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

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Accepted Version

Oat bran, but not its isolated bioactive β-glucans or polyphenols, have a bifidogenic effect in an *in vitro* fermentation model of the gut microbiota[1-4]

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Keywords: oat, polyphenols, β-glucan, bioactive, gut microbiota, 16S rRNA gene sequencing, short chain fatty acids, prebiotic, bifidogenic

PubMed indexing: Kristek, Wiese, Heuer, Kosik, Schär, Soycan, Alsharif, Kuhnle, Walton and Spencer

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Abstract

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

Keywords: oats, β-glucan, polyphenols, gut microbiota, 16S rRNA gene sequencing, short chain fatty acids, prebiotic, bifidogenic

1. Introduction

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

Oats, however, are also rich dietary sources of polyphenols, including avenanthramides and phenolic acids [20]. These are likely to contribute to the health effects of a diet rich in oats [21-23] but have not yet been examined in detail. Phenolic acids are found in three different forms within the oat food matrix: as soluble free acids, as soluble conjugates esterified to low molecular weight components such as sugars, and as insoluble bound acids esterified to high molecular weight components including lignin, cell wall polysaccharides and storage proteins [20]. A large proportion of oat polyphenols are bound via an ester bond, and hence are poorly absorbed in the upper intestine and reach the colon [24], where they may beneficially modulate the microbiota [8]. There are no esterases in human tissues that break these ester links [25]; therefore, the main catalytic site is in the colon through cinnamoyl esterase activity of human faecal microbiota, such as *Escherichia coli* (three isolates), *Bifidobacterium lactis* and *Lactobacillus gasseri* (two strains) [26, 27].

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

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

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

2. Materials and Methods

2.1. Reagents

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

2.2. Oats and Controls

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

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

The method employed was adapted from Mills *et al.* [42]. Oat bran was digested *in vitro* in three phases: the oral phase, the gastric phase and the small intestinal phase. 60 g of oat bran was mixed with 150 ml of sterile and distilled water and homogenised, transferred into a 500 ml Duran bottle, and microwaved for 1 minute. In the oral phase, 20 mg of α-amylase was dissolved in 6.25 ml $CaCl_2$ (1 mM, pH 7.0) and added to the solution, then incubated at 37°C for 30 minutes on a shaker set at 120 *xg*. After incubation, the pH was adjusted to 2.0 with 6 M HCl solution and the gastric phase introduced by adding 2.7 g pepsin in 25 ml HCl (0.1 M) and further incubated for 2 hours under the same conditions. In the small intestinal phase, 560 mg pancreatin and 3.5 g bile were mixed with 125 ml $NaHCO_3$ (0.5 M) and dispensed into the mix. The pH was adjusted to 7.0 then incubated for 3 hours at 37 °C with shaking. Finally, the sample solution was transferred to a seamless semi-permeable 100-500 Dalton molecular weight cut-off regenerated cellulose dialysis tubing and dialysed against NaCl (0.01 M at 5°C) to remove low molecular mass digestion products. After 15 hours, the dialysis fluid was changed and the process continued for an additional 2 hours. The digested oat bran mix was collected and transferred into several 250 ml clear plastic containers, frozen to -80° and freeze-dried for 5 days to remove all fluid content.

