# Specific substrate-driven changes in human faecal microbiota composition contrast with functional redundancy in short-chain fatty acid production

Nicole Reichardt[1,2], Maren Vollmer[1], Grietje Holtrop[3], Freda Farquharson[1], Daniel Wefers[4], Mirko Bunzel[4], Sylvia H. Duncan[1], Janice E. Drew[1], Lynda M. Williams[1], Graeme Milligan[2], Thomas Preston[5], Douglas Morrison[5], Harry J. Flint[1], Petra Louis[1*]

[1]The Rowett Institute, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK

[2]Institute of Molecular, Cell and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

[3]Biomathematics and Statistics Scotland, Foresterhill, Aberdeen AB25 2ZD, UK

[4]Department of Food Chemistry and Phytochemistry, Karlsruhe Institute of Technology (KIT), Adenauerring 20A, 76131 Karlsruhe, Germany

[5]Scottish Universities Environmental Research Centre, University of Glasgow, Rankine Avenue, East Kilbride, G75 0QF, UK

*Corresponding author: Petra Louis; The Rowett Institute, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZD, UK; p.louis@abdn.ac.uk; phone +44 1224 438735.

**Conflict of interest.**

The authors declare that they have no competing interests.

## Abstract

The diet provides carbohydrates that are non-digestible in the upper gut and are major carbon and energy sources for the microbial community in the lower intestine, supporting a complex metabolic network. Fermentation produces the short-chain fatty acids acetate, propionate and butyrate, which have health-promoting effects for the human host. Here we investigated microbial community changes and short-chain fatty acid production during *in vitro* batch incubations of 15 different non-digestible carbohydrates, at two initial pH values with faecal microbiota from three different human donors. To investigate temporal stability and reproducibility, a further experiment was performed one year later with four of the carbohydrates. The lower pH (5.5) led to higher butyrate and the higher pH (6.5) to more propionate production. The strongest propionigenic effect was found with rhamnose, followed by galactomannans, whereas fructans and several α- and β-glucans led to higher butyrate production. 16S rRNA gene-based qPCR analysis of 22 different microbial groups together with 454 sequencing revealed significant stimulation of specific bacteria in response to particular carbohydrates. Some changes were ascribed to metabolite cross-feeding, eg. utilization by *Eubacterium hallii* of 1,2-propanediol produced from fermentation of rhamnose by *Blautia* spp. Despite marked inter-individual differences in microbiota composition, short-chain fatty acid production was surprisingly reproducible for different carbohydrates, indicating a level of functional redundancy. Interestingly, butyrate formation was influenced not only by the overall % butyrate-producing bacteria in the community but also by the initial pH, consistent with a pH-dependent shift in the stoichiometry of butyrate production.

## Introduction

The relationship between the gut microbiota and host health is well established. The highest concentration and diversity of gut microbes is found in the colon, which acts as a fermentor system for dietary compounds that escape the digestive system of the host. Quantitatively, non-digestible dietary carbohydrates (NDCs) are the main energy sources for bacterial growth in the colon. It is estimated that between 20 and 60 g of NDCs, including plant cell wall polysaccharides, resistant starches (RS), oligosaccharides and sugar alcohols, escape the digestive enzymatic breakdown and reach the human colon each day (Cummings and Macfarlane, 1991). Over the last years research has established that gut bacteria possess an enormous variety of carbohydrate-degrading enzyme activities which allow them to access NDCs (Flint *et al.*, 2012). Microbial fermentation of NDCs mainly leads to the production of the short chain fatty acids (SCFA) acetate, propionate and butyrate, and of lactate, succinate, ethanol, methane, carbon dioxide and hydrogen (Cummings and Macfarlane, 1991). SCFAs are of particular interest for maintaining host health as they are known not only to contribute directly to energy metabolism, but also have positive effects on the host's physiology. Butyrate is mainly metabolised by colonic cells (Hamer *et al.*, 2008), whereas acetate and propionate are absorbed and metabolised by the liver and peripheral organs (Den Besten *et al.*, 2013). Besides serving as an energy source, SCFA are associated with a number of health benefits for the host. Whereas butyrate and propionate have been shown to modulate cell differentiation and to exert anti-carcinogenic and anti-inflammatory effects (Hamer *et al.*, 2008; Louis *et al.*, 2014), acetate and propionate are of interest because of their potential to enhance satiety and suppress appetite either through receptor-mediated or other central mechanisms (Frost *et al.*, 2014; Arora *et al.*, 2011).

The intake of NDCs can have direct (primary) and secondary effects on the microbial community in the large intestine, and therefore on the host's physiology. Particular NDCs can lead to the

stimulation of specialised groups of microorganisms that possess the carbohydrate active enzymes necessary for their utilization (Flint *et al.*, 2012). Through cross feeding, NDC breakdown intermediates or fermentation products from primary degraders can serve as substrates for secondary degraders, which are not directly capable of degrading a certain carbohydrate. This has been demonstrated *in vitro* (Belenguer *et al.*, 2006; Rogowski *et al.*, 2015) and reflects the complex nature of the intestinal ecosystem. The decrease of pH in the colon due to the production of SCFAs can also lead to selective effects on the microbial community as has been demonstrated *in vitro* (Walker *et al.*, 2005; Duncan *et al.*, 2009).

The human gut microbiota is composed of several phyla, with the Firmicutes and Bacteroidetes being the most abundant. Firmicutes not only include the major butyrate producing species (Louis *et al.*, 2010), but also include propionate producers and acetogens (Louis *et al.*, 2014; Reichardt *et al.*, 2014). Bacteroidetes possess genes encoding for the succinate pathway and therefore represent the main propionate producers in the gut (Reichardt *et al.*, 2014). Many *Bacteroides* species are able to degrade a wide range of soluble plant cell wall polysaccharides (Flint *et al.*, 2012; Martens *et al.*, 2014). Firmicutes, on the other hand, tend to have fewer genes involved in carbohydrate breakdown (Flint *et al.*, 2012), but specific members appear to play key roles in insoluble polysaccharide degradation (Ze *et al.*, 2013). For example, *Ruminococcus bromii* is of key importance for the degradation of resistant starch (Ze *et al.*, 2015).

It is important to obtain a good understanding of how different NDCs are degraded and how this affects the gut microbiota and its fermentation products in order to reach conclusions on their effects upon the host's health. This study investigated the degradation of 15 different NDCs by human faecal bacteria during *in vitro* fermentations. They included α- and β-glucans, pectins, galactomannans, arabinoxylan and fructans to achieve a good representation of different dietary NDCs. Fermentations were run at two different initial pH values to simulate proximal and distal colon conditions. The aim was to gain a comprehensive overview of the microbial changes and

SCFA production in a complex community of human faecal microbiota in response to NDC breakdown.

## Methods

*In vitro fermentations*

Anaerobic *in vitro* incubations were carried out in a total volume of 10 ml in triplicate in Hungate tubes sealed with butyl rubber stoppers and screw caps (Bellco Glass, Shrewsbury, UK). The medium (details provided in supplementary methods) contained minerals, bile salts, volatile fatty acids, vitamins and 0.2% (wt/vol) of the test NDC. Cysteine was added to the medium following boiling and dispensed into Hungate tubes while they were flushed with $CO_2$. The vitamin solution and the NDCs were added from stock solutions after autoclaving of the medium, directly before inoculation with the faecal suspension. NDC stock solutions were prepared anaerobically by flushing with $CO_2$ at 1% in water and boiled for 1 min. 15 different NDCs (Table 1) were used in fermentation 1 and five in fermentation 2 to assess reproducibility. The supplier of two of the NDCs used in fermentation 2 was changed (Table 1), as this study formed part of a larger project that investigated carbohydrate effects *in vivo* (to be reported elsewhere). Ethical approval for the study was granted by the Rowett Institute Ethical review panel (number 09/005).

Fresh faecal samples were obtained from 4 different donors (fermentation 1, donors 1, 2, 3; fermentation 2, approximately 12 months later, donors 2b, 3b, 4) with no history of gastrointestinal disorders or antibiotic treatment for at least 3 months prior to the study. Faecal samples were processed within 2 h after defecation. Eight ml of pre-reduced phosphate buffered saline were added to 2 g of faecal sample and then homogenised in a Dispomix Drive (Medic Tools, Lussiwag, Switzerland) and 0.5 ml of the homogenised faecal suspension was used as an inoculum for the fermentation tubes (final faecal concentration: 1%). Incubations for each NDC were carried out in

triplicate at pH 5.5, and 6.5 respectively, on a rotator (Stuart SB3, Bibby Scientific, Stone, UK) at 25 rpm for 24 h at 37 ˚C. A no-NDC control was run in triplicate with every fermentation experiment. At 0 h, 6 h and 24 h an aliquot of 2 ml was taken from the fermentation vessels and centrifuged at 10000 x g for 10 min at 4˚C. The supernatant was stored at -20˚C for analysis of SCFA. The cell pellet was re-suspended in 800 µl of sodium phosphate buffer and 122 µl of MT buffer, transferred to a Lysing Matrix E tube (all part of the FastDNA® spin kit for soil, MP Biomedicals, Illkirch, France) and stored at -70˚C until DNA extraction. Six h samples were processed if the 24 h sample was not available, as growth had taken place by then. Only primary data of those samples were included (Tables S1A and S2B/C, shown in grey font), and they were excluded for any statistical analyses.

*DNA extractions*

DNA from the faecal inoculates and the cell pellets from the fermentation experiments (resuspended in buffer and stored as described above) was extracted using the FastDNA® spin kit for soil (MP Biomedicals, Illkirch, France). For the DNA extraction of the faecal inoculates an aliquot of 500 µl of the homogenised faecal suspension was transferred to a Lysing Matrix E tube and 300 µl of sodium phosphate buffer and 122 µl of MT buffer was added. The samples were stored at -70 ˚C until DNA extraction.

*SCFA analysis*

SCFA concentrations were measured in culture supernatants (0.5 ml) using gas chromatography as described previously (Richardson *et al.*, 1989). After derivatisation, 1 µl of sample was analysed using a Hewlett-Packard gas chromatograph fitted with a fused silica capillary column with helium as a carrier gas. The SCFA concentrations were calculated from the relative response factor with respect to the internal standard 2-ethylbutyrate.

148

149 *Quantitative PCR*

150 The pooled triplicate DNA samples from the *in vitro* fermentations 1 and 2 were analysed by
151 quantitative real time PCR as described previously (Fuller *et al.*, 2007; Ramirez-Farias *et al.*, 2009)
152 with the following modifications. Reactions were performed in duplicate with iTaq$^{TM}$ Universal
153 SYBR® Green Supermix (Bio-Rad, Hemel Hempstead, UK) in a total volume of 10 µl with primers
154 at 500 nM and 5 ng of DNA in optical-grade 384-well plates sealed with optical sealing tape in the
155 presence of 1 µg/ml herring sperm DNA (Promega, Madison, WI, USA). Amplification was
156 performed with a CFX384$^{TM}$ Real-time System (Bio-Rad, Hemel Hempstead, UK) with the
157 following protocol: one cycle of 95 ºC for 3 min, 40 cycles of 95 ºC for 5 s and annealing
158 temperature as per Table S3 for 30 s, 1 cycle of 95 ºC for 10 s and a stepwise increase of the
159 temperature from 65 ºC to 95 ºC (at 5 s per 0.5 ºC) to obtain melt curve data. Primers used for the
160 quantification of the specific bacterial groups are given in Table S3. Standard curves consisted of
161 dilution series of amplified bacterial 16S rRNA genes from reference strains. The abundance of 16S
162 rRNA gene was determined from standard curves and bacterial groups were either expressed as a
163 percentage of total bacteria determined by universal primers or as 16S rRNA gene copies per ml
164 culture. The detection limit was determined with negative controls containing only herring sperm
165 DNA.

