifidobacterium adolescentis*  
34 ( $p < 0.05$ ) at 10 and 24 h compared to negative control. Considering the  $\beta$ -glucan treatment  
35 induced an increase in the phylum Bacteroidetes at 24 h, it explains the Bacteroidetes effects  
36 of oats as a food matrix. The polyphenol mix induced an increase in Enterobacteriaceae family  
37 at 24 h. In conclusion, in this study, we found that oats increased bifidobacteria, acetic acid and  
38 propionic acid, and this is mediated by the synergy of all oat compounds within the complex  
39 food matrix, rather than its main bioactive  $\beta$ -glucan or polyphenols. Thus oats as a whole food  
40 led to the greatest impact on the microbiota.

41 Keywords: oats,  $\beta$ -glucan, polyphenols, gut microbiota, 16S rRNA gene sequencing, short  
42 chain fatty acids, prebiotic, bifidogenic

43

## 44 1. Introduction

45 A large body of evidence from prospective and intervention studies suggests that a diet rich in  
46 oats could significantly reduce the risk of bowel disease [1, 2], cardiovascular disease [3-5]  
47 and lowers high blood cholesterol levels [4-7]. Recent reports suggest that oats may act as a  
48 prebiotic, modulating the gut microbiota and impacting on metabolic disease risk [8, 9]. To  
49 date, it is believed that the protective effect of whole grain oats is mainly due to the presence  
50 of dietary fibre, in particular, soluble  $\beta$ -glucan [6, 10-12], and resistant starch [13].  $\beta$ -glucan is  
51 known to lower cholesterol and bile acid absorption through formation of viscous gels in the  
52 upper gut [14] and/or directly binding of cholesterol or bile acids. Resistant starch leads to the  
53 production of short chain fatty acids (SCFA) in the colon and may also increase the growth of  
54 some health promoting bacterial genera within, such as *Bifidobacterium* and *Lactobacillus* [15-  
55 18]. Species of these genera influence the cholesterol metabolism through increasing bile-salt  
56 hydrolase enzyme activity and the deconjugation of bile acids [19].

57 Oats, however, are also rich dietary sources of polyphenols, including avenanthramides and  
58 phenolic acids [20]. These are likely to contribute to the health effects of a diet rich in oats [21-  
59 23] but have not yet been examined in detail. Phenolic acids are found in three different forms  
60 within the oat food matrix: as soluble free acids, as soluble conjugates esterified to low  
61 molecular weight components such as sugars, and as insoluble bound acids esterified to high  
62 molecular weight components including lignin, cell wall polysaccharides and storage proteins  
63 [20]. A large proportion of oat polyphenols are bound via an ester bond, and hence are poorly  
64 absorbed in the upper intestine and reach the colon [24], where they may beneficially modulate  
65 the microbiota [8]. There are no esterases in human tissues that break these ester links [25];  
66 therefore, the main catalytic site is in the colon through cinnamoyl esterase activity of human

67 faecal microbiota, such as *Escherichia coli* (three isolates), *Bifidobacterium lactis* and  
68 *Lactobacillus gasseri* (two strains) [26, 27].

69 Gut microbiota (GM) alterations by whole grain oats have been observed in a number of *in*  
70 *vitro* fermentation models [9, 28-32] and animal studies [13, 33-35]. The fermentation of oats  
71 has led to increased *Bifidobacterium*, *Lactobacillus* [8, 9, 30, 31, 33, 34] and *Bacteroides* [32]  
72 populations and, at the same time decreased *Clostridium* [30, 33].

73 The soluble  $\beta$ -glucans have been one of the most commonly studied components of oats [28,  
74 36, 37]. However, oat-derived isolated  $\beta$ -glucan only impacted on *Bacteroides* growth, not on  
75 *Bifidobacterium* in 24h pH-controlled anaerobic batch culture fermenters [28]. Furthermore,  
76 Crittenden *et al.* [38] reported that  $\beta$ -glucan was fermented by *Bacteroides* spp. but not by  
77 *Lactobacillus* or *Bifidobacterium*. The viscosity and molecular weight of  $\beta$ -glucan may  
78 strongly influence its ability to lead to SCFA production and act as a prebiotic [39].  
79 Additionally, these studies have used the relatively high concentration of 1% v/w of  $\beta$ -glucan  
80 dose [31, 40] which is much higher than the physiological  $\beta$ -glucan concentration delivered  
81 from eating oats. To date, none of the oat or  $\beta$ -glucan *in-vitro* fermentation studies assessed the  
82 polyphenol content of their treatment [9, 28, 32]. Thus, little information exists on the ability  
83 of oat-derived avenanthramides, hydroxycinnamic and hydroxybenzoic acids to influence the  
84 GM.

85 The main purpose of the present *in vitro* study was to examine if a physiologically relevant  
86 dose of polyphenol mix (avenanthramide, hydroxycinnamic acids and benzoic acid  
87 derivatives),  $\beta$ -glucan extract or digested oat bran as whole food matrix beneficially modulates  
88 the GM. Secondly, the study aimed to assess the impact of different doses of digested oat bran  
89 on the GM, notably using an oat bran that was well characterised in terms of physicochemical  
90 properties.

91

92

## 93 2. Materials and Methods

## 94 2.1. Reagents

95 All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co Ltd. (Pool,  
96 Dorset, UK) or Fisher (Loughborough, Leics, UK) unless stated otherwise. Mixed-linkage  $\beta$ -  
97 glucan kit was supplied by Megazyme Co (Wicklow, Ireland). The anaerobic jar (Anaerojar<sup>TM</sup>  
98 2,5L) and gas-generating kit (AnaeroGen <sup>TM</sup>) were obtained from Oxoid Ltd (UK), the  
99 dialysis tube from Spectrum (VWR International). Media and instruments were autoclaved at  
100 121°C for 15 min. HPLC column and guard cartridges were obtained from Phenomenex  
101 (Cheshire, UK). PowerSoil<sup>®</sup>DNA Isolation Kit was purchased from Mo Bio Laboratories, Inc  
102 (USA), the primers for the 16S rRNA gene amplification from Integrated DNA Technologies,  
103 BVBA (Belgium), AccuPrime<sup>TM</sup> SuperMix II from Life Technologies (CA, USA), AMPure  
104 XP beads from Beckman Coulter Genomic (CA, USA).

## 105 2.2. Oats and Controls

106 The oat bran was purchased from White's (Tandragee, Northern Ireland). The oat  
107 macronutrient composition was analysed by Campden BRI laboratories (Total carbohydrate,  
108 Available carbohydrate, Total dietary fibre- AOAC 991.43 method, Total fat – BS 4401, and  
109 protein – AOAC 981.10 method), whereas the detailed polyphenol content was measured in  
110 our laboratory at the University of Reading based on a previous method [41]. The beta-glucan  
111 method used was employed specifically to quantify 1,3:1,4- $\beta$ -D-glucan. Synergy1 the  
112 oligofructose-enriched inulin was supplied by Beneo (Belgium) and 94%- $\beta$ -glucan extract  
113 from Megazyme Co (Wicklow, Ireland). Polyphenols were purchased from Sigma-Aldrich  
114 Chemical Co Ltd. (Pool, Dorset, UK).

