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Research Article

RESEARCH ARTICLE

Impact of carbohydrate substrate complexity on the diversity of the human colonic microbiota

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One sentence summary: Carbohydrate substrate complexity impacts human colonic microbial diversity.

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ABSTRACT

The diversity of the colonic microbial community has been linked with health in adults and diet composition is one possible determinant of diversity. We used carefully controlled conditions *in vitro* to determine how the complexity and multiplicity of growth substrates influence species diversity of the human colonic microbiota. In each experiment, five parallel anaerobic fermenters that received identical faecal inocula were supplied continuously with single carbohydrates (either arabinoxylan-oligosaccharides (AXOS), pectin or inulin) or with a '3-mix' of all three carbohydrates, or with a '6-mix' that additionally contained resistant starch, β-glucan and galactomannan as energy sources. Inulin supported less microbial diversity over the first 6 d than the other two single substrates or the 3- and 6-mixes, showing that substrate complexity is key to influencing microbiota diversity. The communities enriched in these fermenters did not differ greatly at the phylum and family level, but were markedly different at the species level. Certain species were promoted by single substrates, whilst others (such as *Bacteroides ovatus*, LEfSe $P = 0.001$) showed significantly greater success with the mixed substrate. The complex polysaccharides such as pectin and arabinoxylan-oligosaccharides promoted greater diversity than simple homopolymers, such as inulin. These findings suggest that dietary strategies intended to achieve health benefits by increasing gut microbiota diversity should employ complex non-digestible substrates and substrate mixtures.

Keywords: microbial diversity; Bacteroidetes; Firmicutes; CAZymes; dietary carbohydrates

INTRODUCTION

The human large intestine harbors dense microbial communities that collectively possess a remarkable capacity to degrade a wide range of complex dietary carbohydrates that are recalcitrant to digestion by host enzymes (Martens *et al.* 2011; Flint *et al.* 2012b; Kaoutari *et al.* 2013). In healthy adults this is a complex and highly diverse community comprising hundreds of different bacterial species that interact through cross-feeding and competition. The relationship between the host and its gut microbiota is multifarious and the impact of these interactions can have

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profound consequences for human health (Sekirov *et al.* 2010; Flint *et al.* 2012a; Russell *et al.* 2013).

Dietary residues that escape digestion by host enzymes provide energy sources for bacterial growth and metabolism in the colon. Dietary intake of complex non-digestible carbohydrates in the form of plant-derived fibre is widely considered to contribute to the maintenance of a diverse intestinal microbial community that is associated with health (O'Keefe *et al.* 2015; Heiman and Greenway 2016). Interestingly, a cross-over intervention study involving overweight human volunteers found that faecal microbiota diversity was higher during consumption of a wheat bran supplemented diet than with a similar diet in which the main non-digestible component was resistant starch (Salonen *et al.* 2014) indicating that substrate complexity has an impact on microbial community diversity.

Le Chatelier *et al.* (2013) detected a bimodal distribution of 'gene count' for the faecal metagenome within the human population, with low diversity (LGC—low gene count) individuals having a greater likelihood than high gene count individuals of showing symptoms of metabolic syndrome. High diversity could however be restored in LGC individuals through dietary intervention (Cotillard *et al.* 2013). Low gut microbiota diversity is increasingly being seen as a signature for poor health and of many disease states, promoting interest in restoring 'healthier' microbial communities through dietary manipulation (Lozupone *et al.* 2012; Le Chatelier *et al.* 2013; O'Toole and Jeffery 2015).

Diversity in gut microbial communities is likely to be determined by a large number of factors. These range from temporal changes in food supply (Sonnenburg *et al.* 2016) and the gut environment to the consequences of bacteriophage infection (Lim *et al.* 2015; Manrique *et al.* 2016). One obvious factor, however, is the diversity of growth substrates supplied from the diet and in the case of the large intestinal microbial community this means non-digestible carbohydrates and proteins that survive passage through the upper gut. Since many dominant gut bacterial species, especially among the Firmicutes, appear to be nutritionally specialized (Ze *et al.* 2012; Wegmann *et al.* 2014; Ben David *et al.* 2015), it might be anticipated that a single substrate would select for a less diverse community than would be the case with multiple substrates. At the same time, a single chemically complex carbohydrate might lead to greater diversity than a single homo-polymer. We recently showed that apple pectin and inulin promoted different species within the community, but also that the more chemically complex substrate pectin supported a more diverse community than the homo-polymer inulin (Chung *et al.* 2016).

In the present study, we set out to compare the impact of single substrates and combinations of non-digestible carbohydrates upon the microbial community starting from the same faecal inoculum using a model fermenter system approach maintained at a constant controlled pH value. Specifically, in these studies we compared the impact of the single substrates inulin, pectin and arabinoxylan oligosaccharides (AXOS) alone with that of two different carbohydrate mixes upon the microbiota. Inulin is a commonly used prebiotic that is a simple polymer consisting of linear chains of fructose residues. Pectin is a complex polysaccharide that has a galacturonan backbone with side chains of arabinans, galactans and arabinogalactans, and AXOS are oligosaccharides consisting of a backbone of xylose units, which are either unsubstituted (xylo-oligosaccharides) or substituted with arabinose units (arabinoxylo-oligosaccharides). Ferulic acid is ester-linked to some of the arabinose residues of the arabinoxylo-oligosaccharides.

We show here that both the complexity of individual substrates and the multiplicity of these substrates can markedly influence bacterial species composition and diversity. These findings have important consequences for our understanding of nutritional specialization among human colonic bacteria and for predicting how diet composition, including the addition of prebiotics, may be used to manipulate microbiota composition to promote beneficial species and diversity.

MATERIALS AND METHODS

Simulated human colonic fermenter studies

Within each experiment, five single-stage fermenter vessels each containing 250 mL of sterile anaerobic medium pre-heated to 37°C, were inoculated simultaneously from the same faecal sample. During the experiment each vessel received a continuous infusion of fresh medium (one volume replacement per day) with the five vessels being run in parallel. Medium pH was monitored continuously and the pH within each fermenter vessel was maintained at pH 6.1. Medium composition was as follows: 0.3% (w/v) casein hydrolysate, 0.3% (w/v) peptone water, 0.2% (w/v) K_2HPO_4, 0.02% (w/v) $NaHCO_3$, 0.45% (w/v) NaCl, 0.05% (w/v) $MgSO_4.7H_2O$, 0.045% (w/v) $CaCl_2.2H_2O$, 0.0005% (w/v) $FeSO_4.7H_2O$, 0.001% (w/v) haemin, 0.005% (w/v) bile salts, 0.05% (v/v) antifoam A and 0.06% (v/v) resazurin. The five parallel fermenter vessels, however differed in the carbohydrate energy sources added. In three 'single substrate' fermenters the medium contained either apple pectin (Unipectin OB700SB, Cargill), inulin (Oliggo-Fiber DS2, avDP <10, Cargill) or arabinoxylan-oligosaccharides (AXOS) (DP 5, with arabinose to xylose ratio of 0.21 and dry matter 94%, Cargill) at 0.42% (w/v). In the fourth vessel the medium contained a mixture of these three carbon sources (apple pectin, inulin and AXOS, 0.14% (w/v) of each) whilst in the fifth vessel the medium contained a mixture of six carbon sources which comprised apple pectin, inulin and AXOS, plus resistant starch type III (Actistar™, Cargill), galactomannan (Viscogum™, Cargill) and β-glucan (Megazyme) at 0.07% (w/v) each. Fermenter medium was sterilized by autoclaving at 121°C for 15 minutes and cooled under CO_2 gas with constant mixing using a magnetic stirrer. Reducing solution mix containing mineral solution, vitamin solution, cysteine and $NaHCO_3$ were added as a filtered-sterilized solution after autoclaving.