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

Polyphenols were extracted from undigested (raw) and digested (after *in vitro* digestion) oat bran in two separate fractions (i.e. free and conjugated or bound) using the method of Schar *et al.* [41]. The phenolic acids and avenanthramides in oat extracts were identified and quantified using a high-performance liquid chromatography (HPLC) Agilent 1100 series (Agilent Technologies Ltd) equipped with a quaternary pump, autosampler, column thermostat, sample thermostat and photo diode array detector. Compound separation was achieved by a Kinetex biphenyl column (100 A 250 x 4.6 mm length, 5 uM particle size; Phenomenex) and using a gradient elution. Mobile phase A consisted of 0.1 % (v/v) formic acid in HPLC water (A), while mobile phase B was 0.1 % (v/v) formic acid in methanol. The following optimised gradient protocol was run: 0 min, 95% A, 5 % B; 20 min, 75 % A, 25 % B; 25 min, 74 % A, 26 % B; 30 min, 65 % A, 35 % B; 40 min, 64 % A, 36 % B; 53 min, 30 % A, 70 % B; 56 min, 5 % A, 95 % B; 61 min, 5 % A, 95 % B; 62 min, 95 % A, 5 % B; 65 min, 95 % A, 5 % B. The flow rate of the mobile phase was 1.0 ml/min and the sample injection volume was 20 µl [43]. The absorbance was recorded at 254, 280 and 320 nm and quantification was based on 12 point linear calibration curves (mean $R^2 > 0.99$) and as a ratio to the internal standard (i.e. 3,5-dichloro-4-hydroxybenzoic acid) to account for losses during extraction ($R^2 \geq 0.99$).

2.4. Determination of the β-glucan content

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

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

2.5. pH controlled faecal batch culture fermentation

Substrate ability to modulate gut microbiota was determined using anaerobic, stirred, pH and temperature controlled faecal batch cultures. Batch culture fermentation vessels (300 ml volume: one vessel per treatment) were sterilised and filled with 135 ml of sterile basal medium (g/l: 2 g peptone, 2 g yeast extract, 0.1 g NaCl, 0.04 g K_2HPO_4, 0.04 g KH_2PO_4, 0.01 g MgSO4·7H_2O, 0.01 g CaCl2·6H_2O, 2 g $NaHCO_3$, 2 ml Tween 80, 0.05 g Hemin (dissolved in 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 resazurin solution (0.02 %)). Before addition to the vessel, the medium was adjusted to pH 7.0 and autoclaved. The sterile medium was gassed overnight with O_2-free N_2 (15 ml / min) to establish anaerobic conditions. To mimic the proximal region of the human large intestine, pH was held in the range of 6.7 - 6.9 by automatic addition of 0.5 M NaOH or 0.5 M HCl and controlled via pH meter controllers (Electrolab, UK) and the temperature was kept at 37 °C. Faecal samples were collected from three healthy female donors, who were between 25 and 40 years old, with no history of bowel disorders, who had not received antibiotic treatment for at least 6 months before the study and had not consumed pre- or probiotic supplements one month before the study. All donors were informed of the study aims and procedures, and provided their verbal consent for stool samples to be used for the experiments in compliance with the ethics procedures required at the University of Reading. Samples were collected in anaerobic jars and used within one hour of collection. Samples were diluted 1:10 (w/v) with anaerobic phosphate buffer (0.1 M, pH 7.4) and homogenised in a stomacher for 2 mins; the batch culture systems were inoculated with 15 ml faecal slurry from an individual sample. The vessels were treated with the following substrate: 1 % w/v, 1.5 g digested oat bran (OAT1.5); 3 % w/v, 4.5 g digested oat bran (digestibility of oats see McCane et al.[45]) (OAT4.5); 0.12 % w/v, 180 mg 94 % β-glucan extract (BG); 0.01 % w/v, 1.7 mg polyphenol mix (same profile as 4.5 g digested oats) (POLY); 1 % w/v, 1.5 g Synergy1 (positive control, PC); an additional vessel was prepared under the same conditions but without any substrate, negative control (NC) (Table 2). The Synergy 1 is an inulin enriched with oligofructose, has been proved their prebiotic effects [46]. The 3 % w/v oat was chosen as the highest dose since it would be the concentration reached by an average person consuming 60 g oat bran (i. e. assuming 30 g oat bran reaches the colon and colonic total volume is 1L) [45]. Based on this calculation, β-glucan extract and polyphenol mix treatments were matched to the dose present in 3 % w/v oat bran. Treatment with OAT1.5 was added to the experiment to monitor the impact of the oat dose on the prebiotic effect. Five millilitre samples were collected at 0, 5, 10, 24h, of which 1 ml aliquots were centrifuged at 13,000 *xg* for 10 min. Supernatants and pellets were stored separately at -20°C until analysis.