### 115 2.3. *In vitro* digestion of oat bran (from mouth to small intestine)

116 The method employed was adapted from Mills *et al.* [42]. Oat bran was digested *in vitro* in  
117 three phases: the oral phase, the gastric phase and the small intestinal phase. 60 g of oat bran  
118 was mixed with 150 ml of sterile and distilled water and homogenised, transferred into a 500  
119 ml Duran bottle, and microwaved for 1 minute. In the oral phase, 20 mg of  $\alpha$ -amylase was  
120 dissolved in 6.25 ml  $\text{CaCl}_2$  (1 mM, pH 7.0) and added to the solution, then incubated at 37°C  
121 for 30 minutes on a shaker set at 120 *xg*. After incubation, the pH was adjusted to 2.0 with 6  
122 M HCl solution and the gastric phase introduced by adding 2.7 g pepsin in 25 ml HCl (0.1 M)  
123 and further incubated for 2 hours under the same conditions. In the small intestinal phase, 560  
124 mg pancreatin and 3.5 g bile were mixed with 125 ml  $\text{NaHCO}_3$  (0.5 M) and dispensed into the  
125 mix. The pH was adjusted to 7.0 then incubated for 3 hours at 37 °C with shaking. Finally, the  
126 sample solution was transferred to a seamless semi-permeable 100-500 Dalton molecular  
127 weight cut-off regenerated cellulose dialysis tubing and dialysed against NaCl (0.01 M at 5°C)  
128 to remove low molecular mass digestion products. After 15 hours, the dialysis fluid was  
129 changed and the process continued for an additional 2 hours. The digested oat bran mix was  
130 collected and transferred into several 250 ml clear plastic containers, frozen to -80° and freeze-  
131 dried for 5 days to remove all fluid content.

### 132 2.4. Extraction and analyses of polyphenols from undigested and digested oat bran

133 Polyphenols were extracted from undigested (raw) and digested (after *in vitro* digestion) oat  
134 bran in two separate fractions (i.e. free and conjugated or bound) using the method of Schar *et*  
135 *al.* [41]. The phenolic acids and avenanthramides in oat extracts were identified and quantified  
136 using a high-performance liquid chromatography (HPLC) Agilent 1100 series (Agilent  
137 Technologies Ltd) equipped with a quaternary pump, autosampler, column thermostat, sample  
138 thermostat and photo diode array detector. Compound separation was achieved by a Kinetex



139 biphenyl column (100 Å 250 x 4.6 mm length, 5 µm particle size; Phenomenex) and using a  
140 gradient elution. Mobile phase A consisted of 0.1 % (v/v) formic acid in HPLC water (A),  
141 while mobile phase B was 0.1 % (v/v) formic acid in methanol. The following optimised  
142 gradient protocol was run: 0 min, 95% A, 5 % B; 20 min, 75 % A, 25 % B; 25 min, 74 % A,  
143 26 % B; 30 min, 65 % A, 35 % B; 40 min, 64 % A, 36 % B; 53 min, 30 % A, 70 % B; 56 min,  
144 5 % A, 95 % B; 61 min, 5 % A, 95 % B; 62 min, 95 % A, 5 % B; 65 min, 95 % A, 5 % B. The  
145 flow rate of the mobile phase was 1.0 ml/min and the sample injection volume was 20 µl [43].  
146 The absorbance was recorded at 254, 280 and 320 nm and quantification was based on 12 point  
147 linear calibration curves (mean  $R^2 > 0.99$ ) and as a ratio to the internal standard (i.e. 3,5-  
148 dichloro-4-hydroxybenzoic acid) to account for losses during extraction ( $R^2 \geq 0.99$ ).

149

#### 150 2.4. Determination of the $\beta$ -glucan content

151 The  $\beta$ -glucan content was analysed with the 1,3:1,4 mixed-linkage  $\beta$ -glucan kit. The assay uses  
152 lichenase and  $\beta$ -glucosidase to metabolize  $\beta$ -glucan to  $\beta$ -gluco-oligosaccharides and  
153 subsequently glucose. Glucose then reacts with GOPOD reagent and its absorbance was  
154 measured at 510 nm by UltroSpec 1100 photo spectrometer (Scintek Instruments LLC, USA).  
155 The final  $\beta$ -glucan content was calculated by the Megazyme Mega-Calc™ tool [44].

156 The molecular weight of the  $\beta$ -glucan was determined by size-exclusion HPLC. The  
157 chromatography system consisted of three serially connected columns (Shodex SB-G, Shodex  
158 SB-806M, Shodex SB-804 HQ) and an UV-MALLS-Viscometer-dRI detector. The column  
159 temperature was set at 40°C, the mobile phase was MiliQ water (Millipore, Bedford, MA)  
160 containing 0.02 % sodium azide, and the flow rate set at 0.5 ml/min. Samples were prepared at  
161 a concentration of 10 mg/ml, heated at 60°C for 3 hours under constant shaking, syringe filtered  
162 (0.45 µm PVD; Whatman, NY) and diluted to a concentration of 1 mg/ml before injection.

## 163 2.5. pH controlled faecal batch culture fermentation

164 Substrate ability to modulate gut microbiota was determined using anaerobic, stirred, pH and  
165 temperature controlled faecal batch cultures. Batch culture fermentation vessels (300 ml  
166 volume: one vessel per treatment) were sterilised and filled with 135 ml of sterile basal medium  
167 (g/l: 2 g peptone, 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  
168 MgSO<sub>4</sub>·7H<sub>2</sub>O, 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 Hemin (dissolved in  
169 a few drops of 1 M NaOH), 10 µl vitamin K, 0.5 g L-cysteine HCl, 0.5 g bile salts and 4ml of  
170 resazurin solution (0.02 %)). Before addition to the vessel, the medium was adjusted to pH 7.0  
171 and autoclaved. The sterile medium was gassed overnight with O<sub>2</sub>-free N<sub>2</sub> (15 ml / min) to  
172 establish anaerobic conditions. To mimic the proximal region of the human large intestine, pH  
173 was held in the range of 6.7 - 6.9 by automatic addition of 0.5 M NaOH or 0.5 M HCl and  
174 controlled via pH meter controllers (Electrolab, UK) and the temperature was kept at 37 °C.  
175 Faecal samples were collected from three healthy female donors, who were between 25 and 40  
176 years old, with no history of bowel disorders, who had not received antibiotic treatment for at  
177 least 6 months before the study and had not consumed pre- or probiotic supplements one month  
178 before the study. All donors were informed of the study aims and procedures, and provided  
179 their verbal consent for stool samples to be used for the experiments in compliance with the  
180 ethics procedures required at the University of Reading. Samples were collected in anaerobic  
181 jars and used within one hour of collection. Samples were diluted 1:10 (w/v) with anaerobic  
182 phosphate buffer (0.1 M, pH 7.4) and homogenised in a stomacher for 2 mins; the batch culture  
183 systems were inoculated with 15 ml faecal slurry from an individual sample. The vessels were  
184 treated with the following substrate: 1 % w/v, 1.5 g digested oat bran (OAT1.5); 3 % w/v, 4.5  
185 g digested oat bran (digestibility of oats see McCane et al.[45]) (OAT4.5); 0.12 % w/v, 180 mg  
186 94 % β-glucan extract (BG); 0.01 % w/v, 1.7 mg polyphenol mix (same profile as 4.5 g digested  
187 oats) (POLY); 1 % w/v, 1.5 g Synergy1 (positive control, PC); an additional vessel was

188 prepared under the same conditions but without any substrate, negative control (NC) (Table 2).  
189 The Synergy 1 is an inulin enriched with oligofructose, has been proved their prebiotic effects  
190 [46]. The 3 % w/v oat was chosen as the highest dose since it would be the concentration  
191 reached by an average person consuming 60 g oat bran (i. e. assuming 30 g oat bran reaches  
192 the colon and colonic total volume is 1L) [45]. Based on this calculation,  $\beta$ -glucan extract and  
193 polyphenol mix treatments were matched to the dose present in 3 % w/v oat bran. Treatment  
194 with OAT1.5 was added to the experiment to monitor the impact of the oat dose on the prebiotic  
195 effect. Five millilitre samples were collected at 0, 5, 10, 24h, of which 1 ml aliquots were  
196 centrifuged at 13,000  $xg$  for 10 min. Supernatants and pellets were stored separately at  $-20^{\circ}C$   
197 until analysis.