The fermenter culture vessels were maintained under a stream of CO_2 at a constant temperature of 37°C using thermal jackets. The medium reservoir and fermenter culture vessel were mixed by internal stirrer bars powered by external stirring units. The volume of the culture was kept constant at 250 mL with a constant flow of fresh medium at a turnover of 250 mL/d. The pH of the fermenter vessels were monitored and controlled using a pH controller that delivers either 0.1 M HCl or 0.1 M NaOH solutions to maintain the pH at 6.1±0.1 for the full period of the study (20 d).

Two healthy volunteers consuming western diets, one 64 year old male and one 53 year old female (Donors 1 and 2), provided fresh faecal samples, which were prepared within 5 h of donation, to inoculate the fermenters in two separate experiments. The volunteers had no history of colonic disease and had consumed no drugs known to influence the microbiota for at least 3 months prior to the sampling date. For each experiment the inoculum was prepared immediately prior to inoculation using 5 g faeces (wet weight) in 10 mL of 50 mM phosphate buffer (pH 6.5) under O_2-free CO_2 containing 0.05% cysteine homogenized

using gentle MACS M Tubes (MACS Miltenyl Biotec). The same faecal sample was used to inoculate the five fermenter vessels (5 g faecal matter per vessel).

DNA extractions from fermenter samples

Samples for DNA extraction were collected from each fermenter at time point 0, 8 h, 1 d, 2 d, 3 d, 6 d, 9 d, 12 d, 15 d, 18 d and 20 d. In addition, DNA was extracted from the faecal slurry inoculum. DNA was extracted immediately from samples following collection. The samples were processed using the FastDNA Spin kit (MP Biomedicals). For each sample collected, 460 μL was placed in lysing matrix E tubes, 978 μL of sodium phosphate buffer and 122 μL MT buffer were added to each tube, which was processed following the manufacturer's instructions. The DNA was eluted in 50 μL FastPrep elution buffer.

PCR amplification and Illumina MiSeq sequencing

The extracted DNA was used as a template for PCR amplification of the V1-V2 region of bacterial 16S rRNA genes using the barcoded fusion primers MiSeq-27F (5′-AATGATACGGCGACCACC GAGATCTACACTATGGTAATTCCAGMGTTYGATYMTGGCTCAG-3′) and MiSeq-338R (5′-CAAGCAGAAGACGGCATACGAGAT-barcode-AGTCAGTCAGAAGCTGCCTCCCGTAGGAGT-3′), which also contain adaptors for downstream Illumina MiSeq sequencing. Each of the samples was amplified with a unique (12 base) barcoded reverse primer. PCR amplification was undertaken with Q5 High-fidelity DNA polymerase (New England BioLabs) and PCR reactions were prepared as described previously (Chung *et al.* 2016). Following confirmation of adequate and appropriately sized PCR products the quadruplicate reactions were pooled and the amplicons were then quantified using a Qubit 2.0 Fluorometer (Life Technologies Ltd) and a sequencing master-mix was created using equimolar concentrations of DNA from each sample. Sequencing was carried out on an Illumina MiSeq machine, using 2 × 250 bp read length, at the Wellcome Trust Sanger Institute (Cambridgeshire, UK). All sequence data has been deposited in the European Nucleotide Archive and is available under study accession number PRJEB7702, and sample accession numbers ERS580358-ERS580471 (Table S1, Supporting Information).

The sequences obtained were analyzed using the mothur software package (Schloss *et al.* 2009) with the forward and reverse reads assembled into paired read contigs. Any paired contigs that were shorter than 270 bp, longer than 480 bp, contained ambiguous bases or contained homo-polymeric stretches of longer than seven bases were then removed. Unique sequences were aligned against the SILVA reference database. Pre-clustering (diffs = 3) was performed to reduce the impact of sequencing errors. The OTUs were generated at a 97% similarity cut-off level. Chimeric molecules created during PCR amplification, as well as, reads from chloroplast, mitochondria, archaea, eukaryote and unknown sequences were removed from the dataset (Quince *et al.* 2011). As a result, the final dataset had a total of 2 908 622 sequences with a range of 8333 - 44 418 sequences per sample. All samples were rarefied to 8333 to ensure equal sequencing depth for all comparisons. The final OTU-level results are shown in Table S1 (Supporting Information). Significant differences across all cohorts were identified using LEfSe analysis (Segata *et al.* 2011). The Shannon and Inverse-Simpson diversity indices were used to calculate bacterial diversity per sample. Significant difference between fermenter-based samples with differing single carbohydrates and carbohydrate-mixes were tested using independent sample t-tests and one way ANOVA respectively.

Quantitative PCR (qPCR) to estimate total bacterial load

Quantitative real-time PCR (qPCR) was performed with iTaqTM Universal SY BR® Green Supermix (Bio-Rad) in a total volume of 10 μL in optical-grade 384-well plates sealed with optical sealing tape. Amplification was performed with a CFX384TM Real-time System (Bio-Rad) with the following protocol: one cycle of 95°C for 3 min, 40 cycles of 95°C for 5 s and annealing temperature of 60°C for 30 s, 1 cycle of 95°C for 10 s and a stepwise increase of the temperature from 65°C to 95°C (at 5 s per 0.5°C) to obtain melt curve data. As described previously standard curves consisted of 10-fold dilution series of amplified bacterial 16S rRNA genes from reference strains. Samples were amplified with universal primers against total bacteria (UniF) as described previously (Ramirez Farias *et al.* 2009).The abundance of 16S rRNA genes was determined from standard curves. The detection limit was determined with negative controls containing only herring sperm DNA.

Short chain fatty acid (SCFA) analysis

SCFA formation was measured in fermenter samples by gas chromatography as described previously (Richardson *et al.* 1989). Following derivatization of the samples using N- tert-butyldimethylsilyl-N-methyltrifluoroacetamide, the samples were analyzed using a Hewlett Packard gas chromatograph fitted with a fused silica capillary column with helium as the carrier gas.

Statistical analysis

Sequencing (MiSeq) and SCFA data from these experiments were analyzed by ANOVA with donor, time and substrate within donor as random effects, and with substrate, time and their interaction as fixed effects. When an effect was significant ($P < 0.05$) mean values were then compared by post-hoc t-test based on the output from the ANOVA analysis. R (R Core Team 2013) and lme4 (Bates *et al.* 2015) were used to perform a linear mixed effects analysis of the relationship between Shannon diversity index and early and late phases. As fixed effects, early and late phases and substrates were entered into the model. As random effects, intercepts were entered for donor. *P*-values were obtained by likelihood ratio test of the full model with the effect in question against the model without the effect in question.

RESULTS

Experimental design

Five continuous flow fermenters that received an identical faecal inoculum were run in parallel for a period of 20 d at a constant pH (6.1±0.1) under conditions that model active fermentation in the colon (Fig. 1). Three vessels received a continuous input of a single carbohydrate (either inulin, arabinoxylan-oligosaccharides (AXOS) from wheat bran or apple pectin, supplied at 4.2 g/L) while a fourth vessel (3-mix) received all three substrates, each at one-third of the concentration (1.4 g/L of each) used for the single substrate. The fifth vessel (6-mix) received the