166

167 *454 sequencing*

168 Amplicon sequencing of the V1-V3 region of the 16S rRNA genes was performed on GS FLX 454
169 platform by the Centre of Genomic Research of the University of Liverpool and Bioinformatics were
170 conducted in-house using Mothur v. 1.34.4. software platform (Schloss *et al.*, 2009) on the
171 University of Aberdeen's HPC cluster (Maxwell). Full details are given in supplementary methods.
172 Reads per sample varied from 426 to 82 791 (average 9069.1 ± 11044.5). Good's coverage was over

95% for all but one sample (Table S2B) and rarefaction and collectors curves (Fig. S1) showed that a good coverage was achieved for all samples. Exclusion of samples with Good's coverage of less than 97% resulted in very similar results (Table 2) and subsampling to 426 sequence reads per sample revealed that the relative abundance of the top 50 OTUs (representing >88% of all reads) was very similar to the full dataset (Fig. S2). It was therefore decided to work with the full dataset to preserve as much of the data as possible. OTUs were generated at ≥97% sequence identity, which resulted in 1552 OTUs (Table S2C), and the relative abundance was calculated. OTUs with an overall abundance of >100 reads (201 OTUs, 95.6-99.8% of sequence reads per sample) were analysed using the BLAST algorithm (Altschul *et al.*, 1990) and compared to the taxonomy from the SILVA database (Quast *et al.*, 2013). OTUs were then assigned to their corresponding qPCR assays if possible as detailed in supplementary methods and Table S2A.

*Polysaccharide analysis*

All analyses were performed in duplicate. To evaluate authenticity and purity of the NDCs used in this study, their monosaccharide composition was analysed by HPAEC-PAD after acid hydrolysis as described previously (Wefers and Bunzel, 2015). Details of the hydrolysis conditions for the different NDCs are given in supplementary methods. The laminarin from Shaanxi Pioneer Biotech showed significant differences compared to the other polysaccharides, because extremely low levels of glucose (ca. 2 mg/g) were observed. To double-check these results and to get further structural insights, glycosidic linkage types of the two laminarin samples were analysed by methylation analysis as described previously (Wefers and Bunzel, 2015; for details see supplementary methods). The total ion current chromatograms also showed significant differences between the two polysaccharides, with the laminarin from Shaanxi Pioneer Biotech showing only trace amounts of the expected partially methylated alditol acetates (Fig. S3). Thus, based on the analyses described here, a main portion of the NDC purchased from Shaanxi Pioneer Biotech does not appear to be laminarin.

198

199 *Statistical analysis*

200 Data from each fermentation study were analysed by ANOVA with random effect for Donor and
201 fixed effects for NDC, pH, and their interaction, followed by post-hoc t-test. Principal Components
202 Analysis and Partial Least Squares were used to investigate associations between bacteria and SCFA
203 production. Associations of interest were quantified by random effects regression with Donor as
204 random effect and with pH, microbial abundance and their interaction as fixed effects. The
205 agreement between the 454 sequencing and qPCR methods between corresponding bacterial groups
206 (expressed as percentage of total bacteria) was investigated by linear regression. Full details are
207 given in Supplementary methods.

208 For the regression analyses $P<0.05$ was regarded significant. For the ANOVA analyses and
209 subsequent post-hoc comparisons, however, to reduce the reporting of false positives due to the large
210 number of comparisons, an effect was considered significant only when $P<0.001$.

211

212

213 **Results**

214

215 *SCFA from in vitro batch culture incubations*

216 Anaerobic incubations were conducted with 15 different NDCs as energy sources (Table 1; 0.2%
217 w/v) in the presence of human faecal slurries from three healthy donors (fermentation 1) at initial pH
218 values of 5.5 and 6.5. For five NDCs, another fermentation was carried out approximately one year
219 later to assess reproducibility of the responses (fermentation 2, two of the three donors were the same
220 as in fermentation 1). Profiles of net SCFA production after 24 hours of incubation differed between
221 NDCs, especially for those from different NDC classes. Analysis of the chemical composition of
222 NDCs used (shown in Table S4) led to the elimination of one of the sources of laminarin (see

223 Methods) whereas the composition of all other NDCs was consistent with their description. For
224 NDCs used in both fermentations the SCFA profiles were very reproducible (Fig. 1).

225 After 24 hours the cumulative amount of fermentation acids produced was significantly higher at
226 pH 6.5 compared to pH 5.5 in both fermentations (Table S5, $P<0.001$). The main SCFAs produced
227 were acetate, propionate and butyrate, with pH 6.5 leading to higher acetate and propionate and pH
228 5.5 to higher butyrate formation for most NDCs (Fig. 1, $P<0.001$). The branched-chain fatty acids
229 iso-butyrate and iso-valerate as well as formate, valerate, and lactate were only detected in minor
230 amounts (<1.13 mM each), while succinate was not detected in any of the incubations.

231 When individual NDCs were compared to the no-NDC control, starch, β-glucan and
232 inulin/oligofructose gave rise to significantly ($P<0.001$) increased butyrate concentrations when the
233 initial pH was 5.5. At an initial pH of 6.5, this butyrogenic effect was seen for inulin/oligofructose
234 but not for the starches (Fig. 1). Pyrodextrin, laminarin, rhamnose, rhamnogalacturonan and the two
235 galactomannans all gave rise to increased propionate at initial pH 5.5. These same NDCs increased
236 propionate when the initial pH was 6.5, but in addition pullulan, β-glucan and some of the inulin-
237 type NDCs also promoted propionate significantly at the higher pH (all $P<0.001$). The NDC that
238 stands out with regard to propionate production however is rhamnose. The absolute amount produced
239 was between 1.5- and 5.5-fold higher than in the presence of the other NDCs, and the molar ratio
240 (40-42% of total SCFA) was similar to the percentage of acetate in both fermentations and
241 independent of the pH (Fig. 1, Table S5).

242

243 *Microbial population changes detected by qPCR*

244 The microbial composition of the *in vitro* incubations from both fermentation experiments was
245 analysed using qPCR against 21 different bacterial species and groups, in addition to total bacteria
246 and methanogenic Archaea (Table S3). Analysis of the inocula revealed high inter-individual
247 variability of bacterial composition as well as intra-individual differences for the donors used in both

fermentations (donor 2 and 3). In addition to quantitative differences, certain microbial groups were only found in some faecal samples (some ruminococci, *Coprococcus eutactus, Eubacterium eligens*, certain bifidobacteria and methanogenic Archaea), and none of the donors had detectable levels of *Prevotella* spp. (Table S1A). After 24 hours of incubation the total amount of bacteria had increased in all incubations, including the no-NDC control (average fold change of total 16S rRNA gene copies 8.0±2.5). In order to investigate bacterial changes specific to the different NDCs, the data were expressed as the ratio of the absolute 16S rRNA gene copies per ml culture between each carbohydrate incubation and the no-NDC control after 24 hours of incubation (Fig. 2, Table S1B). For several bacterial groups and NDCs, responses were similar in the different donors. Thus, *R. bromii* significantly ($P<0.001$) increased on both types of resistant starch at both pH values, with highest levels reached at pH 5.5 (Fig. 2). Bifidobacteria and several other Firmicutes, especially the *Roseburia* group, also increased on resistant starches and pullulan, in particular at the lower pH value, but due to inter-individual variability this mostly did not reach significance. Barley ß-glucan resulted in significant increases in the *Roseburia* group at both pH values in fermentation 2, whereas *R. inulinivorans* showed a significant increase on laminarin only at pH 6.5 (Fig. 2, all $P<0.001$). At the lower pH value, *Blautia* spp. increased significantly on barley β-glucan in fermentation 1, whereas at the higher pH value, *Bacteroides* spp. increased on two of the three ß-glucan-type incubations (Fig. 2). *Coprococcus eutactus*, which was only detected in one donor, increased dramatically on barley ß-glucan in both fermentations at both pH tested, but not with laminarin (Fig. 2).

Rhamnose led to a significant ($P<0.001$) increase of both *Eubacterium hallii* and *Blautia* spp. at both pH values in fermentation 2, whereas this response was weaker and only observed for *Blautia* spp. at pH 5.5 in fermentation 1 (Fig. 2). NDCs of the pectin class resulted in the highest fold changes relative to the no-NDC control for *F. prausnitzii*, *E. hallii* and *B. bifidum* at pH 5.5, but this did not reach significance. At pH 6.5, *F. prausnitzii* and *E. hallii* showed a significant response on

apple pectin during fermentation 2, whereas *Bacteroides* spp. increased significantly (P<0.001) on rhamnogalacturonan. *Eubacterium eligens*, which was not detected in all donors (Table S1), competed poorly on most of the NDCs tested (fold changes relative to no-NDC control <1; Fig. 2), but showed a numerical increase for the pectin-type NDCs (Fig. 2), especially at the higher pH value. For the two galactomannans, *C. eutactus* (present in only one donor, Table S1) increased relative to no-NDC control, and *Bacteroides* spp. had significantly (P<0.001) higher levels on guar galactomannan at pH 6.5. Bifidobacteria showed the strongest response to arabinoxylan, at pH 5.5, which reached significance for *B. longum*, whereas *Roseburia* spp. increased significantly on arabinoxylan at pH 6.5 only (Fig. 2).

The inulin-type fructans tested resulted in significant (P<0.001) increases of the *Roseburia* group and *A. hadrus* at pH 5.5. Increases were also seen for several other groups, including bifidobacteria, *R. bromii* (pH 5.5 only) and *Blautia* spp., but were mostly not significant (P>0.001, Fig. 2, Table S1B). Interestingly *Bifidobacterium* spp. showed mostly higher increases on oligofructose than on medium- or long-chain inulin (Fig. 2). Individual *Bifidobacterium* species were subject to large inter-individual differences (Fig. 3). For example, *B. adolescentis* and *B. longum*, detected in all donors, responded with higher increases on oligofructose in donor 1 and 3, whereas for donor 2 stronger responses were seen with the longer chain inulin-type fructans. *B. adolescentis* showed a much stronger stimulation in donor 2 compared to *B. longum*, regardless of the type of fructan, whereas *B. longum* achieved high levels of stimulation on various NDCs in the other donors. The *B. catenulatum* group responded strongly to several NDCs in donor 1, whereas *B. bifidum* showed the strongest response to fructan-type NDCs in donor 3 (Fig. 3).

Interestingly, the *R. flavefaciens* group, *Oscillibacter* group, *Dorea* spp. and Negativicutes group were not significantly stimulated by any of the NDCs tested (P>0.001, Fig. 2). Methanogenic Archaea did not exhibit big increases compared to no-NDC control for most incubations they were detected in (Table S1). Regression analysis of SCFA and bacterial groups showed a significant

298 ($P<0.05$) positive correlation of *Bacteroides* spp. and propionate production and of *A. hadrus*, *F.*
299 *prausnitzii* and *Roseburia* group, respectively, and butyrate production (Fig. S4).
300
301 *Relationship between microbiota composition and SCFA formation*
302 In addition to qPCR, the influence of the different NDCs on the microbiota of fermentation 1 was
303 analysed using 454 sequencing. Like qPCR, operational taxonomic unit (OTU) analysis revealed
304 large inter-individual differences in the inocula. In the sample of donor 1 and donor 3 we detected
305 116 and 114 different OTUs, respectively, with an abundance of >100 reads, whereas the inoculum
306 of donor 2 contained 162. OTU 1 (*Blautia obeum*) was the most abundant OTU in donors 2 and 3
307 and the second most abundant in donor 1 after OTU 8 (*A. hadrus*) (Table S2). Statistical analysis of
308 all OTUs that were detected in at least two thirds of all samples after 24 h of incubation revealed that
309 17 OTUs, covering a range of different Bacteroidetes and Firmicutes species, were significantly
310 ($P<0.001$) increased compared to the inoculum on at least one NDC (Table 2).
311 In order to compare the qPCR results with sequencing results, the OTUs were assigned to groups
312 that would be targeted by the qPCR primers used (Fig. S5, Table S2). This led to an assignment rate
313 of 29.2-83.2% per sample (average 59.1%) of all sequences. The results in Fig. S5 show that the
314 changes detected by 454 sequencing agree well with those detected by qPCR. This applies for
315 example to the increase in *Blautia* spp. with rhamnose, in *R. bromii* with RS and in *A. hadrus* with
316 fructans. Correlations were calculated between relative data from qPCR and the sum of OTUs
317 assigned to the corresponding primer set. Significant ($P<0.05$) correlations were found for all OTU
318 groups which could be assigned to corresponding qPCR primers, except for the *Oscillibacter* group
319 (Fig. S6). Weaker correlations likely reflect technical differences arising from either qPCR or
320 sequencing methodology, or a limited understanding of the groups under study, which may affect the
321 accuracy of assigning sequence OTUs to the corresponding qPCR group. *Bifidobacteria* were not

included in this comparison as they are underestimated by 454 sequencing with the primers used here (Walker *et al.*, 2015).