198

## 199 2.6. High throughput sequencing of the gut microbiota

200 The GM compositions and dynamics were determined using tag-encoded 16S rRNA gene  
201 MiSeq-based (Illumina, CA, USA) high throughput sequencing. DNA was extracted from 2 ml  
202 of faecal samples from fermentation vessel using power soil DNA isolation kit. The bead-  
203 beating was performed in 3 cycles of 15 second each at 6.5 pulse / s. (The FastPrep-24<sup>TM</sup> 5G  
204 Instrument, MP Biomedicals). DNA concentrations and purity were determined using  
205 Nanodrop 1000 (ThermoScientific, USA). The V3 region of the 16S rRNA gene was amplified  
206 using primers compatible with the Nextera Index Kit NXt\_338\_F: 5'-  
207 TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACWCCTACGGGWGGCAGCAG  
208 -3' and NXt\_518\_R: 5'-  
209 GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG -3'  
210 [47]. PCR reactions and library preparation were conducted as described in [48]. Briefly, PCR  
211 reactions containing 12  $\mu$ l AccuPrime SuperMix II, 0.5  $\mu$ l of each primer (10  $\mu$ M), 5  $\mu$ l of

212 genomic DNA (~10 ng /  $\mu$ l), and nuclease-free water to a total volume of 20  $\mu$ l were run on a  
213 SureCycler 8800 (Agilent, CA, USA). Applied cycling conditions were: denaturation at 95 °C  
214 for 2 min; 35 cycles of 95 °C for 15 s, 55 °C for 15 s and 68 °C for 40 s; followed by final  
215 elongation at 68°C for 5 min. To incorporate primers with adapters and indices, PCR reactions  
216 contained 12  $\mu$ l Phusion High-Fidelity PCR Master Mix, 2  $\mu$ l P5 and P7 primer, 2  $\mu$ l PCR  
217 product and nuclease-free water for a total volume of 25  $\mu$ l.

218 Cycling conditions applied were: 98°C for 1 min; 12 cycles of 98°C for 10 s, 55°C for 20 s and  
219 72°C for 20 s; elongation at 72°C for 5 min. The amplified fragments with adapters and tags  
220 were purified using AMPure XP beads. Prior to library pooling, clean constructs were  
221 quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) and mixed in  
222 approximately equal concentrations to ensure even representation of reads per sample. 180 bp  
223 pair-ended MiSeq (Illumina) sequencing was then performed according to the instructions of  
224 the manufacturer. The raw dataset containing pair-ended reads with corresponding quality  
225 scores was trimmed using CLC Genomic Workbench (CLC bio, Arhus, Denmark). Trimming  
226 settings were set to a low-quality limit of 0.01, with no ambiguous nucleotides allowed, and  
227 trimming off the primer sequences. Merging overlapped reads was performed using the "Merge  
228 overlapping pairs" tool using default settings. The Quantitative Insight Into Microbial Ecology  
229 (QIIME) tool (version. 1.8.0; Open source software) was used for further analysis [49].

230 Purging the dataset from chimeric reads was performed using USEARCH, while the usearch61  
231 method was used for Operational Taxonomic Units (OTUs) selection [50]. The Greengenes  
232 (version 12.10) 16S rRNA gene database and EzTaxon were used as reference [51, 52].

233 High throughput sequencing yielded 12465219 sequences free from chimeric reads, providing  
234 an average  $\pm$  SD of  $178202 \pm 80036$  sequences per sample (Range 292-447040), five baseline  
235 samples and one 10h sample were excluded due to low reads (292-509).

## 236 2.7. Short-chain fatty acid analysis

237 The defrosted supernatant samples were filtered (0.22  $\mu\text{m}$ , Millipore) and spiked with 50  $\mu\text{l}$   
238 internal standard (2-ethyl butyric acid, 100 mM). SCFAs were measured in an ion exclusion  
239 HPLC system (Agilent 1100 Series) equipped with DAD detector (G-1315B), autosampler  
240 (G1316A) and Aminex HPX-8711 column (300 x 7.8mm) heated to 84°C. Samples (20  $\mu\text{l}$ )  
241 were injected in duplicate, and UV absorption was measured at 214 nm. The mobile phase was  
242 2.5 mM aqueous sulphuric acid run at a flow rate of 0.6 ml/min. Peaks were integrated using  
243 Agilent ChemStation software (Agilent Technologies, Oxford, UK) and single point internal  
244 standard method. Peak identity and quantification were determined using a mixture of  
245 standards of acetic, butyric, iso-butyric, propionic, lactic, formic, valeric and isovaleric acids.  
246 Quantification was based on the 10-point calibration curve of analytical standards ( $R^2 \geq 0.99$ ).

## 247 2.8. Statistics

248 For calculation of alpha and beta diversity measurement of the sequencing data, the d- and e-  
249 values were set to 9800 reads per sample (85 % of the sequence number of the most indigent  
250 sample). Alpha diversity measures expressed with an observed species with Chao1, the  
251 sequence similarity 97 % OTUs value were computed for rarefied OTU tables using the alpha  
252 rarefaction workflow. Differences in alpha diversity were determined using a t-test-based  
253 approach employing the non-parametric (Monte Carlo) method (999 permutations)  
254 implemented in the compare alpha diversity workflow with QIIME.

255 The relative distribution of the genera registered was calculated and summarised at the genus  
256 level OTU tables, followed by Principal Coordinates Analysis (PCoA) plots generated with the  
257 Jackknifed beta diversity workflow based on 10 distance matrices calculated using 10  
258 subsampled OTU tables with QIIME. The *p*-value and the conservative FDR-corrected *p*-value

259 for multiple comparisons are reported. 3D plots were constructed from the three primary PCs  
260 from the PCoA of the MiSeq analysis to visualise group differences in the composition of the  
261 GM.

262 For the rest of the data analysis, GraphPad Prism statistics software package version 7 was  
263 used. One-way ANOVA was used to determine differences between fermentation treatments  
264 (OAT1.5, OAT4.5, POLY, BG) at the same time point (0, 5, 10 or 24 h), followed by the least  
265 significant difference (Bonferroni) post hoc test. A repeated measures ANOVA was used to  
266 explore the differences within the same treatment (OAT1.5, OAT4.5, POLY, BG) with all the  
267 time points (0, 5, 10 and 24 h) with Bonferroni as the post hoc test. In addition to these analyses,  
268 the p values were corrected using false discovery rate (FDR),  $p \leq 0.05$  was considered  
269 statistically significant.