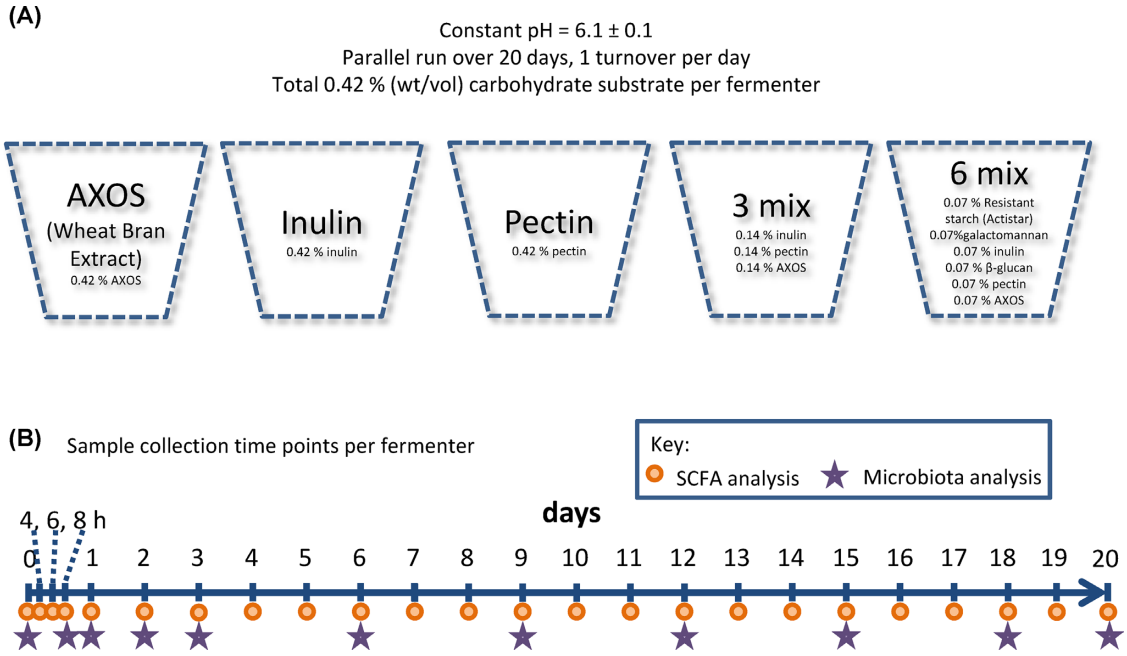

Figure 1. Fermenter study design. **(A)** A schematic diagram showing the design of the fermenter experiments used in this study. Single substrates (inulin, apple pectin or arabinoxylan-oligosaccharide (AXOS) from wheat bran extract (WBE) or carbohydrate mixes (3-mix and 6-mix) were used at a final concentration of 0.42% of total volume (see Methods section). Five vessels were run in parallel at a constant pH (6.1 ± 0.1) with the same faecal inoculum, and two independent experiments were conducted with samples from two different donors. **(B)** Multiple samples were collected for SCFA analysis and DNA extractions, which were used for subsequent amplification of 16S rRNA gene sequences.

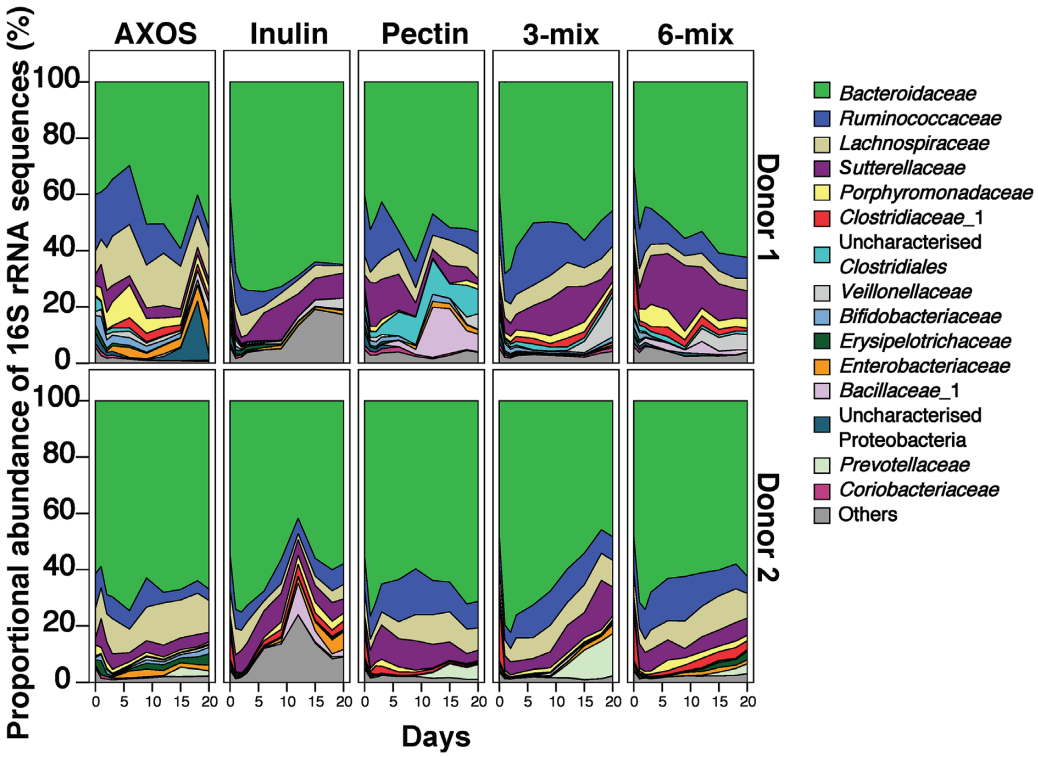

Figure 2. Effect of carbohydrate source on colonic microbial community composition determined by 16S rRNA gene sequencing. Microbiota composition is shown here at the family level, while abundant operational taxonomic units (OTUs) that responded significantly to particular substrate regimes are shown in Table S1 (Supporting Information). A full list of OTUs for all samples is given in Table S3 (Supporting Information).

Table 1. LEfSe analysis showing OTUs derived from 16S rRNA gene sequences that increased significantly in relative abundance with a particular substrate or substrate mix. Only OTUs comprising >0.1% of total sequences are included (see Table S1, S2 (Supporting Information) for listing of all OTUs).

OTU	Substrate	P value	Proportional abundance (%)	MegaBLAST closest match (Representative Seq.)
Otu00001	Inulin	7.8E-15	24.11	*Bacteroides uniformis*
Otu00002	Pectin	1.7E-13	13.41	*Bacteroides vulgatus/dorei*
Otu00003	6-mix	5.6E-10	9.69	*Bacteroides ovatus*
Otu00004	6-mix	1.7E-06	7.38	*Sutterella wadsworthensis*
Otu00005	Pectin	0.02153	4.79	*Bacteroides stercoris*
Otu00006	3-mix	0.00226	2.89	*Bacteroides cellulosilyticus/intestinalis*
Otu00010	AXOS	4.1E-07	1.38	*Parabacteroides distasonis*
Otu00011	Pectin	7.4E-10	0.93	*Eubacterium eligens*
Otu00012	6-mix	0.00402	0.91	*Oscillibacter* sp.
Otu00015	AXOS	4.8E-09	0.71	Unclassified *Lachnospiraceae*
Otu00016	AXOS	0.00018	0.64	*Escherichia/Shigella* spp.
Otu00017	AXOS	1.4E-09	0.62	*Bifidobacterium longum*
Otu00022	Inulin	2.5E-05	0.55	*Enterococcus* sp.
Otu00024	6-mix	0.00011	0.53	*Flavonifractor plautii*
Otu00026	AXOS	2E-12	0.50	*Clostridium* sp.
Otu00030	Pectin	0.01221	0.41	Uncharacterized *Ruminococcaceae*
Otu00033	Pectin	1.6E-07	0.40	*Faecalibacterium prausnitzii* (L2–6)
Otu00042	AXOS	0.01562	0.27	Uncharacterized *Proteobacteria*
Otu00045	AXOS	4.6E-05	0.25	*Oscillibacter valericigenes*
Otu00048	AXOS	8.2E-06	0.24	*Veillonella parvula*
Otu00051	AXOS	0.01927	0.23	Uncharacterized *Lachnospiraceae*
Otu00052	Pectin	7.2E-12	0.22	*Ruminococcus* sp.
Otu00056	AXOS	4.1E-05	0.21	*Ruminococcus* sp.
Otu00058	Inulin	1.1E-05	0.21	*Terrahaemophilus aromaticivorans*
Otu00061	AXOS	1.9E-10	0.19	Uncharacterized *Ruminococcaceae*
Otu00064	Pectin	0.00211	0.19	*Roseburia* sp.
Otu00068	AXOS	6.4E-12	0.18	Uncharacterized *Anaerotruncus*
Otu00071	AXOS	0.02738	0.17	*Coprococcus comes*
Otu00073	Pectin	2.7E-08	0.16	Unclassified *Lachnospiraceae*
Otu00079	AXOS	3.2E-05	0.14	*Clostridium butyricum/beijerinckii*
Otu00080	6-mix	0.00199	0.14	*Bilophila wadsworthia*
Otu00082	AXOS	4E-06	0.12	*Blautia* sp.
Otu00083	AXOS	3.5E-05	0.12	*Bifidobacterium catenulatum*