Heat map analysis of relative OTU abundance revealed high inter-individual variation (Fig. S7, 100 most abundant OTUs). Propionate- and butyrate-producing status was assigned to all classified OTUs (Table S2A; 39-87% of sequence data per sample assigned to fermentation product formation based on at least 97% sequence identity to known species) and heat maps for propionate- and butyrate-producing OTUs, respectively, were generated (Fig. 4). This shows that different OTUs contribute to SCFA production in different donors. However, regression analyses of the sum of all propionate- or butyrate-producing bacteria (as percentage of total sequences) to percentage propionate or butyrate produced over 24 h of incubation showed a strong correlation (Fig. 5, $P<0.001$ for both SCFAs). For propionate the initial pH of the incubations had no effect on this relationship, but for butyrate a strong effect of initial pH was found (Fig. 5, $P<0.001$). Partial least squares regression was carried out on all classified OTUs to reveal any associations with acetate, propionate or butyrate, which revealed some strong associations that mostly were individual-specific (Table S2A). These may reflect not just a direct conversion of NDCs to SCFA, but could also include cross-feeding effects.

Relative qPCR and SCFA data obtained from NDCs that were examined in years one and two after 24 h of incubation were further analysed by principal component analysis. This revealed some clustering by donor, but the samples originating from the same donor in different years showed little overlap, showing a relatively large intra-individual variation (Fig. S8A). Rhamnose incubations in particular clustered separately and were associated with propionate production, *Blautia* spp. and *E. hallii* (Fig. S8B&D). Long-chain inulin also tended to result in a bigger difference to no-NDC control than the other NDCs examined (Fig. S8B). A separation by pH could be observed especially for the NDCs other than rhamnose, which was associated with butyrate formation at pH 5.5 and propionate formation at pH 6.5 (Fig. S8C&D).

## Discussion

This study investigated the impact of 15 different NDCs upon microbiota composition in anaerobic batch cultures inoculated with human faecal samples. The *in vitro* batch culture system provided a fast and cost effective way to study the effects of an extensive set of NDCs on the microbial community from four donors. Because the pH of batch cultures cannot be controlled precisely as acids are produced during incubation, we set the initial pH at two different values (5.5 and 6.5) to simulate prevailing conditions in the healthy proximal and distal colon. Using 0.2% carbohydrate, we anticipate that acid production during incubation will have reduced the pH further by 0.5-1 unit by 24 hours so that growth will have occurred largely under mildly acidic conditions. When pH is controlled at 6.5 using a continuous flow fermentor system, we have shown that *Bacteroides* spp. outcompete Firmicutes and Actinobacteria within the human colonic microbiota for soluble carbohydrate substrates (Walker *et al.*, 2005; Duncan *et al.*, 2003; Chung *et al.*, 2016). By contrast in the present study, this dominance of *Bacteroides* spp. was curtailed by the lower pH conditions, and probably also by the reduction in the peptide content of the medium (to 0.1% casitone and 0.1% yeast extract). This has helped to reveal the response of Firmicutes and Actinobacteria to different carbohydrates. Lowering of gut pH due to increased fermentation may also contribute to the reduced abundance of *Bacteroides* species often observed in human dietary trials with NDC (Martinez *et al.*, 2013; Duncan *et al.* 2009).

An overview of the major microbiota responses on the different carbohydrate classes and corresponding pathways for SCFA formation is provided in Fig. 6. The NDC that promoted by far the highest SCFA proportion of propionate was rhamnose. This can be explained by the fact that rhamnose is fermented via the propanediol pathway in some anaerobic bacteria, yielding propionate and sometimes also propanol (Reichardt *et al.*, 2014; Scott *et al.*, 2006; Louis and Flint, 2017). The

distribution of the propanediol pathway of propionate formation from deoxy sugars is however quite limited, being found so far in *Roseburia inulinivorans* and in *Blautia* spp. (Reichardt *et al.*, 2014). This fits very well with the observed highly specific enrichment of *Blautia* spp. on rhamnose that was detected both by 16S rRNA-based qPCR and sequence analysis. In other bacteria that can utilise deoxy sugars, such as *Bacteroides* spp. (Rodionova *et al.*, 2013) and *Anaerostipes rhamnosivorans* (Bui *et al.*, 2014), propionate is not formed and 1,2-propanediol is an end product. *E. hallii* also showed a high qPCR ratio for rhamnose relative to the no-NDC control. Existing *E. hallii* strains are not known to grow on rhamnose (Holdeman and Moore, 1974), but its stimulation is likely to be indirect, due to cross-feeding of 1,2-propanediol formed from rhamnose by *Bacteroides* spp. and *A. rhamnosivorans*, since a recent study demonstrated the ability of *E. hallii* to metabolise 1,2-propanediol (Engels *et al.*, 2016). *E. hallii* also has the ability to utilize lactate (Duncan *et al.*, 2004), which is a major fermentation product of *Blautia faecis* (Park *et al.*, 2013), the *Blautia* species that was most strongly stimulated by rhamnose in these experiments. The propanediol pathway may also contribute significantly to propionate formation from rhamnose residues on rhamnogalacturonan and pectin (25.3 and 4.8% of monosaccharide composition, Table S4), but for the remaining polysaccharides it is expected that propionate will originate mainly via the succinate pathway found in the Bacteroidetes (Reichardt *et al.*, 2014). Consistent with this, the level of propionate produced showed a significant correlation with the abundance of *Bacteroides* spp. based on qPCR results (Fig. S4). Based on sequencing data, relative propionate production correlated more strongly with the sum of all propionate producers (Fig. 5) than with propionate producers that employ either the succinate or propanediol pathway (data not shown), confirming that both pathways contribute to propionate formation. The percentage of butyrate among SCFA was highest for fructans at both initial pHs, and for pullulan, resistant starch type II and III and β-glucan at pH 5.5. This appeared to reflect the stimulation of known butyrate-producing species, in particular the *Roseburia* group, *F. prausnitzii*, *A. hadrus*, and *C. eutactus*, depending on the NDC.

A number of responses to particular NDCs agreed well with previous reports from *in vivo* and *in vitro* studies. They include stimulation of *R. bromii* on resistant starch (Ze *et al.*, 2013; Ze *et al.*, 2015; Walker *et al.*, 2011; Abell *et al.*, 2008; Martínez *et al.*, 2010), of bifidobacteria (Ramirez-Farias *et al.*, 2009; Scott *et al.*, 2014; Selak *et al.*, 2016; McLaughlin *et al.*, 2015), butyrate-producing *Roseburia/Eubacterium rectale* group and *A. hadrus* (Louis *et al.*, 2010; Scott *et al.*, 2014; van den Abbeele *et al.*, 2011) and *Dorea longicatena* (Taras *et al.*, 2002) on inulin type fructans, and of *Bacteroides* spp., *F. prausnitzii* and *E. eligens* on pectin-type NDCs (Chung *et al.*, 2016; Lopez-Siles *et al.*, 2012; Salyers *et al.*, 1977). Arabinoxylan increased bifidobacteria at the lower pH, which reached significance for *B. longum* based on qPCR results. This is in agreement with another *in vitro* study that investigated pure culture growth of different *Bifidobacterium* species and found good growth on arabinoxylan only for strains belonging to *B. longum* (McLaughlin *et al.*, 2015).

SCFA production was surprisingly reproducible for the different NDCs investigated here compared to the high microbiota variation between donors, which indicated that different OTUs contributed to NDC breakdown and SCFA formation in the different donors. For example, OTU 11 (closest relative *R. bromii*, 94% identity) responded strongly to resistant starch in donor one, whereas it was low in donor 3 and OTU 9 (*R. bromii*, 99%) responded strongly to resistant starch in this donor (Fig. S7). When looking specifically at propionate- or butyrate-producing bacteria, it becomes clear that they show a heterogeneous response to different carbohydrates (Fig. 4), but their combined response correlates very strongly with the corresponding SCFA output (Fig. 5), revealing functional redundancy in the microbiota. The activities of each microbiota member will be dependent not only on their genetic potential to degrade certain NDCs and produce certain SCFA, but also on their interaction with other microbes and their competitive fitness. This likely underlies the different response seen for some OTUs in different donors (for example, OTU 8, *A. hadrus* (100%) showing an increase on arabinoxylan in donor 1 and 3, but not donor 2). The poor response of *R. inulinivorans* on inulin and rhamnose likely also reflects its poor ability to compete effectively in the

422 complete microbiota, despite the fact that it can grow on those NDCs in pure culture (Reichardt *et*
423 *al.*, 2014; Scott *et al.*, 2014; Duncan *et al.*, 2006). This agrees with a human intervention study,
424 which also failed to see an increase in this species after inulin supplementation in subjects with high
425 baseline levels of this species (Louis *et al.*, 2010).

426 Our data also reveal that while the % butyrate among SCFA products was related to the
427 proportion of butyrate-producing bacteria, the initial pH altered this relationship considerably (Fig.
428 5). It is known that species such as *F. prausnitzii* and *Roseburia* spp. that use the butyryl-
429 CoA:acetate CoA-transferase route for butyrate formation, exhibit a shift in fermentation
430 stoichiometry in pure culture at lower pH (5.5) in favour of greater butyrate production and greater
431 acetate consumption per mol of carbohydrate consumed (Louis and Flint, 2017; Kettle *et al.*, 2015).
432 The relationships seen in Fig. 5 indicate that this shift in stoichiometry applies also to butyrate
433 production by the mixed community, while propionate production was simply related to the %
434 propionate-producing bacteria regardless of the initial pH.

435 In conclusion, the work presented here is one of few *in vitro* studies that compares the impact of a
436 large variety of NDCs on the composition and metabolic activity of the human faecal microbiota.
437 Some of the NDCs investigated here are currently classed as prebiotics, but this study does not reveal
438 a clear distinction between those and NDCs currently classed as dietary fibre, in terms of a selective
439 stimulation of specific bacteria. Prebiotic NDCs are considered to have consequences for health
440 mainly through their impact upon the gut microbiota. These impacts can be ascribed to two types of
441 mechanism. First, as shown here, prebiotics can promote the growth of a limited number of bacterial
442 species, boosting their populations and their representation within the gut microbiota, although the
443 pattern of stimulation can vary between microbiota from different individuals. Some of these bacteria
444 may interact with the host's immune system, but we can expect that inter-individual variability in the
445 microbiota and the selective effects of different NDCs may result in wide variation in health
446 consequences. Second, we know that the metabolites produced by the microbial community have

important consequences for health. Here our results suggest, at least for short chain fatty acids, that the consequences of NDC fermentation are likely to be more consistent between individuals. This is explained by the large number of gut anaerobes capable of producing the major SCFA, resulting in functional redundancy that tends to mask inter-individual variation at the species level. Thus we have shown that, in spite of inter-individual differences in microbiota composition, SCFA profiles were very similar for each individual and for a given NDC. We should also note however that for metabolites whose production is limited to a smaller number of species, individual variability is likely to be correspondingly greater. In addition, if keystone species are absent, the capacity of the microbiota to ferment NDC can be greatly reduced, as shown for individuals lacking *R. bromii* on diets high in RS (Walker *et al.*, 2011). There is currently much debate on the prebiotic concept, and the stipulation that they have to selectively stimulate certain microbes is increasingly challenged (Louis *et al.*, 2016; Bindels *et al.*, 2015; Steinert *et al.*, 2016). The data presented here are in support of a more general definition with regard to the modulation of the gut microbiota in order to achieve a beneficial effect on the host.