2.6. High throughput sequencing of the gut microbiota

The GM compositions and dynamics were determined using tag-encoded 16S rRNA gene MiSeq-based (Illumina, CA, USA) high throughput sequencing. DNA was extracted from 2 ml of faecal samples from fermentation vessel using power soil DNA isolation kit. The bead-beating was performed in 3 cycles of 15 second each at 6.5 pulse / s. (The FastPrep-24™ 5G Instrument, MP Biomedicals). DNA concentrations and purity were determined using Nanodrop 1000 (ThermoScientific, USA). The V3 region of the 16S rRNA gene was amplified using primers compatible with the Nextera Index Kit NXt_338_F: 5’-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGACWCCTACGGGWGGCAGCAG -3’ and NXt_518_R: 5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGATTACCGCGGCTGCTGG -3’" [47]. PCR reactions and library preparation were conducted as described in [48]. Briefly, PCR reactions containing 12 µl AccuPrime SuperMix II, 0.5 µl of each primer (10 µM), 5 µl of genomic DNA (~10 ng / µl), and nuclease-free water to a total volume of 20 µl were run on a SureCycler 8800 (Agilent, CA, USA). Applied cycling conditions were: denaturation at 95 °C 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 elongation at 68°C for 5 min. To incorporate primers with adapters and indices, PCR reactions contained 12 µl Phusion High-Fidelity PCR Master Mix, 2 µl P5 and P7 primer, 2 µl PCR product and nuclease-free water for a total volume of 25 µl.

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

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

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

2.7. Short-chain fatty acid analysis

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

2.8. Statistics

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

The relative distribution of the genera registered was calculated and summarised at the genus level OTU tables, followed by Principal Coordinates Analysis (PCoA) plots generated with the Jackknifed beta diversity workflow based on 10 distance matrices calculated using 10 subsampled OTU tables with QIIME. The *p*-value and the conservative FDR-corrected *p*-value for multiple comparisons are reported. 3D plots were constructed from the three primary PCs from the PCoA of the MiSeq analysis to visualise group differences in the composition of the GM.

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

3. Results

3.1. The composition of undigested and digested oat bran

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

3.2. Polyphenols and β-glucan content in oat bran and extract

The OAT4.5 contained 1.73 mg polyphenols (total amount of free + conjugated and bound), the POLY intervention were matched to OAT4.5 (Table 2). OAT1.5 and OAT4.5 delivered 56.67 mg and 170 mg β-glucan, respectively; the BG vessel to match OAT4.5 180 mg of 94% β-glucan extract was added (Table 2). The molecular weight of the β-glucan in the digested oat bran and the extract were similar (mean ± SD 335.5 ±1.0 % kDa and 387.3±1.0 % kDa; $p >$ 0.05).

3.2. Changes in alpha and beta diversity

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

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

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

3.3. Compositional Shifts

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

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

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

At the lower taxonomic level and across all samples, 59 genera and 69 species were identified when setting the cut-off at a relative abundance above 0.1 % of the community (Table 3). 5 h, the OAT4.5 treatment led to a significantly lower abundance of *Ruminococcus* (0.1 %), and *Coprococcus* (0.1 %) genus and, at the same time, increased the proportion of unassigned *Enterobacteriaceae* (61.2 %) compared to NC (25.8 %) (Table 3). Most notably, OAT1.5 increased the abundance of *Bifidobacterium unassigned* (1.5 % vs. 0.2 for NC, $p < 0.05$) at 10 h and *Bifidobacterium adolescentis* at 10 h (1.9 % vs. 0.3 % for NC, $p < 0.04$) and at 24 h (2 % vs. 0.4 for NC, $p < 0.02$) (Table 3), whereas OAT4.5 did not significantly impact the relative abundance of these species due to the high standard deviation among donors. Using EzTaxon database, we were able to further identify the species designation of several OTUs at 24 h with BG and POLY treatments. At 24 h, the BG treatment had higher abundances in *Bacteroides ovatus* (13.5 %), *B. uniform* (12.9 %) and *B. xylaniosolvens* (3.7 %) species, while POLY led to the most abundant levels of *Enterobacteriaceae* species, *Hafnia alvei* (21.7 %). However, these changes mediated by BG and POLY treatments were not significantly different compared to NC.