same three substrates plus three additional substrates (starch, β-glucan and galactomannan), each at one-sixth of the concentrations (0.7 g/L of each) used for the single substrates. The whole experiment was subsequently repeated using a different faecal inoculum from a second donor.

Dominant bacterial species (OTUs) and total bacterial load

Microbiota composition changes for the two sets of experiments were assessed using Illumina MiSeq sequencing of 16S rRNA gene amplicons (Table S1, Supporting Information) and qPCR to determine total bacterial load (Table S2A, Supporting Information), which showed no significant difference in the total bacterial abundance across substrates and the two donors. The faecal inocula showed considerable overlap in the dominant OTUs between the two donor communities. Of the top 50 OTUs detected in the faecal samples, 36, which included nine Bacteroidetes and 22 Firmicutes OTUs, were found in both donors (Table S2B, Supporting Information). During the subsequent 20 d incubation period, several significant differences were detected at the family level between the microbial communities present in the five parallel vessels supplied with different substrates or substrate mixes (Fig. 2). In particular the Bacteroidaceae family was most abundant when inulin was added as the sole carbohydrate source ($P = 0.001$) and also shown in Table S2A (Supporting Information). Moreover, LEfSe analysis identified a number of OTUs that were significantly more proportionally abundant with certain substrate regimes. Out of the top 92 OTUs (those comprising >0.1% of all sequences), 16 OTUs were significantly stimulated in relative abundance by AXOS, eight OTUs by pectin and three OTUs by inulin (Table 1; Table S3, Supporting Information). Moreover, five OTUs were significantly promoted by the 6-mix and one OTU (*Bacteroides cellulosilyticus/intestinalis*) by the 3-mix.

Selective stimulation in relative abundance of *B. vulgatus*, *B. stercoris* and *Eubacterium eligens* by pectin and of *B. uniformis* by inulin agrees well with previous findings (Chung *et al.* 2016) despite the fact that the present study involved a different source of apple pectin. The two donors providing samples in this study were also involved in the previous study (D1 and D2 in this study correspond to D1 and D3 in Chung *et al.* 2016).

Ten OTUs were identified from the Bacteroidetes phylum in the top 26 most proportionally abundant OTUs (Fig. S1A, Supporting Information). *Bacteroides uniformis* accounted for 73% of total bacterial 16S rRNA gene sequences throughout the 20 d with inulin as substrate. Thirteen OTUs were identified from the Firmicutes phylum in the top 26 OTUs (Fig. S1B, Supporting Information). The proportional abundance of *E. eligens* was stimulated in pectin fermenters (LEfSe, $P <$

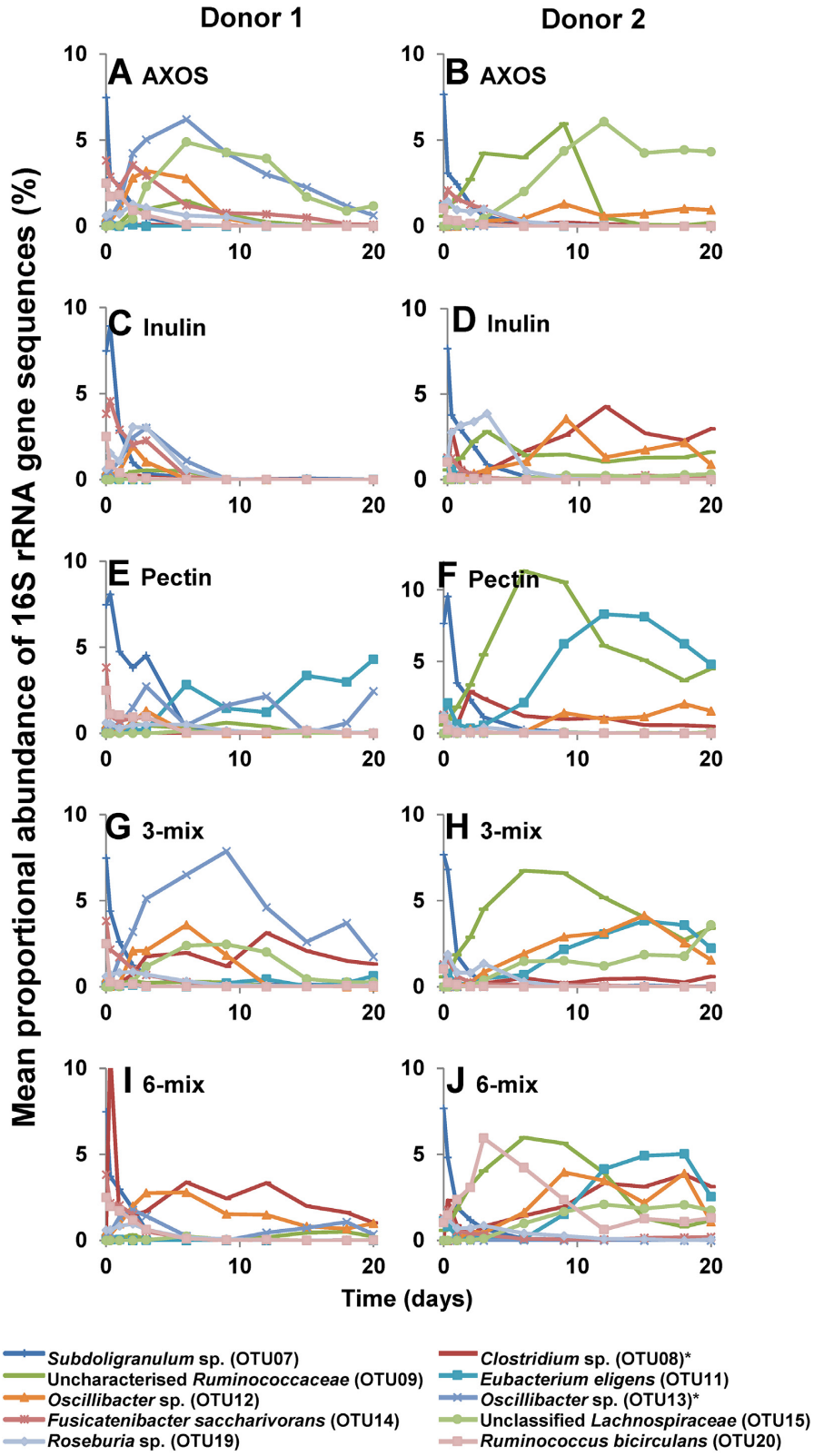

Figure 3. Firmicutes changes in proportional abundance over time (20 d) at the operational taxonomic unit (OTU) level for three single substrates, a three-mix and six-mix run in parallel. Separate data are shown for two different donors. **(A, B)** AXOS, **(C, D)** inulin, **(E, F)** pectin, **(G, H)** 3-mix and **(I, J)** 6-mix with mixed faecal microbiota from two different donors. Data for donors 1 and 2 are shown separately.