## 270 3. Results

### 271 3.1. The composition of undigested and digested oat bran

272 The macronutrient, fibre and phenolic content of undigested and digested oat bran are reported  
273 in Table 1. The *in vitro* oral, gastric and small intestine digestion reduced the carbohydrate  
274 content by 53 %, available carbohydrate by 62 %, while the dietary fibre and  $\beta$ -glucan content  
275 remained stable, losing only 2 % and 12 %, respectively. Free and conjugated, bound  
276 polyphenol compounds decreased by 48 % and 26 %, respectively.

### 277 3.2. Polyphenols and $\beta$ -glucan content in oat bran and extract

278 The OAT4.5 contained 1.73 mg polyphenols (total amount of free + conjugated and bound),  
279 the POLY intervention were matched to OAT4.5 (Table 2). OAT1.5 and OAT4.5 delivered  
280 56.67 mg and 170 mg  $\beta$ -glucan, respectively; the BG vessel to match OAT4.5 180 mg of 94%  
281  $\beta$ -glucan extract was added (Table 2). The molecular weight of the  $\beta$ -glucan in the digested oat

282 bran and the extract were similar (mean  $\pm$  SD 335.5  $\pm$ 1.0 % kDa and 387.3 $\pm$ 1.0 % kDa;  $p >$   
283 0.05).

### 284 3.2. Changes in alpha and beta diversity

285 Independent of the donor, alpha diversity decreased with oats treatments over the 24h  
286 fermentation period (Chao1 2618 $\pm$ 15 at 0h vs. 1577 $\pm$ 22 at 24h for OAT1.5) but was constant  
287 over time in the negative control (Chao1 2639 $\pm$ 87 at 0h vs. 2488 $\pm$ 77 at 24h) and POLY (Chao1  
288 2655 $\pm$ 138 at 0h vs. 2241 $\pm$ 320 at 24h).

289 PCoA analyses based on unweighted UniFrac distance matrices of all samples showed a clear  
290 donor effect (Figure 1.A). However, the donor effect was no longer significant when using  
291 weighted UniFrac distance matrix analysis (ANOSIM test, Donor 1. Vs. Donor 2  $p = 1$ , Donor  
292 1 vs Donor 3  $p = 1$ , Donor 2. Vs. Donor 3  $p = 1$ , Figure 1.B).

293 Across all samples, seven bacterial phyla were classified and one phylum designated as  
294 unassigned. Phyla composition was similar for all donors ( $p < 0.06$ ), although at baseline the  
295 third donor had lower Tenericutes ( $p < 0.01$ ), while the second donor had higher Actinobacteria  
296 ( $p < 0.05$ ). At baseline, the bacterial communities, were dominated by Firmicutes (57-67 %)  
297 and Bacteroidetes (32-41 %), while the remaining five phyla including Actinobacteria (0.8-1.4  
298 %), Verrucomicrobia, Cyanobacteria, Tenericutes and Proteobacteria (0.4-0.9 %) constituted  
299  $< 1.5$  % of the community (Figure 2.).

### 300 3.3. Compositional Shifts

301 Supplementation with OAT4.5 led to a lower abundance of Firmicutes ( $p < 0.02$ ) at 5 to 24 h  
302 and to an increased abundance of Proteobacteria ( $p < 0.01$ ) and Bacteroidetes at 24 h compared  
303 to NC. A similar trend was observed for OAT1.5 ( $p$  values)(Figure 2).

304 The OAT1.5 treatment had a significant effect on the relative abundance of Actinobacteria at  
305 10 h. The relative abundance of Actinobacteria differed between OAT1.5 and OAT4.5 at  
306 baseline (1.1 % and 0.8 %, respectively). Following 10 h fermentation, Actinobacteria were  
307 almost four times higher in OAT1.5 compared to OAT4.5 (4.9 % and 1.3 %, respectively),  
308 however, differences did not persist over 24 h (4.2 % vs. 3.4 %, respectively). The treatment  
309 of BG and POLY did not promote the growth of Actinobacteria, even though their dose was  
310 matched to the OAT4.5 treatment. The PC, Synergy1 resulted in the strongest bifidogenic  
311 effect and the relative abundance of Actinobacteria (baseline 1.1 % vs. 5 h at 31.1 %, 10 h at  
312 11.5 %,  $p < 0.05$ ) (Figure 2.).

313 Focusing on changes over 24 h, the abundance of Bacterioidetes increased with BG (49.3 % vs.  
314 NC for 21 %), whereas Proteobacteria significantly decreased in comparison to the NC (14 %  
315 vs. 33.5 %). POLY treatment, the abundance of Proteobacteria increased over the course of  
316 fermentation (45 % vs. NC for 33.5 %) and decreased for Bacterioidetes ( $p < 0.04$ ) (18.6 % vs.  
317 46.8 % for OAT4.5). The oats and BG treatments promoted the growth of Bacterioidetes (Figure  
318 2.).

319 At the lower taxonomic level and across all samples, 59 genera and 69 species were identified  
320 when setting the cut-off at a relative abundance above 0.1 % of the community (Table 3). 5 h,  
321 the OAT4.5 treatment led to a significantly lower abundance of *Ruminococcus* (0.1 %), and  
322 *Coprococcus* (0.1 %) genus and, at the same time, increased the proportion of unassigned  
323 *Enterobacteriaceae* (61.2 %) compared to NC (25.8 %) (Table 3). Most notably, OAT1.5  
324 increased the abundance of *Bifidobacterium unassigned* (1.5 % vs. 0.2 for NC,  $p < 0.05$ ) at 10  
325 h and *Bifidobacterium adolescentis* at 10 h (1.9 % vs. 0.3 % for NC,  $p < 0.04$ ) and at 24 h (2  
326 % vs. 0.4 for NC,  $p < 0.02$ ) (Table 3), whereas OAT4.5 did not significantly impact the relative  
327 abundance of these species due to the high standard deviation among donors. Using EzTaxon



328 database, we were able to further identify the species designation of several OTUs at 24 h with  
329 BG and POLY treatments. At 24 h, the BG treatment had higher abundances in *Bacteroides*  
330 *ovatus* (13.5 %), *B. uniform* (12.9 %) and *B. xylaniosolvans* (3.7 %) species, while POLY led  
331 to the most abundant levels of *Enterobacteriaceae* species, *Hafnia alvei* (21.7 %). However,  
332 these changes mediated by BG and POLY treatments were not significantly different compared  
333 to NC.