3.4. Production of SCFA

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

ACKNOWLEDGMENTS

We kindly thank Prof Dennis Sandris Nielsen Department of Food Science, University of Copenhagen, Denmark for his support to conduct DNA extraction and amplification in his laboratory.

CONTRIBUTIONS

The authors’ contributions were as follows: AK, MYS and GW designed the study; OK analysed the β-glucan molecular weight, AK and PH conducted the *in vitro* fermentation experiments; MW and AK conducted the 16S rRNA gene sequencing, AK and MW conducted the statistical analyses. AK, MW, MYS, GW, GK, GS, SA and JS interpreted the data and AK, MW, MYS, GM and JS drafted the manuscript. AK had primary responsibility for the final content. All authors agreed on the final version of the manuscript.

The authors declare no conflict of interest

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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
Free+conjugated polyphenols	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
Bound polyphenols	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	β-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), β-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), β -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[1]						
					OAT 1.5	OAT 4.5	BG	POLY	PC	NC	*p value*
Actinobacteria	Bifidobacteriaceae	*Bifidobacterium*	*adolescentis*	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	*Ruminococcus*	*unassigned*	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	*Coprococcus*	*unassigned*	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	*unassigned*	*unassigned*	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	*Bifidobacterium*	*unassigned*	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	*Bifidobacterium*	*adolescentis*	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	*unassigned*	*unassigned*	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	*unassigned*	*unassigned*	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	*Blautia*	*unassigned*	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	*unassigned*	*unassigned*	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	*Bifidobacterium*	*adolescentis*	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

[1] 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 (69taxa) 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), β-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), β-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), β-glucan extract (BG), polyphenol mix (POLY), Synergy 1 (PC) as the substrates. Samples were analysed at 0, 5, 10, 24 h

Figure 1.