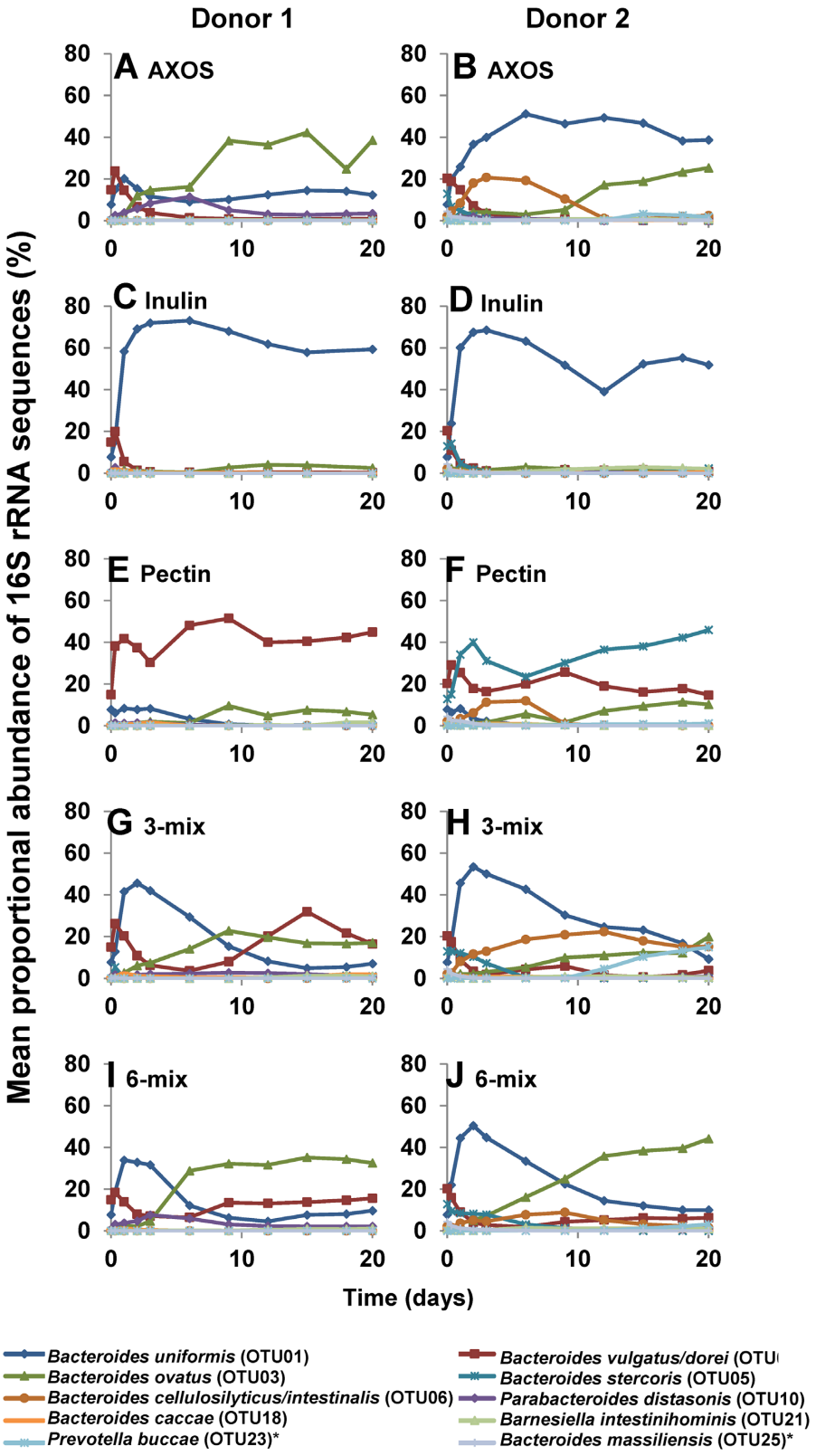

Figure 4. Bacteroidetes changes in proportional abudance over time (20 d) at the operational taxonomic unit (OTU) level for three single substrates, a 3-mix and 6-mix run in parallel. Separate data are shown for two different donors. **(A, B)** AXOS, **(C, D)** inulin, **(E, F)** pectin, **(G, H)** 3-mix and **(I, J)** 6-mix following inoculation with mixed faecal microbiota from two different donors. Data for donors 1 and 2 are shown separately.

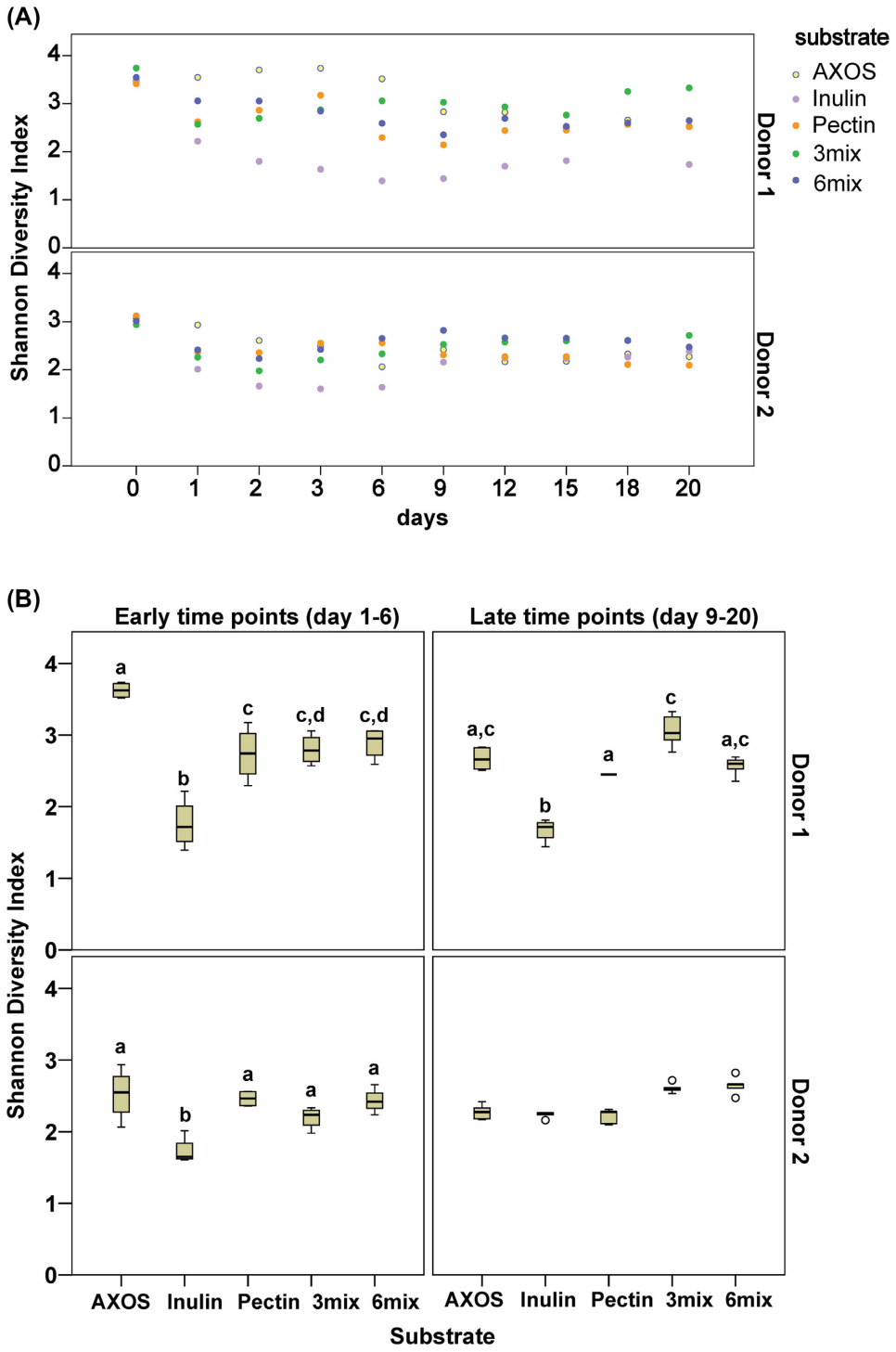

Figure 5. Alpha diversity as measured by the Shannon diversity index are shown in **(A)** for individual time points over 20 d for each of the donors. In **(B)** mean indices are shown for week one (day 1–6) and weeks two and three (day 9–20) for each donor. Treatments that do not share a superscript letter are significantly different at the level $P < 0.01$.