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603

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## Figure legends

**Figure 1:** Net SCFA production after 24 h incubation of human faecal samples with different NDCs. Average of three donors in 2 fermentations (f1 and f2) at pH 5.5 and 6.5 (t=24 h minus t=0, standard error of the difference and percentages given in Table S5). Faecal donors were donor 1, 2 and 3 in f1; and 2, 3 and 4 in f2. Analysed by ANOVA with donor as random effect and with NDC, pH and their interaction as fixed effects. NDCs that differ ($P<0.001$) from the no-NDC control for each of the two pH levels are indicated by *.

**Figure 2:** Increase of bacterial groups analysed by qPCR after 24 h incubation of human faecal samples with different NDCs. Average of three donors in 2 fermentations (f1 and f2) at pH 5.5 and pH 6.5 in relation to the increases with no NDC (given as relative fold change). Faecal donors were donor 1, 2 and 3 in f1 (for pH 5.5 only donors 1 and 3 were included, as no-NDC control was available at 24 h for donor 2; data for all donors are shown in Table S1) and 2, 3 and 4 in f2. Bacterial 16S rRNA gene copies/ml culture were expressed relative to the no-NDC control for each donor and pH. The log-transformed ratios were analysed by ANOVA with donor as random effect and with NDC, pH and their interaction as fixed effects. Presented here are the back-transformed mean log ratios. Test NDCs that differ ($P<0.001$) from the no-NDC control for each of the two pH levels are shown in bold and with a border. Rhamnogal.ur., rhamnogalacturonan; gal.man., galactomannan; MC, medium-chain; LC, long-chain. †, bacterial group not detected in all donors.

**Figure 3:** Growth response of individual *Bifidobacterium* species on α-glucans, arabinoxylan and fructans. Data shown are from individual faecal incubations (fermentation 1 donor 1, 2, 3; fermentation 2 donor 2b, 3b, 4) analysed by qPCR after 24 h incubation at pH 5.5. At 0 h, *Bifidobacterium* species levels in all donors ranged from $6.1\times10^4$ to $9.7\times10^6$/ml (see Table S1).

630

631 **Figure 4:** Relative abundance of propionate- and butyrate-producing OTUs with at least 97%
632 sequence identity to known bacterial species after 24 h incubation of human faecal samples from
633 fermentation 1 with different NDCs (white – black: panel A, Propionate producing OTUs 0 – 34%;
634 panel B, Butyrate producing OTUs 0 – 24%). Rationale for assignment of SCFA production capacity
635 is given in Table S2A. Relative production of the corresponding SCFA is given at the top of each
636 heat map (white – black: panel A, Propionate % 0 - 43%; panel B, Butyrate % 0 – 47%). OTUs
637 showing a significant ($P<0.001$) increase under certain conditions, compared to inoculum, are
638 indicated by * (for details see Table 2). AP, apple pectin; AX, arabinoxylan; BG, ß-glucan; Ca, carob
639 galactomannan; Gu, guar galactomannan; i, inoculum; I-GP, medium-chain inulin; I-HP, long-chain
640 inulin; La, laminarin; no, no-NDC; OF, oligofructose; Pu, pullulan; Py, pyrodextrin; RG,
641 rhamnogalacturonan; Rh, rhamnose; RSII, type II resistant starch; RSIII, type III resistant starch.
642 Panel A: Acr, acrylate pathway; Pdu, propanediol pathway.

643

644 **Figure 5:** Relationship between sum of all propionate- or butyrate-producing OTUs with at least
645 97% sequence identity to known bacterial species after 24 h of incubation of human faecal samples
646 from fermentation 1, expressed as percentage of total sequences, and SCFA production. Relative
647 propionate or butyrate production (percentage of total SCFA produced) was regressed on percentage
648 of propionate or butyrate producers, using mixed effect models with donor as random effect and with
649 fixed effects for pH, percentage producers, and their interaction. pH 5.5, crosses; pH 6.5, triangles.
650 Lines correspond to the fit for each pH where a significant effect of pH was observed (B, solid line
651 pH 5.5, dashed line pH 6.5).

652

653 **Figure 6**: Overview of known fermentation pathways for SCFA formation in human gut bacteria.
654 NDC class colour is based on whether they mainly stimulated propionate (red) or butyrate (blue)

production or both (purple) (see Fig. 1). Responses of gut bacteria to different NDCs detected in this study are shown above each NDC class; significant responses detected by qPCR (see Fig. 2, compared to no-NDC) and 454 sequencing (see Table 2, compared to inoculum, only OTUs with >97% identity to known species given) are shown in black, non-significant qPCR-based responses with a fold change >10 are shown in grey.

**Supplementary Information**

**Fig. S1**: Rarefaction curves of 454 sequencing data. The inset shows collectors curves of observed OTUs for the two samples with the lowest number of sequences.

**Fig. S2**: Relative abundance of 50 most abundant OTUs after 24 h incubation of human faecal samples from fermentation 1 with different NDC from full sequence dataset (A) compared to corresponding OTUs after subsampling to 426 sequence reads (B).

**Fig. S3**: Total ion current chromatograms of laminarin purchased from Sigma (A) and Shaanxi Pioneer Biotech (B). Marked partially methylated alditol acetates were identified by their mass spectra and semiquantitatively determined by GC-FID**.**

**Fig. S4**: Relationship between log bacterial 16S rRNA gene copies/ml culture and propionate or butyrate production. Combined qPCR data from fermentations f1 and f2 for *Bacteroides* spp. (A), *A. hadrus* (B), *F. prausnitzii* (C) and the *Roseburia* groups (D). SCFA production was regressed on log bacterial 16S rRNA gene copies/ml culture observed at 24 h using a mixed effects model with donor and year within donor regarded as random effects, and with fermentation, pH, log bacterial 16S rRNA gene copies/ml culture, and their interactions as fixed effects. Significant effects ($P<0.05$) are

680 listed above each plot. pH 5.5: blue, pH 6.5: red, fermentation 1: circles, fermentation 2: +. Lines
681 correspond to the fit for each year and pH combination (solid lines: fermentation 1, dashed lines:
682 fermentation 2).
683
684 **Fig. S5**: Relative abundance of OTUs after 24 h incubation of human faecal samples with different
685 NDCs. Average data from three donors from fermentation 1 analysed by 454 sequencing. OTUs are
686 grouped together per corresponding qPCR assay. Assignment of individual OTUs to corresponding
687 qPCR groups are given in Table S2A. This figure also provides a comparison of the microbiota
688 composition between the inoculum and no-NDC (24 h incubation) control. Certain groups, notably
689 *Blautia* spp., *Roseburia* and *E. hallii*, appear to have decreased in relative abundance from the
690 inoculum to the 24 h incubation (no-NDC) at both pH values. Possibly these groups may be less able
691 to replicate and/or become more prone to cell lysis in the absence of an added carbohydrate energy
692 source; this effect may therefore have amplified some of the changes shown in Fig. 2, which
693 compares 24 h incubations with and without added NDCs.
694
695 **Fig. S6**: Linear regression analysis between microbial composition as determined by qPCR and 454
696 sequencing. The analysis was performed for bacterial groups that were detected by both methods (*C.*
697 *eutactus* was only found in D3 by qPCR, corresponding OTU-137 was found in 6 and 3 of 31
698 samples, respectively, in D1 and D2, see Table S2). Blue, donor 1; green, donor 2; purple, donor 3;
699 lighter colours pH 5.5; darker colours pH 6.5.
700
701 **Fig. S7**: Relative abundance of 100 most abundant OTUs after 24 h incubation of human faecal
702 samples from fermentation 1 with different NDCs (white - black: 0 - 51%). Relative production of
703 the corresponding SCFA is given at the top of each heat map (white - black: 0 - 47%). OTUs

showing a significant increase ($P<0.001$) under certain conditions, compared to inoculum, are indicated by * (for details see Table 2). NDC abbreviations as per Fig. 4.

**Fig. S8**: Principal component analysis of relative SCFA and qPCR data of NDCs included in both fermentation years. A: scores plot colour-coded by donor and year. B: scores plot colour-coded by NDC. C: scores plot colour-coded by pH. NDC abbreviations as per Fig. 4; pH 6.5, italics. D: loading plot of variables. Ac, acetate; But, butyrate; Prop, propionate; Ahad, *A. hadrus*; Bact, *Bacteroides* spp; Bif, *Bifidobacterium* spp.; Blaut, *Blautia* spp.; Dorea, *Dorea* spp.; Ehal, *E. hallii*; Fprau, *F. prausnitzii*; Neg, Negativicutes; Osc, *Oscillibacter* group; Rbro, *R. bromii*; Rfla, *R. flavefaciens* group; Rinul, *R. inulinivorans*; Ros, *Roseburia* group.

**Table S1**: qPCR analysis of faecal incubations. A: 16S rRNA gene copies per ml culture for each donor in the inoculum and after 24 h of incubation (grey font: 6 h of incubation as 24 h sample was not available). Key for bacterial groups given to right of table; colour shading by conditional formatting per bacterial group (yellow low - green high values); nd: not detected. B: Average fold change and confidence interval (for groups present in all donors) after growth on different NDCs compared to no-NDC control after 24 h of incubation (6 h samples were excluded).

**Table S2**: 454 sequencing analysis of faecal incubations of fermentation experiment 1. A: Average relative abundance of operational taxonomic units (OTUs, 97% sequence identity; abundance > 100 reads; complete dataset given in C below, row 839) after 24 h of incubation of human faecal samples (n=3, fermentation 1, 6 h samples were excluded; individual donor data are given under B below, row 213) with different NDCs at pH 5.5 and 6.5. Assignment to butyrate or propionate producing status based on closest known species is given in columns AR-AY; Partial least squares regression of association with acetate, propionate or butyrate in columns AX-BJ. B: Relative abundance of

operational taxonomic units per individual donor after 24 h incubation (grey font: 6 h of incubation as 24 h sample was not available; empty cells: not enough sequences obtained for those samples). C: Number of sequence reads obtained per sample for all 1552 OTUs (97% identity; grey font: 6 h of incubation as 24 h sample was not available).

**Table S3**: Quantitative PCR primers and annealing temperatures used in this study.

**Table S4:** Monosaccharide composition (mol%) of the NDCs used in this study. Monosaccharides were analysed by HPAEC-PAD after methanolysis followed by TFA hydrolysis (apple pectin and rhamnogalacturonan I), mild TFA hydrolysis (oligofructose, medium-chain inulin, and long-chain inulin), and sulfuric acid hydrolysis (all other samples).

**Table S5**: Net SCFA production and proportions after 24 h incubation of human faecal samples with different NDCs. Average and individual data from 2 fermentations (f1: d1, 2, 3 and f2: d2, 3, 4) at pH 5.5 and 6.5.