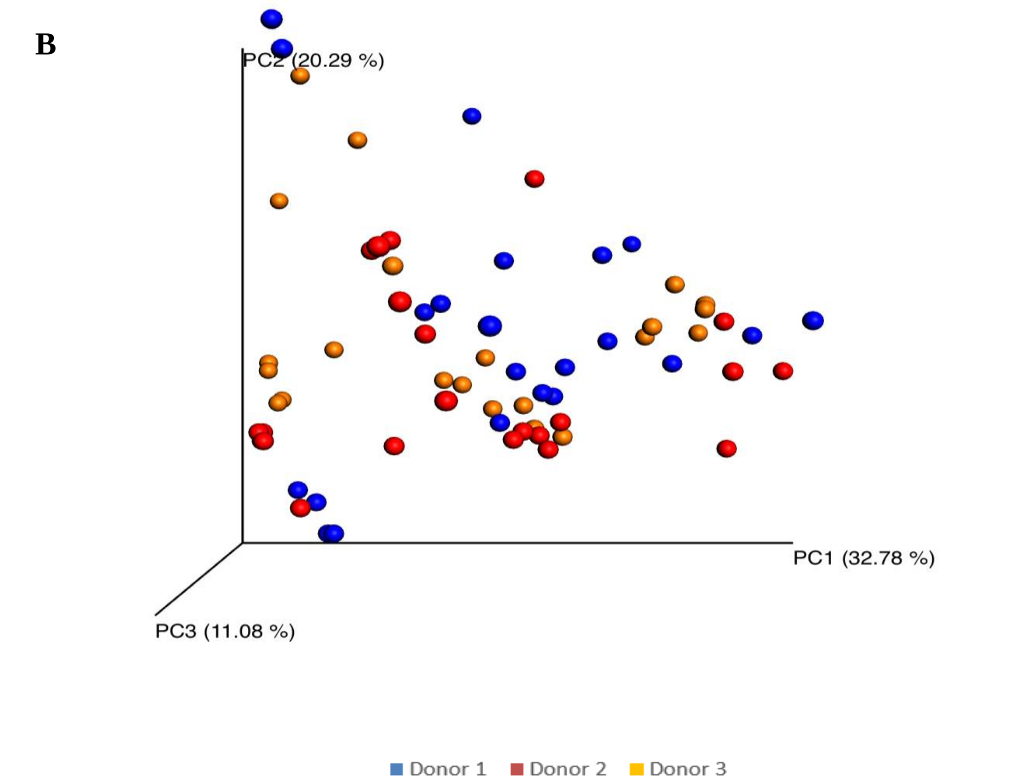

A
PC2 (15.58 %)
PC1 (15.71 %)
PC3 (10.72 %)
B
PC2 (20.29 %)
PC1 (32.78 %)
PC3 (11.08 %)
Donor 1
Donor 2
Donor 3

Figure 2.