0.0001) for both D1 and D2 (Fig. 3; Fig. S1, Supporting Information). Similarly an unidentified Lachnospiraceae (OTU00015) was stimulated by AXOS in both experiments with samples provided by the two donors. Among the Actinobacteria, *B. longum* (OTU00017) and *B. catenulatum* (OTU00083) were proportionally more abundant in the AXOS fermenters (LEfSe, $P < 0.0001$). Many other changes appeared donor-specific, with an unidentified Ruminococcaceae (OTU00009) increasing in proportional abundance initially with AXOS, pectin, the 3-mix and 6-mix only in the D2 incubations, and an *Oscillibacter* OTU (OTU00013) becoming prominent in D1 incubations in the AXOS and 3-mix fermenters (Fig. 3).

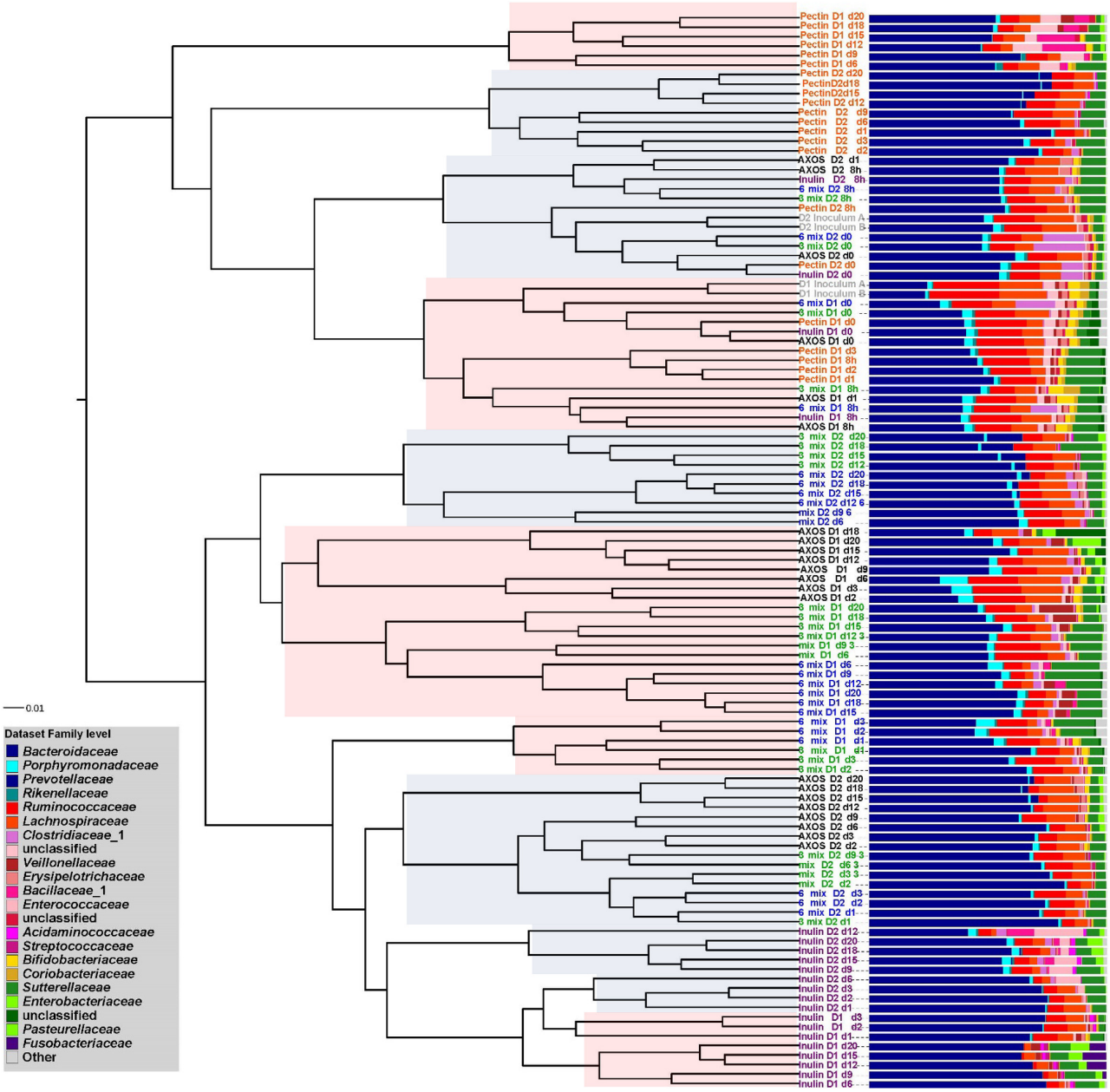

Figure 6. Bray–Curtis dissimilarity dendrogram showing beta-diversity between samples with bacterial composition at the family level shown. Sample labels are color coded with different substrates: pectin (orange), AXOS (black), inulin (purple), 3-mix (green), 6-mix (blue) and branches are color coded with donor 1 shown in red and donor 2 in light blue.

Compositional shifts over time

The time courses revealed some major shifts in microbiota profiles when viewed at the OTU level (Fig. 3, Fig. 4). In the experiments with the donor 2 (D2) inocula, proportional abundances of *B. ovatus* increased and *B. cellulosilyticus/ intestinalis* decreased between 10 and 20 d both in the AXOS-fed and pectin-fed fermenters, although neither was the most dominant OTU. In the 6-mix fermenters for both donors, *B. uniformis* was dominant over the first 5 d, but was progressively replaced by *B. ovatus* thereafter (Fig. 4). These changes might be explained by the emergence and selection of *B. ovatus* strains with increased competitiveness during the experiment. It is worth noting that the five vessels were run in parallel for each inoculum (D1 and D2) and that the dominant OTU for the inulin-fed fermenters (*B. uniformis*) remained at relatively stable levels (accounting for between 40% and 80% of total sequences) throughout the 20 d in both cases. This apparently constant selection for the same species in the case of inulin provides a striking contrast with the pattern of multiple competing species that was seen for the substrate mixtures.

Impact of substrate complexity on microbial diversity

Bacterial diversity within each sample (alpha diversity) was assessed using the Shannon index and inverse Simpson's index (Fig. 5; Fig. S2, Supporting Information). The average Shannon index across all time points revealed that the inoculum (day

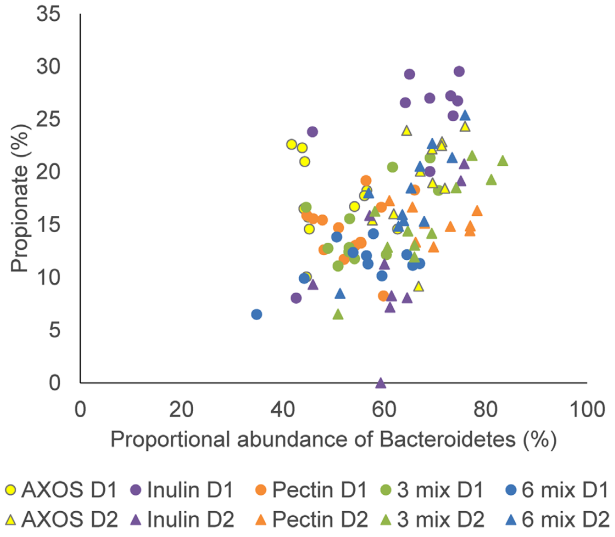

Figure 7. Correlation between propionate (%) and Bacteroidetes proportion (%). Each point represents the propionate levels (%) of the total short chain fatty acid concentration and the abundance of Bacteroidetes (%) of the total microbiota on single substrates and substrate mixes following incubations in fermenters inoculated with slurries from two different donors (D1 and D2).