334

### 335 3.4. Production of SCFA

336 OAT4.5 led to a high production of total SCFA compared to NC at 10 h ( $98.2 \pm 22$  mM vs  $12.8$   
337  $\pm 4$  mM respectively) and 24 h ( $151.5 \pm 43$  mM vs  $28.1 \pm 5$  mM). The concentration of butyric  
338 acid was significantly increased after 24 h fermentation of OAT1.5 ( $p < 0.05$ ), and PC at 10 h  
339 ( $p < 0.05$ ) and 24 h ( $p < 0.01$ ) compared to NC (Figure 3). Acetic acid was the most abundant  
340 SCFA. Both oat treatments resulted in a significant increase of acetic acid at 10 h ( $p < 0.01$ ),  
341 and 24 h ( $p < 0.01$ ) compared with baseline and NC (Figure 3.). At 24 h, OAT4.5, OAT1.5 and  
342 PC significantly increased the concentration of propionic acid compared to NC ( $48 \pm 24$  mM,  
343  $16.7 \pm 3$  mM,  $21 \pm 9$  mM and  $4.5 \pm 0.3$  mM, respectively  $p < 0.01$ ,  $0.05$  and  $0.05$ , respectively).  
344 Lactic and formic acids increased upon fermentation of OAT4.5 after 10 h compared to  
345 baseline, but did not reach significance due to the large variation among donors. BG led to a  
346 small production of acetic and butyric acids (not significant) whereas POLY did not induce  
347 SCFA production. Overall, there was a dose-response effect between the two oats doses in total  
348 SCFA at 10 h ( $98.2 \pm 22$  mM vs  $58 \pm 19$  mM respectively,  $p < 0.05$ ) and 24 h ( $151.5 \pm 43$  mM  
349 vs  $86.1 \pm 27$  mM,  $p < 0.05$ ).

350

#### 351 4. Discussion

352 This study aimed to identify the impact of different doses of oats and its isolated bioactive  
353 compounds (i.e.  $\beta$ -glucan or polyphenols) on the faecal gut microbiota using *in vitro* systems.  
354 Digested oat bran decreased alpha diversity and had a bifidogenic effect but isolated  $\beta$ -glucan  
355 or polyphenol mix given at a matched dose did not induce a similar effect. (Figure 2.). This is  
356 likely to be because these compounds alone did not provide enough energy for bacterial growth.  
357 We have demonstrated that OAT1.5 significantly increased the proliferation of  
358 *Bifidobacterium adolescentis* (Table 3). An increase in *Bifidobacterium* (genus level) was  
359 reported by Connolly *et al.* [9] and Kedia *et al.* [30] after fermentation of 1 % and 5 % oat  
360 grains in similar *in vitro* studies. *B. adolescentis* has a number of benefits including prevention  
361 of the development of diabetes by stimulating insulin secretion. Furthermore *B. adolescentis*  
362 has the ability to synthesise and secrete the neuroactive substance gamma-aminobutyric acid  
363 (GABA). GABA facilitates communication between bacteria and the human nervous system,  
364 enabling release of other neurotransmitters from specific epithelial intestinal cells [53, 54]. Our  
365 main findings suggest that oat bran could have beneficial effects on the host through increasing  
366 the relative abundance of *B. adolescentis*.

367 In accordance with the literature [55, 56], the compositional analysis of digested oat bran  
368 showed limited degradation of  $\beta$ -glucan in the stomach and the small intestine (Table 1). BG  
369 (180 mg – 387 kDa) did not introduce changes to Actinobacteria phyla (Figure 2.), which is  
370 supported by previous studies on  $\beta$ -glucan [28, 38]. However, BG tended to promote the growth  
371 of beneficial *Bacteroides uniformis*, *Bacteroides ovatus* and *Bacteroides xylaniosolvans*  
372 compared with NC (Table 3), which is in line with reports of Wang *et al.* [57]. Additionally,  
373 Hughes *et al.* [28] used a similar anaerobic batch culture system for fermentation of 0.5 g  $\beta$ -  
374 glucan and also showed an increase in a member of Bacteroidetes, specifically in the

375 *Bacteroides-Prevotella* group following 24 h fermentation. However, the dose of  $\beta$ -glucan used  
376 by Hughes *et al.*, was almost three times higher in concentration compared with what was  
377 found in 60 g oat bran (containing 0.17 g  $\beta$ -glucan), which would equate to consumption of  
378 150 g oat bran.

379 While human digestive enzymes cannot degrade plant cell wall polysaccharides, gut  
380 xylanolytic bacteria (e.g. *B. xylaniosolvans*, *B. uniformis* and *B. ovatus*) can, while producing  
381 SCFA with potential health-beneficial effects [58-60]. Certain strains of *B. uniformis*  
382 downregulate gene and protein expression of pro-inflammatory cytokines, notably iNOS and  
383 PPAR- $\gamma$ , IFN- $\gamma$ , resulting in reduced inflammatory status [61], suggesting that oat  $\beta$ -glucan  
384 could have beneficial effects on human health. More powered studies should be applied to  
385 confirm this effect.

386 *In vitro* upper gut digestion (mouth to small intestine) reduced polyphenols in the free -  
387 conjugated, bound fractions by 48 % and 26 %, respectively (Table 1). This is in agreement  
388 with findings by Dall`Asta *et al.* [62] on phenolic bioaccessibility in durum wheat aleurone  
389 fractions where caffeic and sinapic acids appeared as the most bioaccessible (83.3 % and 79.5  
390 %) while total ferulic acid and *p*-coumaric acid were less bioaccessible (29.5 % and 40.7%)  
391 with *in vitro* digestion. Moreover, several phenolic metabolites, including vanillic acid, 4 - and  
392 3 - hydroxyhippuric acids, sulfate-conjugates of benzoic and ferulic acids are derived from the  
393 hepatic and microbial metabolism of oat brans [41]. Schar *et al.* [41] found relevant  
394 concentrations in urinary excretion of 30 different phenolics, and amounted to a total excretion  
395 of  $33.7 \pm 7.3 \mu\text{mol}$ , suggesting that a high proportion of oat phenolics are bioavailable with  
396 absorption occurring both in the small intestine and then in the large intestine within eight hours  
397 of consumption.

398 In the current study, POLY did not change the abundance of the Actinobacteria phyla, which  
399 is similar to the research conducted by Gwiazdowska *et al.* [63], which showed that 20 µg/ml  
400 polyphenols had an effect on *Bifidobacterium* after 1 h incubation, but no effect at 24 h. The  
401 effects of polyphenols on microbial composition, may also be related to the fact that there is  
402 no carbohydrate energy available for this fermentation. Therefore, at least part of the change  
403 may also be due to utilisation of protein as an energy source. However, in the current study a  
404 POLY- induced increase in Enterobacteriaceae phyla and *Hafnia alvei* species was detected  
405 [64]. Several studies connected this group with polyphenol-degrading metabolism [65, 66].  
406 While Wen Gu *et al.* [67] and Hunter *et al.* [68] observed that Enterobacter strains could  
407 transform ferulic acid to vanillin via the non-oxidative decarboxylation, Kuntz *et al.* [69]  
408 believed that the *H. alvei* significantly attenuated the expression of adhesion molecules and  
409 cytokine secretion (IL-8 and IL-6), resulting in reduced inflammation [69]. The higher relative  
410 abundance of Enterobacteriaceae phyla and *H. alvei* species in POLY and digested oat bran  
411 treatments might be associated with the metabolic activity of these microbial groups towards  
412 plant-derived polyphenols and saccharides [70].

413 Previous oat fermentation studies have not shown significant increases in the growth of  
414 Enterobacteriaceae phyla and *H. alvei* species [30], most likely because the fluorescence *in situ*  
415 hybridisation (FISH) method used did not target Enterobacteriaceae or allow analysis at the  
416 species level. We have detected an increased presence of Proteobacteria phyla (Figure 2) in all  
417 3 vessels including NC, which might be due to the artificial conditions of batch culture systems  
418 [71, 72]. Facultative anaerobic microbes are indeed less abundant in the human colon [73] but  
419 this batch culture model may not be able to achieve a strict anaerobic environment, causing an  
420 increase in Proteobacteria phyla [72].