**Table 1:** NDCs used for anaerobic *in vitro* incubations with human faecal samples in fermentation (f) 1 and 2 and their suppliers.

| Class | NDC | Commercial name and supplier | f1 | f2 |
|---|---|---|---|---|
| α-glucans | Pyrodextrin | Fibersol-2; gifted by Matsutani, Itami-City, Japan | x | |
| | Pullulan | Megazyme, Bray, Ireland (Cat No P-PULLN) | x | |
| | Resistant starch type II | Hylon VII, National Starch & Chemical Comp., Bridgewater, USA | x | |
| | Resistant starch type III | Novelose330, National Starch & Chemical Comp., Bridgewater, USA | x | |
| ß-glucans | β-Glucan from barley | Megazyme, Bray, Ireland (Cat No P-BGBL) | x | |
| | β-Glucan from barley | Glucagel, PolyCell Technologies, Crookston, USA | | x |
| | Laminarin | Sigma Aldrich, UK (Cat No L9634) | x | |
| | "Laminarin"[1] | Shaanxi Pioneer Biotech, China | | x |
| Methyl-pentose | Rhamnose | Sigma Aldrich, UK (Cat No W373011) | x | x |
| Pectins | Rhamnogalacturonan from potato | Megazyme, Bray, Ireland (Cat No P-RHAM1) | x | |
| | Apple pectin | Sigma Aldrich, UK (Cat No 76282) | x | x |
| Galactomannans | Carob galactomannan | Megazyme, Bray, Ireland (Cat No P-GALML) | x | |
| | Guar galactomannan | Megazyme, Bray, Ireland (Cat No P-GGMMV) | x | |
| Hemicellulose | Arabinoxylan | Megazyme, Bray, Ireland (Cat No P-WAXYL) | x | |
| Inulin-type fructans | Oligofructose, DP=2-8 | Orafti P95, gifted by Beneo, Tienen, Belgium | x | |

| | | | | |
|---|---|---|---|---|
| | Medium-chain inulin, average DP≥10 | Orafti GR, Beneo, Tienen, Belgium | x | |
| | Long-chain inulin, average DP ≥23 | Orafti HP, gifted by Beneo, Tienen, Belgium | x | x |

[1]Chemical analysis suggests that a large portion of this NDC does not seem to be bona fide laminarin (for details see Methods)

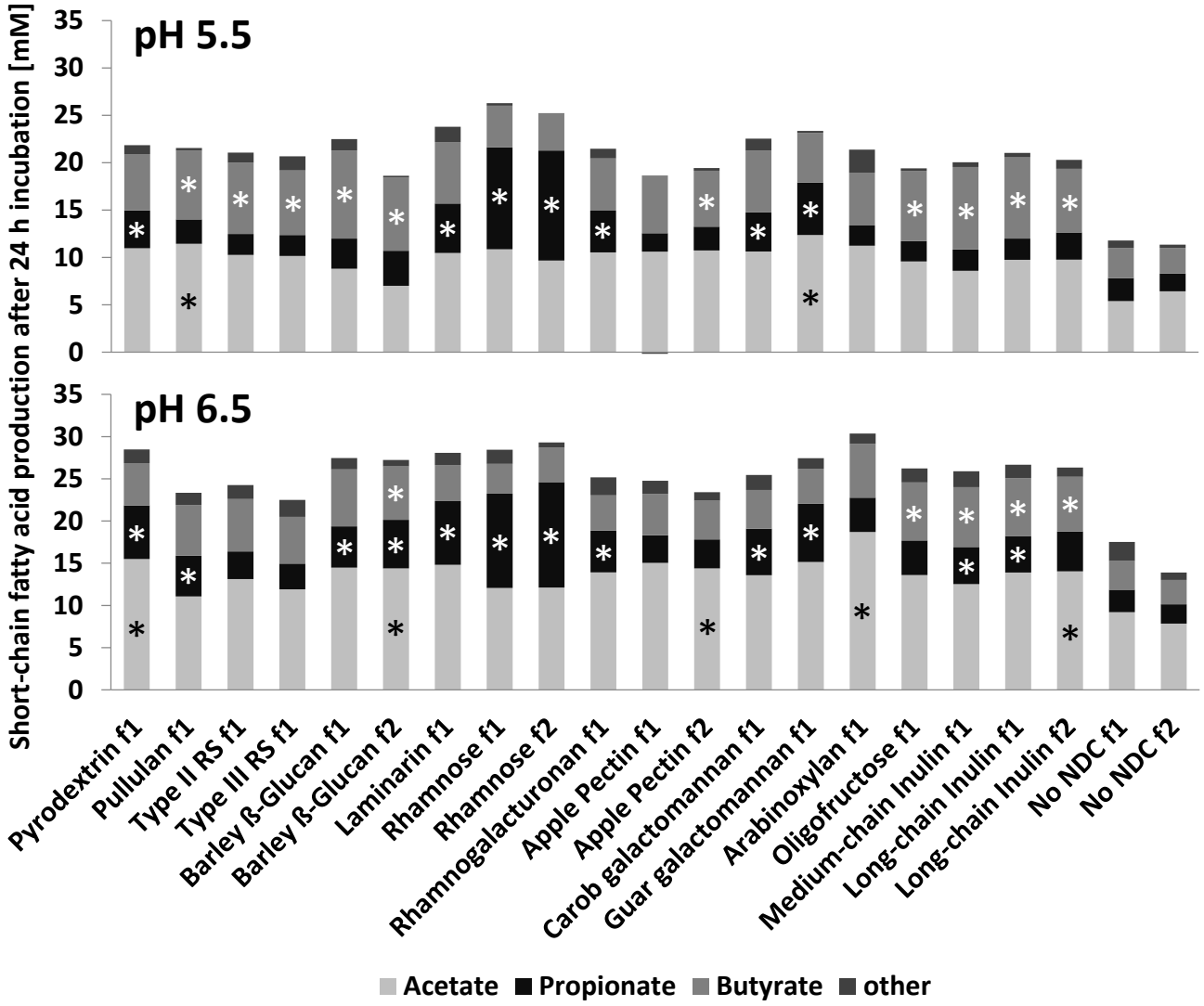
**Table 2:** Operational taxonomic units **(**OTUs) from 454 sequencing analysis of fermentation 1 exhibiting a significant increase on specific NDCs and pH values (P<0.001, see Table S2).

| OTU No. | closest relative bacterial species (BLAST) | Identity | Non-digestible carbohydrate pH 5.5 | pH 6.5 |
|---|---|---|---|---|
| Otu0002 | *Faecalibacterium prausnitzii* | 99% | apple pectin[1,2] | apple pectin[1,2] |
| | | | | carob galactomannan[1,2] |
| Otu0003 | *Clostridium spiroforme* | 93% | laminarin[1,2] | |
| Otu0005 | *Bacteroides uniformis* | 100% | | pyrodextrin[1,2] |
| | | | | laminarin[1,2] |
| | | | | guar galactomannan[1,2] |
| Otu0006 | *Blautia faecis* | 99% | rhamnose[1,2] | rhamnose[1,2] |
| Otu0010 | *Fusicatenibacter saccharivorans* | 99% | laminarin[1] | |
| | | | carob galactomannan[1,2] | |
| Otu0013 | *Subdoligranulum variabile* | 99% | arabinoxylan[1,2] | |
| Otu0017 | *Oscillibacter ruminantium* | 96% | | rhamnogalacturonan[1] |
| Otu0018 | *Dorea longicatena* | 99% | pullulan | |
| | | | guar galactomannan | |

| | | | | |
|---|---|---|---|---|
| | | | medium-chain inulin[1,2] | |
| Otu0024 | *Lactobacillus rogosae* | 96% | rhamnogalacturonan[1] | |
| | | | guar galactomannan | |
| Otu0026 | *Parabacteroides merdae* | 98% | | guar galactomannan[1] |
| Otu0027 | *Bacteroides thetaiotaomicron* | 99% | | barley β-glucan[1] |
| Otu0031 | unclassified | | carob galactomannan[1] | |
| | | | guar galactomannan | guar galactomannan[1] |
| Otu0037 | unclassified | | rhamnogalacturonan[1] | |
| Otu0041 | *Flavonifractor plautii* | 96% | pullulan[1] | rhamnogalacturonan[1] |
| Otu0043 | *Eubacterium ventriosum* | 98% | pyrodextrin | |
| | | | pullulan[1] | |
| | | | laminarin[1] | |
| Otu0045 | *Bacteroides ovatus* | 100% | | barley β-glucan[1] |
| Otu0055 | *Clostridium bolteae* | 97% | rhamnogalacturonan | |

[1]also significant after removal of samples with <97% Good's coverage

[2]also significant after subsampling to lowest coverage (426 sequence reads)


pH 5.5
pH 6.5
Short-chain fatty acid production after 24 h incubation [mM]
0
5
10
15
20
25
30
35
Pyrodextrin f1
Pullulan f1
Type II RS f1
Type III RS f1
Barley ß-Glucan f1
Barley ß-Glucan f2
Laminarin f1
Rhamnose f1
Rhamnose f2
Rhamnogalacturonan f1
Apple Pectin f1
Apple Pectin f2
Carob galactomannan f1
Guar galactomannan f1
Arabinoxylan f1
Oligofructose f1
Medium-chain Inulin f1
Long-chain Inulin f1
Long-chain Inulin f2
No NDC f1
No NDC f2
Acetate
Propionate
Butyrate
other