0) was significantly more diverse than the day 1–20 fermenter communities (versus 3-mix $P = 0.038$, 6-mix $P = 0.031$, AXOS $P = 0.049$, inulin $P = 0.003$ and pectin $P = 0.016$) (Fig. 5A). Analysis of samples from the first week (days 1–6) showed that for Donor 1, the AXOS-fed condition resulted in significantly higher community diversity ($P < 0.002$), and inulin significantly lower diversity ($P < 0.001$), than the other substrates (Fig. 5B). Community diversity continued to be lowest for the inulin-fed fermenter during days 9–20 ($P < 0.007$). In the Donor 2 experiment, community diversity was significantly lower for the inulin-fed fermenter only in the early phase (day 1–6) ($P < 0.008$) (Fig. 5B).

The inverse Simpson's index also indicated that the overall effect of substrate is dependent on the length of the time that the microbiota had been subjected to the various substrates (ANOVA, $P = 0.008$) (Fig. S2, Supporting Information). The diversity in the AXOS-fed D1 inoculated fermenters changed with time from inoculation (ANOVA, $P = 0.001$), with the highest diversity observed at the early time points (day 1–6) (Fig. S2, Supporting Information).

The similarity and diversity across the samples were also calculated using the Bray–Curtis dissimilarity index (Fig. 6). Samples were separated into two main clusters, one group consists of inoculum and early time points and another group with mainly later time points. Bacterial communities were significantly different across substrates (Analysis of Molecular Variance AMOVA, $P < 0.001$) and individual clusters were observed by donor (AMOVA, $P < 0.001$).

Fermentation products

Total SCFA concentrations were relatively stable over time in individual fermenters (Fig. S3, Supporting Information). There were, however, differences between the two experiments, for example in the proportion of propionate when inulin was the substrate. This appears to reflect the higher percentage *Bacteroides* in the Donor 1 compared to Donor 2 inulin fermenters. Bacteroidetes were by far the most proportionally abundant group of propionate-producing bacteria present in these incubations and there was a significant correlation ($P < 0.001$) between percentage propionate among total SCFA and percentage Bacteroidetes (slope by regression 0.1861) within the community (Fig. 7). When inulin was the sole substrate, there was strong selection for *B. uniformis* (OTU00001) for both donors ($P = 0.001$).

CAZyme profiles of dominant polysaccharide-utilizing *Bacteroides* species

Table 2 shows the complement of glycoside hydrolase, polysaccharide lyase and carbohydrate esterase genes potentially involved in degradation of AXOS, pectin and inulin (derived from the CAZY database; URL http://www.cazy.org/) within the genomes of seven *Bacteroidetes* species that were found to be stimulated by the different substrates and substrate combinations in these experiments. It appears that CAZyme profiles of previously isolated strains do not provide a straightforward prediction of the competitive success of that species on a given substrate, although some general patterns were in agreement. For example, *B. uniformis*, the most successful inulin degrader in our experiments, is predicted to encode the greatest number of GH32 genes required for inulin metabolism. When considering pectin, complements of putative pectin-degrading genes range from 7, 8 and 17 in *B. uniformis, P. distasonis* and *B. stercoris* respectively up to 44, 52, 55 and 69 in *B. vulgatus, B. intestinalis, B. dorei* and *B. ovatus* respectively. In agreement with the fermenter experiments, two of the three species with comparatively low predicted pectin degrading ability, *B. uniformis* and *P. distasonis*, did not increase in relative abundance in fermenters solely fed with pectin. However for the third species, *B. stercoris*, sequences were significantly promoted by pectin within the mixed microbiota derived from one of the faecal donors.

DISCUSSION

Many factors influence the diversity of gut microbial communities *in vivo* including the supply of substrates, growth factors and pH (Duncan *et al.* 2009; Walker *et al.* 2011; David *et al.* 2014; Magnúsdóttir *et al.* 2015). Microbial diversity as measured in faecal samples is particularly complex to interpret as it represents a historical record of shifts in transient communities derived from different regions of the large intestine.

By using conditions of constant pH and substrate supply *in vitro* we have been able to focus here solely on the impact of carbohydrate substrate diversity and complexity upon microbial community diversity and composition with a limited number of donors providing the faecal inoculum. A number of studies employing chemostats have recommended that several weeks are allowed for 'stabilization' of the community to occur (McDonald *et al.* 2013). While this may be desirable where the system is being used to test imposed perturbations, such an approach would have little value when instigating the impact of substrate complexity on microbial diversity. As we report, there was an initial decrease in diversity for all fermenter communities compared with the inoculum. Since t_0 samples, taken within 30 minutes of inoculation, showed community profiles that clustered with those of the inoculum (Fig. 6), we can conclude that this is the result of selection within the fermenter. The observed decrease in alpha diversity is expected as a result of the greater constancy of environmental conditions and substrate supply, together with a much more limited range of substrates, *in vitro*, as compared with the situation *in vivo*. The result

Table 2. Genes encoding carbohydrate active enzymes (CAZymes) potentially involved in AXOS, inulin and pectin degradation in six species of Bacteroidetes that showed significantly higher proportional abundances with a specific substrate or substrate mix. Shading reflects number of domains.

	LEfSe significant increase	AXOS	Pectin	Inulin	Pectin	Pectin	3 mix	6 mix	
	Enzyme families	*P. distasonis* ATCC 8503	*B. stercoris* ATCC 43183	*B. uniformis* ATCC 8492	*B. vulgatus* ATCC 8482	*B. dorei* DSM 17855	*B. intestinalis* DSM 17393	*B. ovatus* ATCC 8483	
AXOS degrading enzymes	GH3	7	5	23	5	5	21	21	xylan 1,4-β-xylosidase/α-L-arabinofuranosidase
	GH5	0	0	6	0	1	5	5	endo-β-1,4-xylanase/endo-β-1,4-glucanase
	GH10	0	0	0	1	1	6	8	endo-1,4-β-xylanase
	GH30	2	0	3	5	2	7	4	endo-β-1,4-xylanase/β-xylosidase
	GH39	0	0	0	0	0	1	0	β-xylosidase
	GH43	6	2	17	22	28	50	35	β-xylosidase/arabinanase/xylanase
	GH51	3	0	3	3	3	5	4	endo-β-1,4-xylanase/β-xylosidase
	GH67	0	0	0	1	1	1	2	xylan α-1,2-glucuronidase
	GH115	0	0	1	0	5	6	8	xylan α-1,2-glucuronidase
	CE1	2	1	5	1	2	8	1	acetyl xylan esterase
	CE2	0	0	0	0	0	1	0	acetyl xylan esterase
	CE4	4	2	3	3	5	4	4	acetyl xylan esterase
	CE6	0	0	0	0	0	3	3	acetyl xylan esterase
	CE7	0	1	2	1	2	2	3	acetyl xylan esterase
	CE15	0	0	0	1	1	1	0	4-O-methyl-glucuronoyl methylesterase
Total AXOS degradation		***24***	***11***	***63***	***43***	***56***	***121***	***98***	
Inulin degrading enzymes	GH32	1	2	4	1	1	3	2	endo-inulinase/exo-inulinase
	GH91	0	0	0	0	0	0	2	inulin lyase
Total inulin degradation		***1***	***2***	***4***	***1***	***1***	***3***	***4***	
Pectin degrading enzymes	GH28	1	5	2	13	19	15	14	rhamnogalacturonase
	GH78	7	0	3	5	5	6	8	α-L-rhamnosidase
	GH105	0	2	2	7	7	16	12	unsaturated rhamnogalacturonyl hydrolase
	GH106	0	1	0	3	6	4	4	α-L-rhamnosidase
	PL1	0	3	0	2	2	2	9	pectate lyase/exo-pectate lyase
	PL9	0	0	0	0	0	0	2	pectate lyase/exo-polygalacturonate lyase
	PL10	0	1	0	2	3	1	1	pectate lyase
	PL11	0	1	0	3	3	2	5	exo-unsaturated rhamnogalacturonan lyase
	CE8	0	3	0	4	4	2	6	pectin methylesterase
	CE12	0	1	0	5	6	4	8	pectin acetylesterase
Total pectin degradation		***8***	***17***	***7***	***44***	***55***	***52***	***69***	
Total GH/PL/CE domains		***114***	***120***	***200***	***201***	***252***	***368***	***378***	