421 Despite *in vitro* enzymatic digestion (mimicking the digestion in the upper intestine prior to  
422 colon fermentation), the available carbohydrate content of the oat bran remained high (62 %)  
423 (Table 1). Wholegrain oats are a source of starch (60 % of the total dry matter of the oat grain),  
424 consisting of 7 % rapidly digestible starch, 22 % slowly digestible and 25 % resistant starch  
425 [74] all of which contribute to the available carbohydrates. The findings of Englyst *et al.*  
426 suggest that human digestive enzymes do not break down dietary resistant starch [75].  
427 Strikingly, 30 % resistant starch type 4 also led to species level alterations in a colon and  
428 increases in *B. adolescentis*. [76]. A previous study [40] reported that 1 % (w/v) arabinoxylan,  
429 a dietary fibre found in wholegrain including oats, has a bifidogenic effect using a similar 24 h  
430 batch culture fermentation *in vitro* model, yet oats generally have about 3.8 – 13.2 % of  
431 arabinoxylan [20]. This, in combination with our findings, suggests that interactions among a  
432 wide range of dietary polysaccharides may explain the bifidogenic effect of oats [77].

433 The current results indicate that digested oat bran treatments increase SCFA production dose-  
434 dependently, with a higher dose inducing higher acetate and propionate production but lower  
435 butyrate (Figure 3.). Lactic acid disappeared at 24 h, possibly because it can be further  
436 metabolised by bacteria to acetic and propionic acids [78]. Acetate and lactate are widely  
437 produced by different bacteria; however, bifidobacteria have a distinct pathway, transforming  
438 glucose to acetate and lactate [79]. SCFA can create acidic conditions in the human colon  
439 inhibiting the growth of pathogens and reducing harmful enzyme activities in the human  
440 intestine, while they also act as an energy source for gut epithelial cells [80, 81]. Furthermore,  
441 propionate derived from carbohydrate fermentation is a substrate for gluconeogenesis in  
442 humans, and also inhibits the utilisation of acetate for cholesterol synthesis in the colon and  
443 liver [82, 83]. Therefore oat bran may have a beneficial impact on human health through SCFA  
444 production.

445 In previous studies [84, 85] differences were observed between the activity of pure and of food  
446 matrix derived  $\beta$ -glucan on small intestine digestion and lipolysis. Purified  $\beta$ -glucan was  
447 observed to have less gel forming capacity and a lesser effect on lipolysis and cholesterol  
448 metabolism, than the matrix derived  $\beta$ -glucan. In addition, dietary fibre induced SCFA  
449 production has been shown to interact with phenolics [86], where butyric acid increased the  
450 ferulic acid absorption in the colon, potentially translating to higher concentrations of ferulic  
451 acid in systemic circulation *in vivo*. These findings indicate positive interactions of fibres and  
452 polyphenols within the food matrix on cholesterol metabolism and the bioavailability of  
453 phenolics, and also highlights the importance of considering the structure and physicochemical  
454 properties of foods, and not just the nutrient content.

455 In conclusion, our study has shown that oat bran as a complex food matrix beneficially  
456 increases the number of *B. adolescentis* and the amount of SCFA production. In contrast, a  
457 matched dose of the isolated bioactive compounds,  $\beta$ -glucan and other polyphenols did not  
458 show any effect on the abundance of Actinobacteria. However, by regulating the Bacteroides  
459 and Enterobacteriaceae families, they may also contribute to further health benefits.

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#### 464 CONTRIBUTIONS

465 The authors' contributions were as follows: AK, MYS and GW designed the study; OK  
466 analysed the  $\beta$ -glucan molecular weight, AK and PH conducted the *in vitro* fermentation  
467 experiments; MW and AK conducted the 16S rRNA gene sequencing, AK and MW conducted  
468 the statistical analyses. AK, MW, MYS, GW, GK, GS, SA and JS interpreted the data and AK,

469 MW, MYS, GM and JS drafted the manuscript. AK had primary responsibility for the final  
 470 content. All authors agreed on the final version of the manuscript.

471 The authors declare no conflict of interest

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690

Table 1. Macronutrient, fibre and phenolic content of oat bran before and after *in vitro* digestion and bioaccessibility (%) through digestion

Components	Before digestion	After digestion	Bioaccessibility %
Amount (g)	60	40	33
Total Carbohydrate (g)	37.8	17.6	53
Available Carbohydrate (g)	32.6	12.5	62
Total dietary fibre(g)	5.2	5.1	2
β-glucan (g)	1.7	1.5	12
Total protein (g)	7	5	29
Fat (g)	3	1.1	63
<i>Free+conjugated polyphenols</i>	6.2	3.2	48
Hydroxybenzoic acids (mg)	1.2	1.1	8
Hydrocinnamic acids (mg)	4.6	2.1	54
Avenanthramides (mg)	0.4	0.1	75
<i>Bound polyphenols</i>	16.4	12.2	26
Hydroxybenzoic acids (mg)	1	0.4	60
Hydrocinnamic acids (mg)	15.4	11.8	23
Avenanthramides (mg)	ND	ND	-
Total polyphenols (mg)	22.6	15.4	32

**Bioaccessibility** - evaluated following *in vitro* upper gut digestion procedures, we have used the term *bioavailability* to indicate the percentage of compound remaining, thus available to the large gut microbiota. This term was first defined by Dall'Asta et al. in *Nutrients* 2016, 8(1), 42.

Table 2. *In vitro* fermentation treatments

Treatment	$\beta$ -glucan (mg)	Polyphenol (mg)
OAT1.5	56.67	0.58
OAT4.5	170	1.73
BG (94% of extract)	180	0.1
POLY	ND	1.73
PC	ND	ND
NC	ND	ND

*Digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5),  $\beta$ -glucan extract (BG), polyphenol mix (POLY), Synergy 1 positive control (PC) and negative control (NC), not detected (ND)*

**Table 3. Significant changes (FDR  $p < 0.05$ ) in relative abundance (%) of bacterial taxa at 5h, 10h and 24h of *in vitro* batch culture fermentation inoculated with faeces and administered with digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5),  $\beta$ -glucan extract (BG), polyphenol mix (POLY), Synergy 1 (PC) and negative control (NC) as substrates. This experiment was conducted three times, with a different faecal donor used for each run.**