| | NDC | f | *Bacteroides spp.* | *F. prausnitzii* | *R. bromii* | *R. albus gr.†* | *R. flavefaciens gr.* | *Oscillibacter gr.* | *Roseburia gr.* | *R. inulinivorans* | *E. hallii* | *A. hadrus* | *Blautia spp.* | *Dorea spp.* | *C. eutactus†* | *E. eligens†* | *Negativicutes* | *Bifidobacterium spp.* | *B. adolescentis* | *B. longum* | *B. catenulatum gr.†* | *B. bifidum†* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH 5.5 | Pyrodextrin | 1 | 0.9 | 1.5 | 1.2 | 0.7 | 1.1 | 0.5 | 1.6 | 1.0 | 0.8 | 1.4 | 1.7 | 0.7 | 1.2 | 0.7 | 0.8 | 1.5 | 1.2 | 1.0 | 1.9 | 3.4 |
| | Pullulan | 1 | 0.6 | 1.9 | 3.0 | 0.7 | 0.7 | 0.3 | 3.1 | 1.6 | 1.3 | 2.1 | 1.8 | 0.8 | 4.3 | 0.5 | 0.9 | 3.5 | 5.7 | 4.5 | 1.9 | 2.4 |
| | Type II RS | 1 | 0.8 | 1.9 | **12.8** | 1.1 | 1.1 | 0.5 | **3.6** | 1.3 | 2.1 | 2.5 | 1.8 | 1.1 | 5.5 | 0.7 | 1.0 | 8.3 | 14.9 | 3.4 | 7.3 | 3.8 |
| | Type III RS | 1 | 0.8 | 2.5 | **13.8** | 1.1 | 1.1 | 0.4 | 2.6 | 1.5 | 1.6 | 1.3 | 1.2 | 1.2 | 3.2 | 0.8 | 0.8 | 3.5 | 6.7 | 1.7 | 2.8 | 2.6 |
| | ß-Glucan | 1 | 0.6 | 2.5 | 1.2 | 0.8 | 1.5 | 0.3 | 2.4 | 2.0 | 1.4 | 3.3 | **4.2** | 0.8 | 18.4 | 0.6 | 1.1 | 1.2 | 0.8 | 0.8 | 1.6 | 2.9 |
| | ß-Glucan | 2 | 1.6 | 2.2 | 1.5 | nd | 0.6 | 0.6 | **5.7** | 2.7 | 1.7 | 2.2 | 2.6 | 0.7 | 10.6 | 0.5 | 0.7 | 3.1 | 3.0 | 2.3 | 2.8 | 1.1 |
| | Laminarin | 1 | 1.2 | 1.6 | 1.6 | 1.2 | 0.7 | 0.7 | 1.6 | 2.3 | 1.7 | 2.1 | 1.3 | 2.3 | 2.0 | 0.6 | 0.8 | 0.7 | 0.8 | 0.8 | 1.0 | 2.6 |
| | Rhamnose | 1 | 1.0 | 1.2 | 1.2 | 1.2 | 0.9 | 0.4 | 1.3 | 1.5 | 3.2 | 0.7 | **5.7** | 0.6 | 2.3 | 0.6 | 0.7 | 0.7 | 0.6 | 0.8 | 1.0 | 2.2 |
| | Rhamnose | 2 | 1.7 | 1.0 | 1.3 | nd | 0.3 | 0.8 | 1.1 | 1.1 | **12.8** | 0.6 | **13.3** | 0.8 | 2.0 | 0.4 | 0.4 | 1.1 | 1.1 | 1.0 | 1.0 | 0.9 |
| | Rhamnogal.ur. | 1 | 1.5 | 2.9 | 1.2 | 0.8 | 1.2 | 0.5 | 1.5 | 0.9 | 2.6 | 1.0 | 1.4 | 1.1 | 1.1 | 1.5 | 0.6 | 1.0 | 1.1 | 1.2 | 1.5 | 3.6 |
| | Apple Pectin | 1 | 0.9 | 3.4 | 2.3 | 0.7 | 0.9 | 0.4 | 1.4 | 1.2 | 1.5 | 1.9 | 1.3 | 1.1 | 2.7 | 1.5 | 1.1 | 2.0 | 2.1 | 1.9 | 2.6 | 5.3 |
| | Apple Pectin | 2 | 1.3 | 2.8 | 1.4 | nd | 0.3 | 1.1 | 1.2 | 1.2 | 2.8 | 0.9 | 1.6 | 1.6 | 1.4 | 1.7 | 0.5 | 2.3 | 2.2 | 1.6 | 1.9 | 1.4 |
| | Carob gal.man. | 1 | 0.4 | 0.8 | 0.4 | 0.7 | 0.3 | 0.1 | 0.9 | 0.5 | 0.4 | 0.4 | 0.5 | 0.5 | 12.8 | 0.2 | 0.3 | 0.5 | 0.3 | 0.2 | 1.2 | 2.2 |
| | Guar gal.man. | 1 | 1.2 | 1.2 | 1.7 | 4.6 | 0.7 | 0.8 | 1.8 | 1.1 | 0.7 | 0.6 | 1.4 | 1.7 | 3.7 | 0.6 | 0.7 | 0.7 | 0.7 | 0.7 | 0.8 | 1.9 |
| | Arabinoxylan | 1 | 0.6 | 0.9 | 1.2 | 0.8 | 0.9 | 0.3 | 1.8 | 1.4 | 1.1 | 2.6 | 1.2 | 0.7 | 1.9 | 0.5 | 0.7 | 5.3 | 5.5 | **12.0** | 4.5 | 2.9 |
| | Oligofructose | 1 | 0.8 | 1.8 | 4.4 | 1.0 | 1.3 | 0.5 | 3.5 | 1.6 | 2.4 | **4.7** | 1.9 | 1.7 | 4.1 | 0.7 | 1.2 | 7.0 | 8.0 | 5.5 | 7.3 | 11.5 |
| | MC Inulin | 1 | 0.5 | 2.4 | 4.2 | 0.6 | 1.2 | 0.3 | **4.2** | 1.6 | 1.8 | **5.2** | 2.2 | 1.2 | 4.2 | 0.5 | 1.2 | 1.7 | 1.8 | 1.6 | 2.2 | 6.7 |
| | LC Inulin | 1 | 0.5 | 2.2 | 4.2 | 0.7 | 1.0 | 0.2 | **3.7** | 1.4 | 1.8 | **5.2** | 2.8 | 1.2 | 4.7 | 0.5 | 0.9 | 1.5 | 1.5 | 1.4 | 1.9 | 5.9 |
| | LC Inulin | 2 | 0.9 | 1.9 | 2.3 | 1.1 | 1.3 | 0.3 | **3.5** | 1.1 | 1.5 | **7.4** | 3.1 | 0.8 | 2.7 | 0.4 | 0.5 | 5.3 | **5.3** | 2.0 | 1.5 | 2.1 |
| pH 6.5 | Pyrodextrin | 1 | 2.2 | 0.3 | 0.5 | 0.8 | 0.2 | 0.4 | 1.1 | 0.6 | 0.1 | 0.1 | 0.2 | 1.4 | 1.0 | 0.4 | 0.3 | 0.1 | 0.1 | 0.7 | 0.1 | nd |
| | Pullulan | 1 | 2.1 | 1.2 | 1.8 | 1.3 | 0.8 | 0.3 | 2.4 | 1.2 | 2.0 | 1.2 | 1.6 | 1.0 | 2.7 | 0.8 | 1.2 | 2.1 | 8.3 | 2.5 | 0.0 | 0.8 |
| | Type II RS | 1 | 1.0 | 1.4 | **6.3** | 1.0 | 0.9 | 0.4 | 4.1 | 1.9 | 2.3 | 1.3 | 2.3 | 1.2 | 1.0 | 0.6 | 0.6 | 3.6 | 4.6 | 1.7 | 4.7 | 0.9 |
| | Type III RS | 1 | 1.2 | 0.7 | **7.0** | 0.9 | 0.3 | 0.5 | 2.0 | 0.9 | 0.4 | 0.2 | 0.3 | 1.1 | 2.1 | 0.4 | 0.3 | 0.4 | 0.6 | 0.6 | 0.1 | 0.0 |
| | ß-Glucan | 1 | 1.5 | 0.7 | 0.4 | 1.2 | 0.5 | 0.3 | 3.2 | 3.2 | 0.3 | 0.3 | 0.5 | 0.7 | 9.8 | 0.4 | 0.4 | 0.2 | 0.1 | 0.4 | 0.1 | nd |
| | ß-Glucan | 2 | **2.6** | 2.2 | 0.9 | nd | 0.3 | 0.8 | **4.6** | 4.5 | 3.2 | 1.7 | 1.8 | 0.8 | 9.5 | 0.7 | 1.2 | 2.5 | 2.5 | 2.4 | 1.8 | 1.1 |
| | Laminarin | 1 | **2.8** | 1.0 | 1.0 | 0.9 | 0.8 | 0.5 | 1.0 | **9.0** | 1.9 | 1.2 | 1.5 | 1.3 | 0.8 | 0.6 | 1.8 | 1.2 | 0.8 | 1.7 | 1.1 | 0.7 |
| | Rhamnose | 1 | 1.6 | 0.6 | 0.4 | 0.9 | 0.3 | 0.3 | 0.7 | 0.6 | 0.8 | 0.2 | 0.7 | 0.2 | 1.5 | 0.3 | 0.3 | 0.2 | 0.2 | 0.4 | 0.1 | 0.0 |
| | Rhamnose | 2 | 1.5 | 1.3 | 2.0 | nd | 0.3 | 1.2 | 1.0 | 0.9 | **17.9** | 0.8 | **12.4** | 1.0 | 0.9 | 0.8 | 0.7 | 1.4 | 1.3 | 1.1 | 1.0 | 0.8 |
| | Rhamnogal.ur. | 1 | **2.6** | 0.7 | 0.3 | 0.7 | 0.2 | 0.7 | 0.5 | 0.6 | 0.5 | 0.2 | 0.2 | 1.2 | 1.4 | 4.1 | 0.4 | 0.1 | 0.1 | 0.5 | 0.2 | nd |
| | Apple Pectin | 1 | 1.2 | 1.9 | 0.9 | 0.9 | 0.9 | 0.5 | 1.1 | 1.1 | 1.7 | 1.1 | 1.5 | 1.2 | 0.9 | 2.7 | 0.8 | 1.6 | 1.4 | 1.4 | 1.5 | 1.7 |
| | Apple Pectin | 2 | 1.7 | **5.9** | 1.3 | nd | 0.1 | 1.5 | 1.2 | 0.9 | **7.4** | 1.0 | 1.5 | 1.5 | 1.1 | 1.9 | 0.7 | 2.4 | 2.3 | 1.9 | 1.7 | 1.3 |
| | Carob gal.man. | 1 | 1.6 | 0.6 | 0.5 | 1.3 | 0.3 | 0.3 | 1.5 | 0.7 | 0.3 | 0.4 | 0.4 | 1.0 | 5.4 | 0.3 | 0.6 | 0.4 | 0.4 | 0.9 | 0.3 | nd |
| | Guar gal.man. | 1 | **2.2** | 1.0 | 1.0 | 2.2 | 0.6 | 0.5 | 2.5 | 0.8 | 1.0 | 0.9 | 1.3 | 1.0 | 4.7 | 0.7 | 1.4 | 0.4 | 1.0 | 1.8 | 0.8 | 0.9 |
| | Arabinoxylan | 1 | 1.2 | 0.3 | 0.3 | 0.7 | 0.1 | 0.3 | **5.0** | 1.2 | 0.2 | 0.7 | 0.2 | 1.4 | 1.3 | 0.3 | 0.5 | 0.2 | 0.1 | 1.7 | 0.6 | nd |
| | Oligofructose | 1 | 1.4 | 1.7 | 1.8 | 1.5 | 1.1 | 0.4 | 2.4 | 1.2 | 3.7 | 2.5 | 2.2 | 1.5 | 2.2 | 0.9 | 1.0 | 2.8 | 9.1 | 3.2 | 8.0 | 3.0 |
| | MC Inulin | 1 | 1.3 | 1.9 | 1.6 | 1.3 | 1.3 | 0.4 | 3.1 | 1.3 | 2.9 | 2.6 | 2.8 | 1.4 | 2.6 | 0.8 | 1.2 | 1.6 | 7.0 | 2.3 | 0.1 | 2.8 |
| | LC Inulin | 1 | 1.0 | 1.1 | 0.8 | 1.3 | 0.4 | 0.3 | 2.1 | 1.1 | 0.4 | 0.8 | 0.7 | 1.3 | 2.2 | 0.4 | 0.5 | 0.6 | 1.1 | 0.9 | 0.1 | nd |
| | LC Inulin | 2 | 1.6 | 3.1 | 1.4 | nd | 1.1 | 0.8 | 2.1 | 0.9 | 4.3 | **3.1** | 3.3 | 1.0 | 1.0 | 0.6 | 1.2 | **5.8** | 4.9 | 2.3 | 1.1 | 1.5 |

**fold-change:**

- ≤1
- >1
- >2.5
- >5
- >10

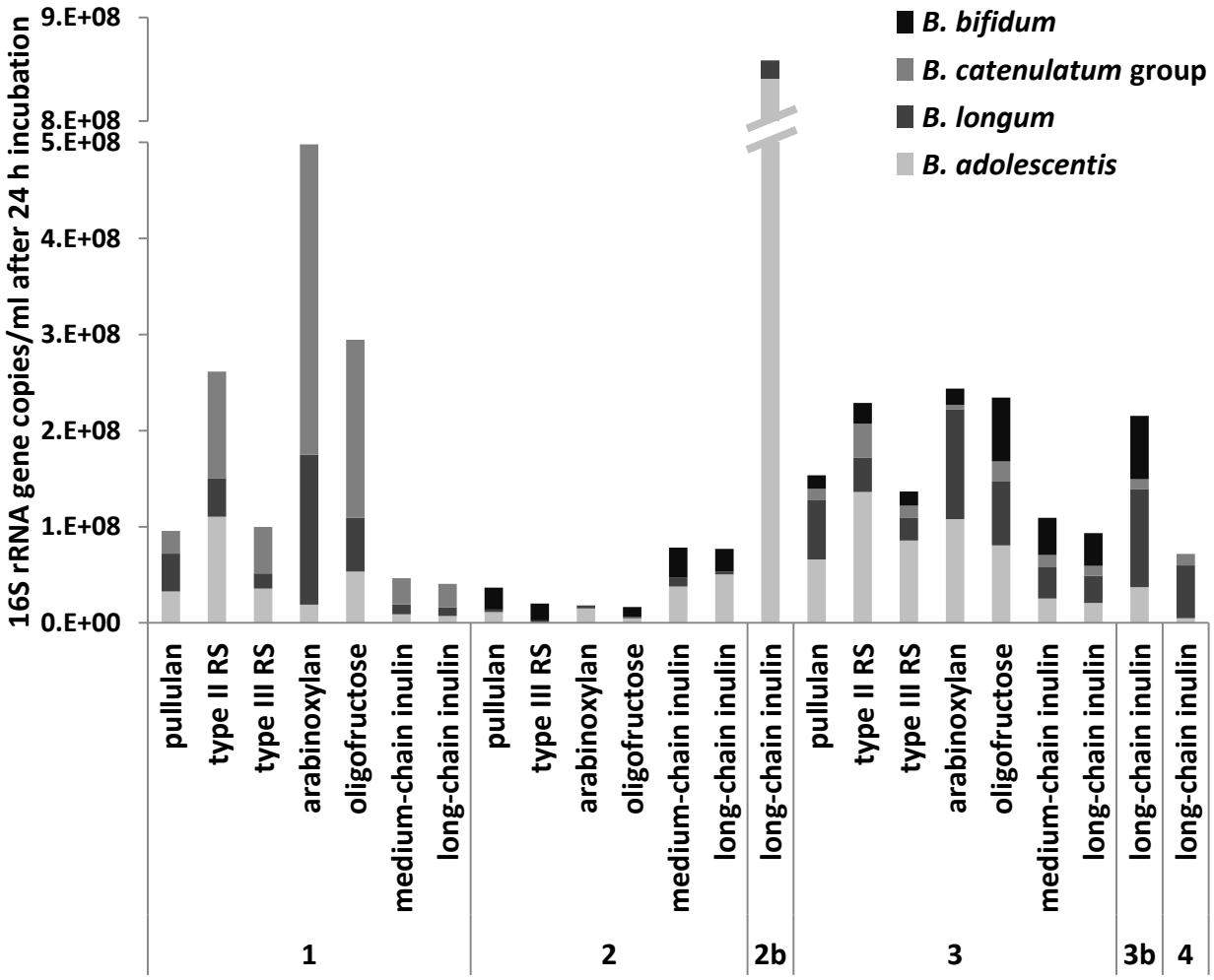

16S rRNA gene copies/ml after 24 h incubation
9.E+08
8.E+08
5.E+08
4.E+08
3.E+08
2.E+08
1.E+08
0.E+00
B. bifidum
B. catenulatum group
B. longum
B. adolescentis
pullulan
type II RS
type III RS
arabinoxylan
oligofructose
medium-chain inulin
long-chain inulin
1
2
2b
3
3b
4