is selection for the most competitive strains under the constant conditions of flow rate, pH and substrate supply within each fermenter vessel (Kettle *et al.* 2015; Chung *et al.* 2016). By introducing the alternative substrate regimes without a delay, we maximize the diversity of strains that are subject to selection by the substrates and substrate combinations employed. In contrast, a 'fermenter-adapted' community established after a 'stabilization' period of 2 weeks or more would have lost much of the initial species diversity, as shown previously (McDonald *et al.* 2013). For this reason we chose to start with the closest available approximation to the *in vivo* community, as represented by the faecal inoculum, rather than with a less diverse, 'fermenter-adapted', community. The key point here is that the fermenters were run simultaneously and in parallel from the same inoculum, thus allowing direct comparison of community changes resulting from different substrates and substrate mixes.

Comparison of the three fermenters supplied with single substrates, which were run in parallel from the same inoculum, showed significantly lower overall diversity of OTUs in the inulin-fed fermenters within the first week compared with those fed with AXOS or pectin. This is likely to reflect the fact that inulin is a simple homopolymer, comprised of fructose residues, while the greater chemical complexity of AXOS and pectin (Caffall and Mohnen 2009) may create more nutritional niches. This suggests that the complexity of individual substrates has a real impact upon community diversity. It might be anticipated that increasing the number of substrates, while keeping the total carbohydrate input constant, would further increase community diversity. In reality, however, we could find little evidence that the '6-mix' substrate combination increased diversity above that seen with the single AXOS substrate although the '3-mix' did result in the highest diversity indices at the final time point. It is feasible that increasing the total level of carbohydrate may also result in increased diversity.

As in previous studies, we found that *Bacteroides* spp. were dominant in these fermenters (Duncan *et al.* 2003; Chung *et al.* 2016). This is likely to reflect the supply of soluble polysaccharides together with the high peptide content of the medium and the controlled pH of 6.1 was evidently not low enough to curtail *Bacteroides* growth (Walker *et al.* 2005). As reported previously (Chung *et al.* 2016) proportional abundances of different species were promoted by the individual substrates, with *B. uniformis* favored by inulin and *B. vulgatus/dorei* and *B. stercoris* by pectin. AXOS, not included in the previous study, promoted another *Bacteroidetes* species, *Parabacteroides distasonis*. Of particular interest, however, is the finding that *B. ovatus* was significantly favored by the '6-mix' and *B. intestinalis/cellulosilyticus* by the '3-mix' substrate combinations. Species representing these two OTUs encode particularly large numbers of Carbohydrate Active Enzymes (CAZymes) (>350 each) (Table 2) and this suggests that such large complements of degradative enzymes may be of particular benefit to these species in competing for energy sources when a variety of alternative complex polysaccharides is available. This conclusion agrees with a study conducted using gnotobiotic mice in which *B. cellulosilyticus* was found to be exceptionally competitive within an artificial consortium of 12 human colonic anaerobes that included seven *Bacteroides* species (McNulty *et al.* 2013). On the other hand, several of the *Bacteroidetes* species that were most successful in fermenters supplied with single substrates tended to have smaller CAZyme complements (<250 genes) and appear relatively more specialized. For example, *B. uniformis* possesses four genes (GH32) likely to be involved in inulin degradation, but relatively small numbers of genes likely to be involved in pectin utilization. By contrast *B. vulgatus*, which was the most competitive pectin-utilizer, has 44 potential pectin utilization genes compared with only seven in *B. uniformis*, but encodes only one GH32 enzyme. It should be noted, however, that the extent of within-species or

strain variation in CAZyme profiles has not been investigated in detail and we cannot be certain that the isolated strains for which genomes are available are representative of the strains that became enriched in these experiments. Under the constant selection conditions prevailing in our chemostats it seems likely that the affinities of the relevant systems for sequestering and taking up soluble polysaccharides (PUL-encoded Sus protein systems in the case of *Bacteroides* spp.) will be critical in determining competitive success. While the molecular architecture of *sus* systems in increasingly well understood for a few strains, detailed kinetic data are less well documented (Martens *et al.* 2009).

While two *Bifidobacterium* species were significantly stimulated by AXOS (Table 1) in these experiments, bifidobacteria did not achieve the dominance that has been reported in faecal samples from many *in vivo* studies following dietary supplementation with inulin or AXOS (Bouhnik *et al.* 2007; Ramirez Farias *et al.* 2009). As we have suggested previously (Chung *et al.* 2016) the low pH values that result from active fermentation and short chain fatty acid production in the proximal colon *in vivo* may be important in creating conditions that allow bifidobacteria to compete with other inulin-utilizing bacteria, notably *Bacteroides* species. Our data suggest that the proximal colonic pH may need to be lower than the value of 6.1 employed here to result in high proportions of *Bifidobacterium* species. It may also be that media containing high peptide levels select against *Bifidobacterium* species under chemostat conditions, as suggested by the work of Walker *et al.* (2005).

The two donors employed showed relatively similar bacterial profiles with 72% of the top 50 most abundant OTUs being common to both inocula. In spite of this, there were some notable differences in responses at the species and metabolite level between the two experiments, as discussed earlier. Our expectation in designing this study was that impacts of substrate complexity upon microbiota diversity would be generic and largely independent of the detailed composition of the microbiota and the emphasis was therefore placed on sampling a large number of time points rather than a large number of donors. Nevertheless, it would clearly be of interest to examine a larger number of microbiota donors in future studies.

Overall, this study suggests that the complexity of different non-digestible dietary polysaccharide substrates can have an important impact on gut microbiota diversity. Combinations of partially purified substrates may also increase microbiota diversity, but these showed a less clear-cut effect here. It should be noted, however, that this work has focussed on soluble polysaccharides and it remains to be established what the effect on microbiota diversity is of insoluble fibrous substrates, notably plant cell walls, which possess a very high degree of both chemical and structural complexity. Recent work suggests that such insoluble substrates are likely to create additional niches for specialized primary degraders, which are to be found especially among the Firmicutes (Ze *et al.* 2012; Ben David *et al.* 2015; Duncan *et al.* 2016). The ability to deconstruct complex, recalcitrant substrates requires attachment mechanisms and enzyme systems that appear to be present in a limited number of species whose activities release substrates that can become available to other members of the community (Ze *et al.* 2012; Ben David *et al.* 2015). Insoluble fibre may therefore also play a role in increasing and maintaining microbiota diversity within the colonic microbiota of healthy human adults.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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