Phylum	Family	Genus	Species	Time	Treatment <sup>1</sup>						<i>p</i> value
					OAT 1.5	OAT 4.5	BG	POLY	PC	NC	
Actinobacteria	Bifidobacteriaceae	<i>Bifidobacterium</i>	<i>adolescentis</i>	5h	2.73±1.9	0.82±0.7	0.31±0.1	0.38±0.1	18.48±3.5*	0.39±0.2	0.001
Firmicutes	Ruminococcaceae	<i>Ruminococcus</i>	<i>unassigned</i>	5h	0.2±0*	0.1±0.1	0.52±0.1	0.67±0.1	0.32±0.1	0.74±0.1	0.03
Firmicutes	Lachnospiraceae	<i>Coprococcus</i>	<i>unassigned</i>	5h	0.2±0.1*	0.12±0*	0.48±0.2	0.44±0	0.76±0.3	0.54±0	0.02
Proteobacteria	Enterobacteriaceae	<i>unassigned</i>	<i>unassigned</i>	5h	55.46±8.4*	61.2±9*	23±6	31.9±8.6	11.33*±3.7	25.9±11	0.05
Actinobacteria	Bifidobacteriaceae	<i>Bifidobacterium</i>	<i>unassigned</i>	10h	1.17±10.1*	1.1±0.9	0.19±0.1	0.17±0.1	4.12±2.1*	0.27±0.1	0.03
Actinobacteria	Bifidobacteriaceae	<i>Bifidobacterium</i>	<i>adolescentis</i>	10h	1.93±0.5*	0.66±0.5	0.19±0.1	0.27±0.1	6.7±2.6*	0.26±0	0.02
Firmicutes	Ruminococcaceae	<i>unassigned</i>	<i>unassigned</i>	10h	4.26±5	0.05±0*	8.96±2.8	8.68±1.7	9.76±10	8.85±0.8	0.03
Firmicutes	Lachnospiraceae	<i>unassigned</i>	<i>unassigned</i>	10h	5.14±4	0.08±0*	10.1±2.4	8.14±5	5.11±1.2	10.1±0.2	0.03
Firmicutes	Lachnospiraceae	<i>Blautia</i>	<i>unassigned</i>	10h	0.35±0.1*	0.02±0*	1.39±0.3	1.21±0.4	1.15±0.8	1.14±0.4	0.03
Proteobacteria	Enterobacteriaceae	<i>unassigned</i>	<i>unassigned</i>	10h	50.2±12.2	67.2±14*	12.75±6*	36.1±9.2	6.47±3.7*	29.2±1.4	0.02
Actinobacteria	Bifidobacteriaceae	<i>Bifidobacterium</i>	<i>adolescentis</i>	24h	2.03±0.1*	1.91±1.8	0.31±0.1	0.31±0.2	6.87±3.7	0.41±0	0.05

<sup>1</sup> The difference in relative abundance of taxa between treatments within the same time points was assessed by ANOVA. The *p*-value after correction for multiple tests (69 taxa) with the FDR method.

\* indicate significant differences ( $p < 0.05$ ) to NC at the same time point. Values are mean ± SE





## Figure Legend

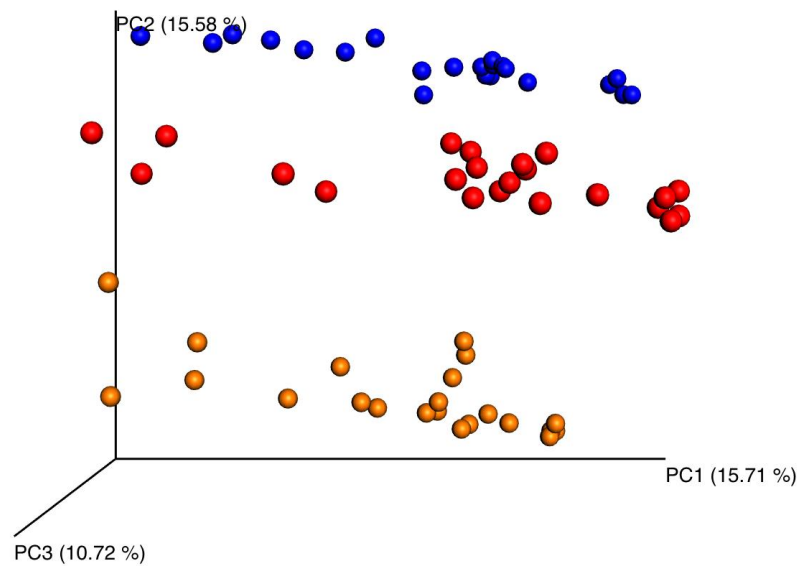
Figure 1. Principal coordinates analysis (PCoA) plots of 16S rRNA gene profiles based on (A) unweighted and (B) weighted phylogenetic Unifrac distance matrices calculated from 10 rarefied OTU tables (9800 reads per samples) unweighted (A) showing clear clustering according to donors (ANOSIM test,  $p = 0.01$ ). The degree of variation between 10 jackknifed replicates of PCoA is displayed with confidence ellipsoids around each sample. Weighted (B) quantitative information used to generate the bacterial relative abundance of donors showed no clear clustering (ANOSIM test,  $p > 0.05$ ) for a whole dataset (24h *in vitro* batch culture fermentation inoculated with 3 healthy donors faeces and administered with digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5),  $\beta$ -glucan extract (BG), polyphenol mix (POLY), Synergy 1 (PC) and negative control (NC) as the substrates). Each colour represents a different donor

Figure 2. Changes in bacterial phyla (relative abundances (%)) 0, 5, 10 and 24h *in vitro* batch culture fermentation. This experiment was conducted three times, with a different faecal donor used for each run. The media was supplemented with digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5),  $\beta$ -glucan extract (BG), polyphenol mix (POLY), Synergy 1 (PC) and negative control (NC). Samples were analysed at 0, 5, 10, 24 h. Values are mean (%).

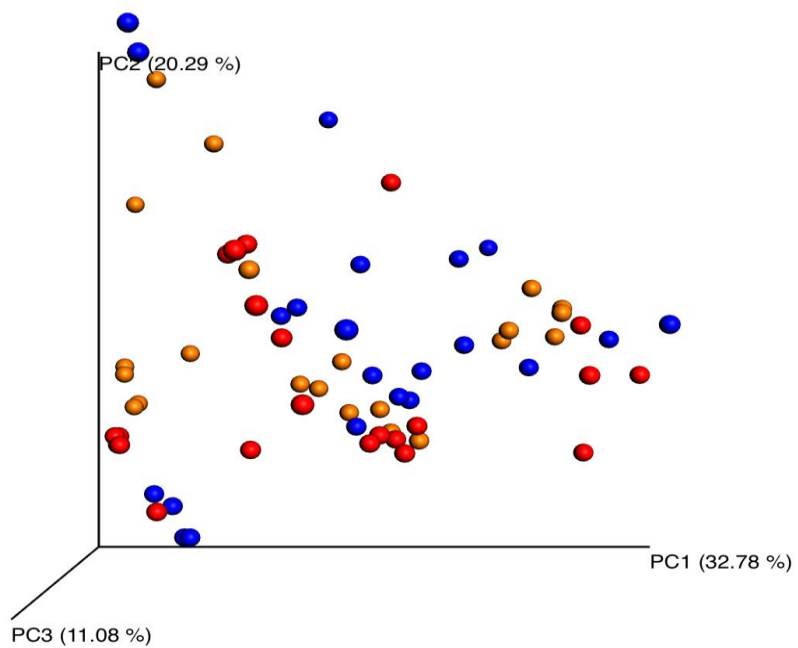
Figure 3. Change in concentrations of acetic acid (A), propionic acid (B), butyric acid (C) from negative control (mM) throughout 24h *in vitro* batch culture fermentation. This experiment was conducted three times, with a different faecal donor used for each run. The media supplemented with digested oat bran 1.5g (OAT1.5) and 4.5g (OAT4.5),  $\beta$ -glucan extract (BG), polyphenol mix (POLY), Synergy 1 (PC) as the substrates. Samples were analysed at 0, 5, 10, 24 h

Figure 1.