Fig. 4

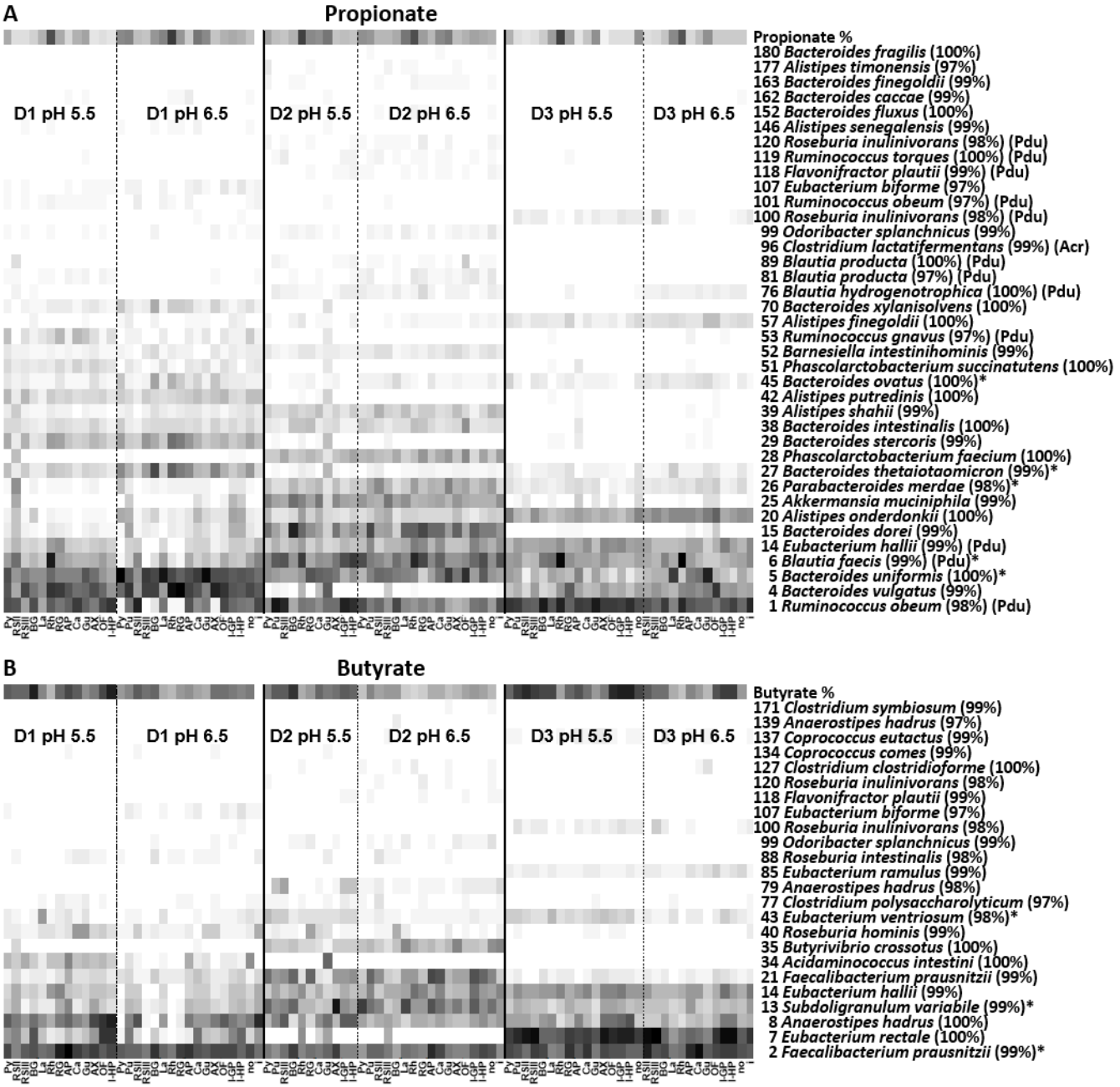
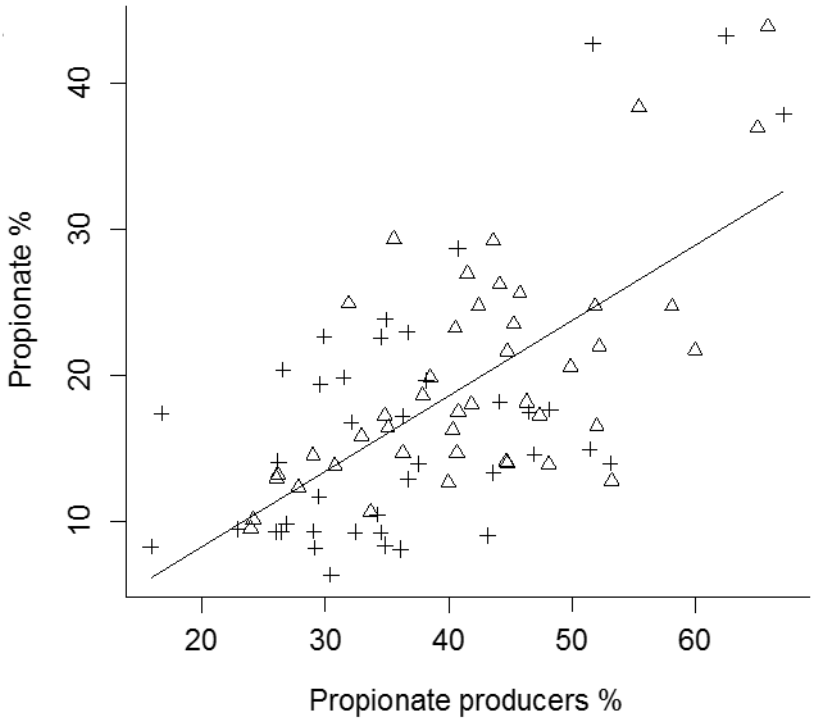
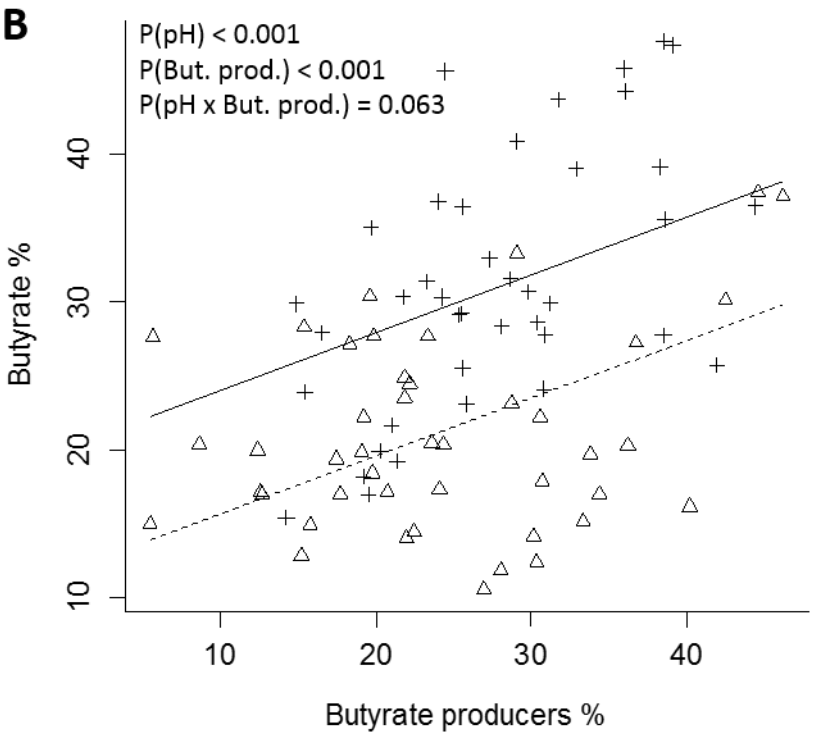
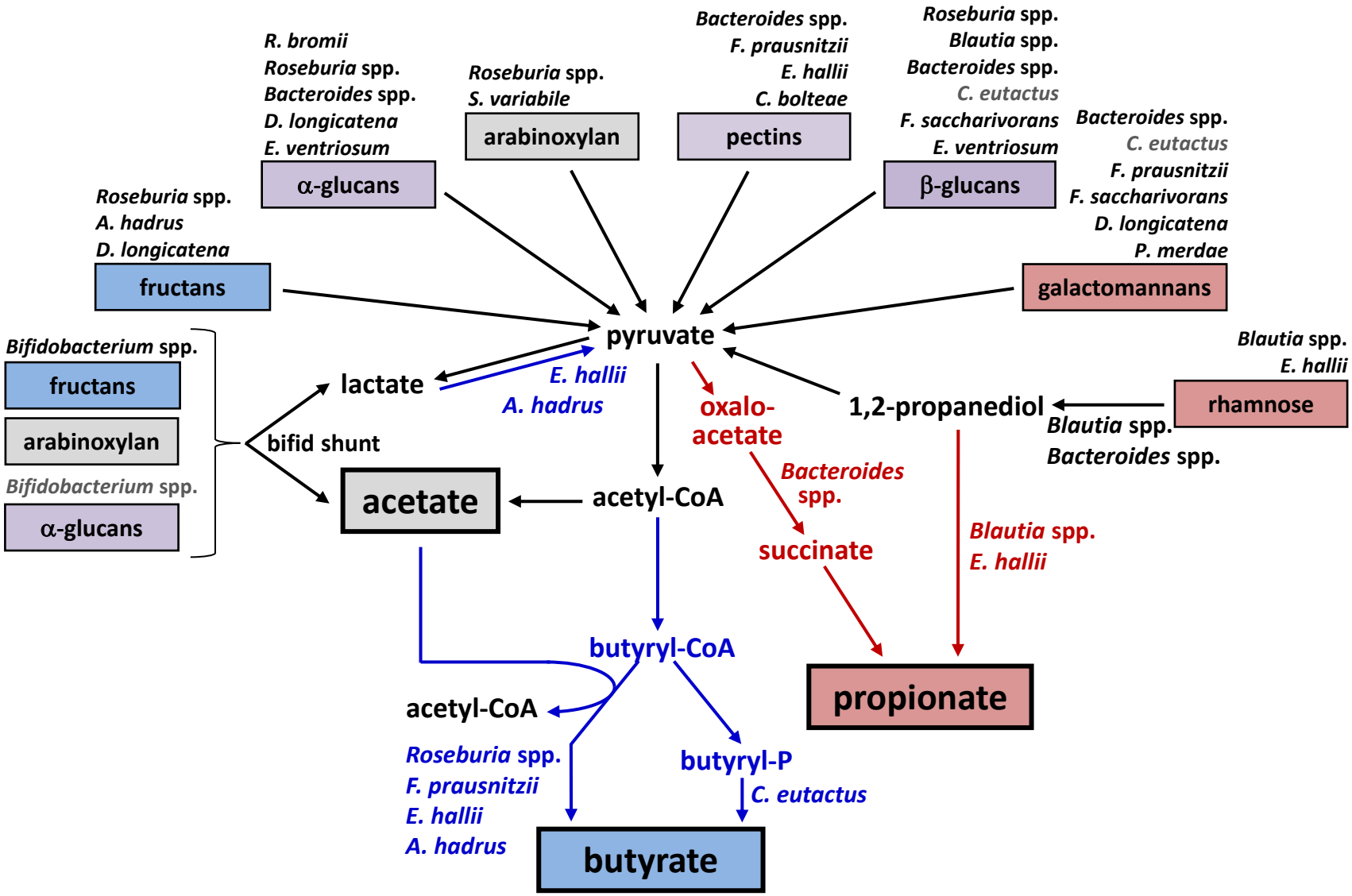