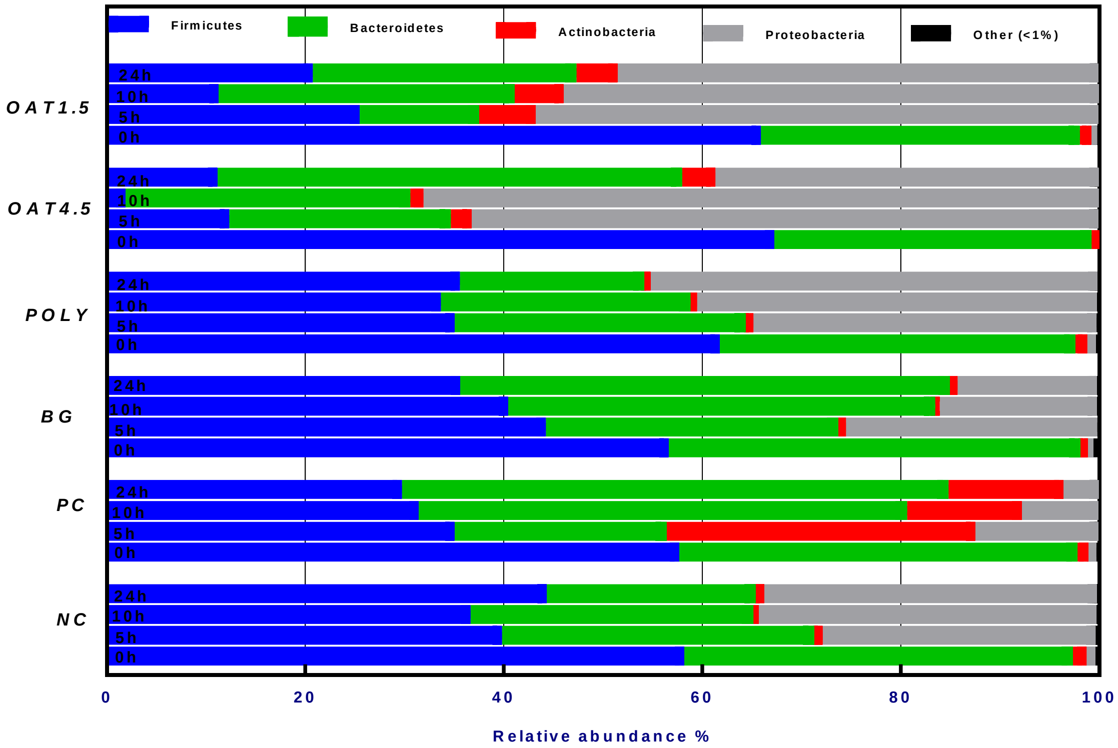
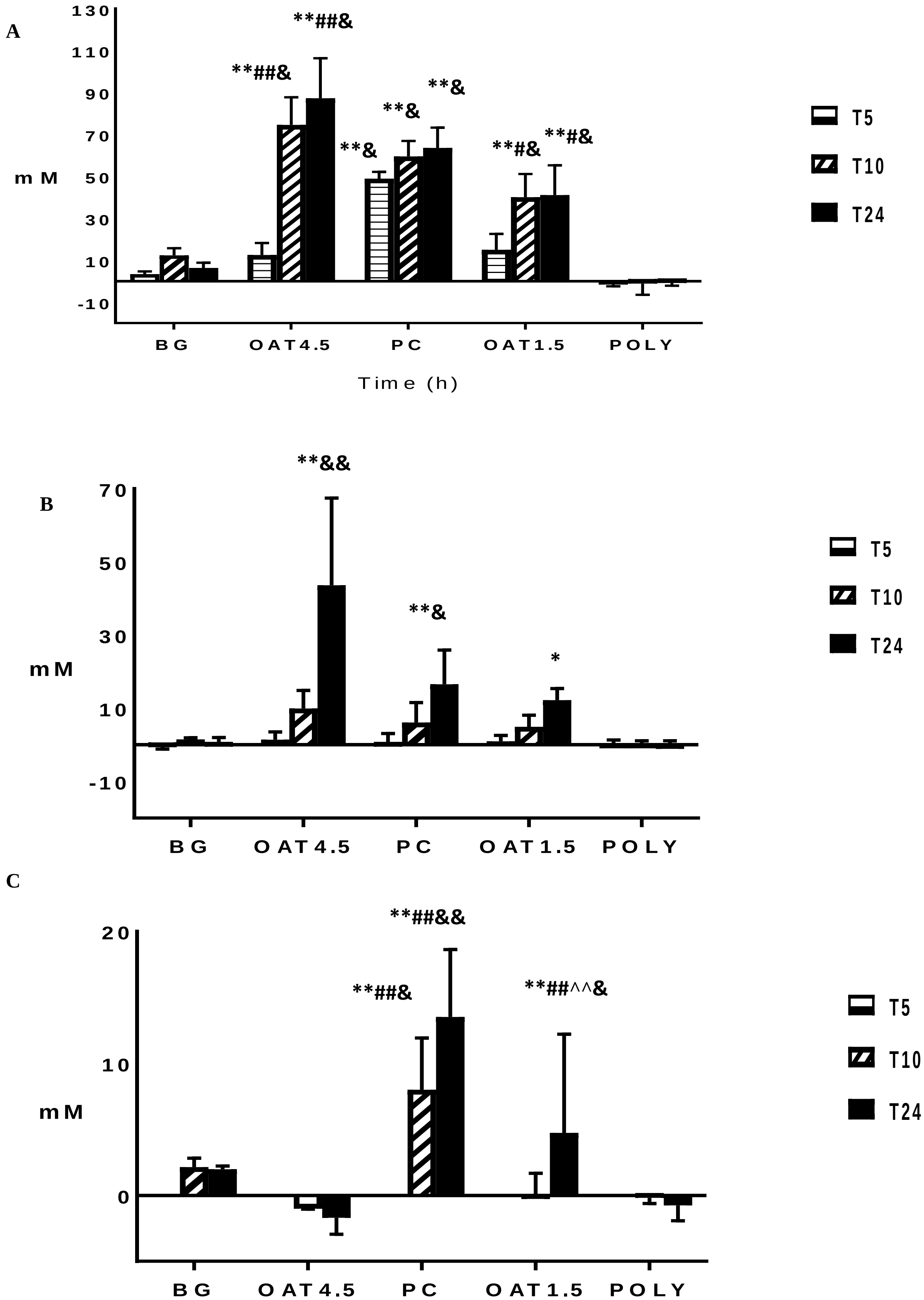

Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
Other (<1%)
OAT1.5
OAT4.5
POLY
BG
PC
NC
24h
10h
5h
0h
0
20
40
60
80
100
Relative abundance %

Figure 3.


A
130
110
90
70
50
30
10
-10
mM
**##&
**##&
**&
**&
**&
**#&
**#&
BG
OAT4.5
PC
OAT1.5
POLY
Time (h)
T5
T10
T24
B
70
50
30
10
-10
mM
**&&
**&
*
BG
OAT4.5
PC
OAT1.5
POLY
T5
T10
T24
C
20
10
0
mM
**##&&
**##&
**##^^&
BG
OAT4.5
PC
OAT1.5
POLY
T5
T10
T24

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