A



B



■ Donor 1 ■ Donor 2 ■ Donor 3

Figure 2.

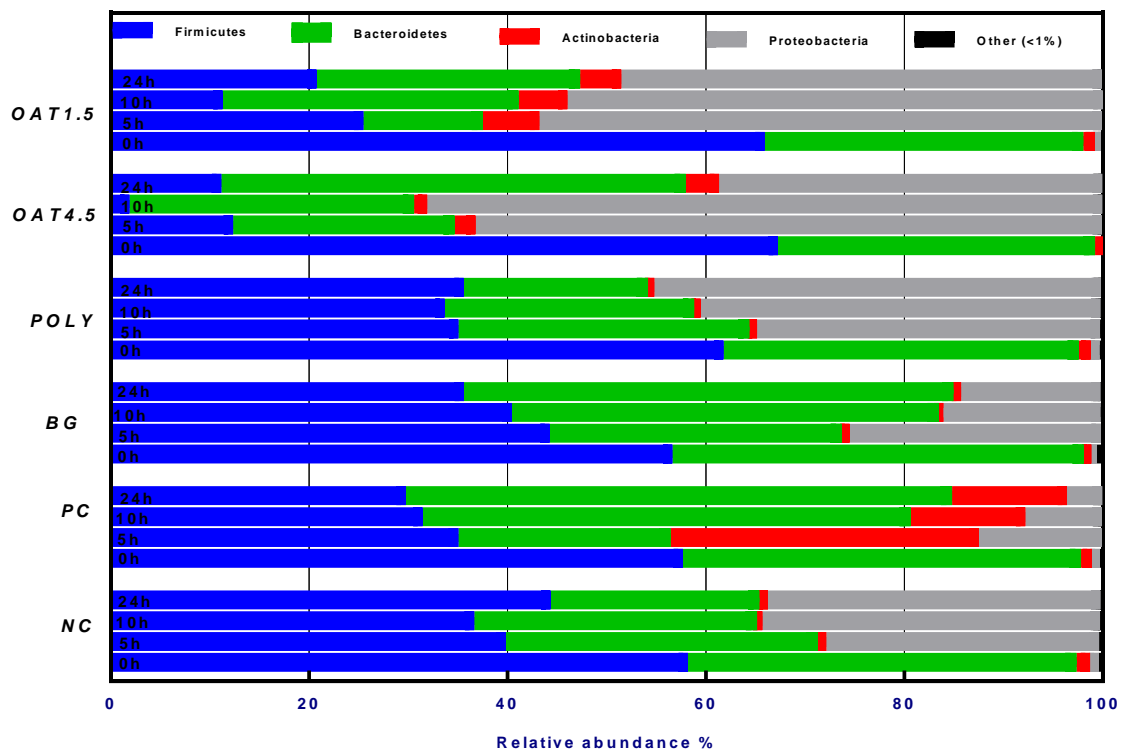
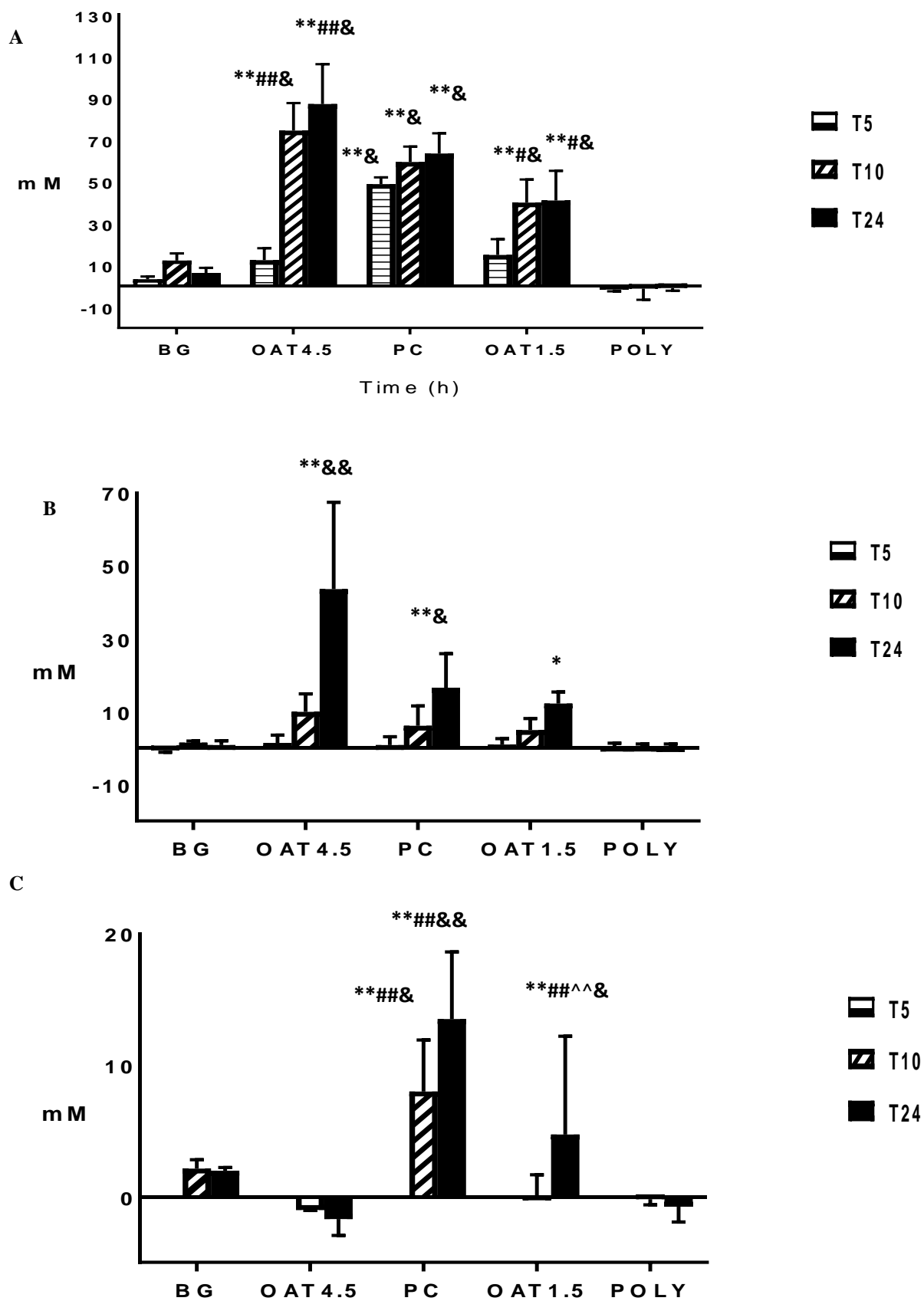


Figure 3.



*Values are reported after subtracted of negative control value*

*Significant from initial value \* $p < 0.05$ , \*\* $p < 0.01$ ; Significant from 5h value # $p < 0.05$ , ## $p < 0.01$ ,*

*Significant from 10h value ^ $p < 0.05$ , ^^ $p < 0.01$ ; Significant from negative control value at the same time point & $p < 0.05$ , && $p < 0.01$*