A
Propionate
D1 pH 5.5
D1 pH 6.5
D2 pH 5.5
D2 pH 6.5
D3 pH 5.5
D3 pH 6.5
Propionate %
180 Bacteroides fragilis (100%)
177 Alistipes timonensis (97%)
163 Bacteroides finegoldii (99%)
162 Bacteroides caccae (99%)
152 Bacteroides fluxus (100%)
146 Alistipes senegalensis (99%)
120 Roseburia inulinivorans (98%) (Pdu)
119 Ruminococcus torques (100%) (Pdu)
118 Flavonifractor plautii (99%) (Pdu)
107 Eubacterium biforme (97%)
101 Ruminococcus obeum (97%) (Pdu)
100 Roseburia inulinivorans (98%) (Pdu)
99 Odoribacter splanchnicus (99%)
96 Clostridium lactatifermentans (99%) (Acr)
89 Blautia producta (100%) (Pdu)
81 Blautia producta (97%) (Pdu)
76 Blautia hydrogenotrophica (100%) (Pdu)
70 Bacteroides xylanisolvens (100%)
57 Alistipes finegoldii (100%)
53 Ruminococcus gnavus (97%) (Pdu)
52 Barnesiella intestinihominis (99%)
51 Phascolarctobacterium succinatutens (100%)
45 Bacteroides ovatus (100%)*
42 Alistipes putredinis (100%)
39 Alistipes shahii (99%)
38 Bacteroides intestinalis (100%)
29 Bacteroides stercoris (99%)
28 Phascolarctobacterium faecium (100%)
27 Bacteroides thetaiotaomicron (99%)*
26 Parabacteroides merdae (98%)*
25 Akkermansia muciniphila (99%)
20 Alistipes onderdonkii (100%)
15 Bacteroides dorei (99%)
14 Eubacterium hallii (99%) (Pdu)
6 Blautia faecis (99%) (Pdu)*
5 Bacteroides uniformis (100%)*
4 Bacteroides vulgatus (99%)
1 Ruminococcus obeum (98%) (Pdu)
B
Butyrate
D1 pH 5.5
D1 pH 6.5
D2 pH 5.5
D2 pH 6.5
D3 pH 5.5
D3 pH 6.5
Butyrate %
171 Clostridium symbiosum (99%)
139 Anaerostipes hadrus (97%)
137 Coprococcus eutactus (99%)
134 Coprococcus comes (99%)
127 Clostridium clostridioforme (100%)
120 Roseburia inulinivorans (98%)
118 Flavonifractor plautii (99%)
107 Eubacterium biforme (97%)
100 Roseburia inulinivorans (98%)
99 Odoribacter splanchnicus (99%)
88 Roseburia intestinalis (98%)
85 Eubacterium ramulus (99%)
79 Anaerostipes hadrus (98%)
77 Clostridium polysaccharolyticum (97%)
43 Eubacterium ventriosum (98%)*
40 Roseburia hominis (99%)
35 Butyrivibrio crossotus (100%)
34 Acidaminococcus intestini (100%)
21 Faecalibacterium prausnitzii (99%)
14 Eubacterium hallii (99%)
13 Subdoligranulum variabile (99%)*
8 Anaerostipes hadrus (100%)
7 Eubacterium rectale (100%)
2 Faecalibacterium prausnitzii (99%)*

Fig. 5


Propionate %
10
20
30
40
20
30
40
50
60
Propionate producers %


B
P(pH) < 0.001
P(But. prod.) < 0.001
P(pH x But. prod.) = 0.063
Butyrate %
10
20
30
40
10
20
30
40
Butyrate producers %


R. bromii
Roseburia spp.
Bacteroides spp.
D. longicatena
E. ventriosum
α-glucans
Roseburia spp.
S. variabile
arabinoxylan
Bacteroides spp.
F. prausnitzii
E. hallii
C. bolteae
pectins
Roseburia spp.
Blautia spp.
Bacteroides spp.
C. eutactus
F. saccharivorans
E. ventriosum
β-glucans
Roseburia spp.
A. hadrus
D. longicatena
fructans
Bacteroides spp.
C. eutactus
F. prausnitzii
F. saccharivorans
D. longicatena
P. merdae
galactomannans
pyruvate
Bifidobacterium spp.
fructans
arabinoxylan
Bifidobacterium spp.
α-glucans
bifid shunt
lactate
E. hallii
A. hadrus
oxalo-
acetate
1,2-propanediol
Blautia spp.
E. hallii
rhamnose
Blautia spp.
Bacteroides spp.
acetate
acetyl-CoA
Bacteroides
spp.
succinate
Blautia spp.
E. hallii
butyryl-CoA
propionate
acetyl-CoA
Roseburia spp.
F. prausnitzii
E. hallii
A. hadrus
butyryl-P
C. eutactus
butyrate

**Supplementary information**

**Supplementary methods**

*In vitro fermentations*

The medium contained (per L): 1 g casein hydrolysate, 1 g yeast extract, 2 g $K_2HPO_4$, 3.2 g $NaHCO_3$, 4.5 g NaCl, 0.5 g $MgSO_4.7H_2O$, 0.4 g $CaCl_2.2H_2O$, 0.005 g $FeSO_4.7H_2O$, 0.01 g haemin, 0.05 g bile salts, 0.6 ml resazurin (0.1%), 0.5 g cysteine HCl, 3.6 ml volatile fatty acid (VFA) solution (containing per 40 ml: 17 ml acetic acid, 1 ml *n*-valeric acid, 1 ml *iso*-valeric acid, 1 ml *iso*-butyric acid), 20 ml NaOH (10 mM), 2 ml mineral solution (containing per L: 500 mg EDTA, 200 mg $FeSO_4.7H_2O$, 10 mg $ZnSO_4.7H_2O$, 3 mg $MnCl_2.7H_2O$, 30 mg $H_3BO_3$, 20 mg $CoCl_2.6H_2O$, 1 mg $CuCl_2.2H_2O$, 2 mg $NiCl_2.6H_2O$, 3 mg $NaMoO_4.2H_2O$, 7.5 mg $NaSeO_3$), 1.4 ml vitamin solution (containing per L: 1 g menadione, 2 g biotin, 2 g pantothenate, 10 g nicotinamide, 0.5 g cobalamine, 4 g thiamine, 5 g *p*-aminobenzioc acid; filter-sterilised) and 0.2% (wt/vol) of the test NDC. NDC stock solutions were prepared anaerobically by flushing with $CO_2$ at 1% in water and boiled for 1 min. Cysteine was added to the medium following boiling and dispensed into Hungate tubes while they were flushed with $CO_2$. The vitamin solution and the NDCs were added from stock solutions after autoclaving of the medium, directly before inoculation with the faecal suspension.

*454 sequencing*

DNA concentrations from the cell pellets of the fermentations were measured using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies, Paisley, UK) and microplate reader (TECAN Safire II, TECAN, Reading, UK). As triplicate DNA samples showed comparable concentrations they were pooled into one sample using 10 µl of each

DNA/sample, DNA concentrations measured again by NanoDrop (NanoDrop Technologies, Wilmington, DE, USA) and diluted to 30 ng/µl. PCR of the V1-V3 region of the 16S rRNA genes was performed in quadruplicate for each pooled sample (template concentration 60 ng) using primers 7F (5'AGAGTTTGATYMTGG-3'; note that this primer is not optimal for amplification of *Bifidobacterium* species) and 534R (5'ATTACCGCGGCTGCTGG-3') fitted with the Roche adaptor A (reverse primer) and B (forward primer) fused to the 5' end of the primer. The reverse primer additionally contained a 12 nucleotide long unique barcode sequence. Quadruplicate PCR amplicons were combined and gel purified using the Wizard® SV *Gel* and PCR Clean-Up System (Promega, Madison, USA) and DNA concentration measured using PicoGreen as described above. Amplicons were pre-pooled (50 samples/pool, 2 ng DNA/sample) and sequenced on a GS FLX 454 platform by the Centre of Genomic Research of the University of Liverpool. Bioinformatics were conducted in-house using Mothur v. 1.34.4. software platform (Schloss *et al.*, 2009) on the University of Aberdeen's HPC cluster (Maxwell). All data extraction, pre-processing, analysis of operational taxonomic units (OTUs), and classifications were performed using modules implemented in the Mothur v. 1.34.4. software platform. In total 1 652 684 reads were generated of which 879 706 reads remained in the dataset after several QC steps (denoising and filtering poor quality reads; removal of chimeric molecules and reads from chloroplast, mitochondria, archaea, eukaryote and unknown sequences, (Quince *et al.*, 2011)). Two samples failed to generate any sequences and three samples with reads <20 were excluded from analysis (Table S2B). OTUs were generated at ≥97% sequence identity, which resulted in 1552 OTUs (Table S2C), and the relative abundance was calculated on the whole dataset. Rare OTUs only present in a few samples cannot be analysed statistically for their response to different NDCs, therefore the reference sequences of OTUs with an overall abundance of >100 reads (201 OTUs, 95.6-99.8% of sequence reads per sample) were analysed using the BLAST algorithm

(Altschul *et al.*, 1990) and compared to the taxonomy from the SILVA database (Quast *et al.*, 2013) used in the Mothur analysis and assigned accordingly. OTUs were then assigned to their corresponding qPCR assays if possible. Any OTUs with BLAST assignment to species-level primers below 97% and group-specific primers below 93% were analysed using the BLAST algorithm against the complete NCBI database (Altschul *et al.*, 1990) and the top 100% identity sequence was inspected for primer binding sites (for details see Table S2A). Sequencing data generated during this study are available in the SRA database under SRA accession SRP078412 and is accessible at http://www.ncbi.nlm.nih.gov/sra/SRP078412.

*Polysaccharide analysis*

All analyses were performed in duplicate. To evaluate authenticity and purity of the NDCs used in this study, their monosaccharide composition was analysed by HPAEC-PAD after acid hydrolysis as described previously (Wefers & Bunzel, 2015). The fructan samples (oligofructose, medium-chain inulin, and long-chain inulin) were hydrolysed with 1 M trifluoroacetic acid (TFA) for 30 min at 70°C, because fructose is degraded at elevated temperatures and high acid concentrations (Carpita et al, 1991). For the two pectin samples (apple pectin and rhamnogalacturonan I), methanolysis (1.25 M methanolic HCl, 80°C, 16 h) followed by TFA hydrolysis (2 M TFA, 121°C, 1 h) was performed (Wefers & Bunzel, 2015; De Ruiter et al, 1992). All other samples were analysed by sulfuric acid hydrolysis as described previously (Wefers & Bunzel, 2015; Saeman et al, 1945). Briefly, the polysaccharides were swollen in 12 M sulfuric acid for 2.5 h, diluted, and hydrolysed for 3 h at 100°C.

Laminarin samples were analysed by methylation analysis as described previously (Wefers & Bunzel, 2015). Briefly, laminarin samples from Sigma Aldrich and Shaanxi Pioneer Biotech

were dissolved in dimethylsulfoxide and methylated by using powdered sodium hydroxide and methyl iodide. After extraction, trifluoroacetic acid hydrolysis, $NaBD_4$ reduction, and acetylation, the partially methylated alditol acetates were analysed by GC-MS and GC-FID. The compounds were identified by their mass spectra and retention times and quantified by using FID response factors described previously (Sweet et al, 1975).

*Statistical analysis*

Each of the two fermentation studies provided data from three donors, with 15 (fermentation 1) or four (fermentation 2) NDCs plus a no-NDC control tested at two pH levels. The metabolite data were analysed by ANOVA for each study separately, with donor as random effect and with NDC, pH and their interaction as fixed effects. To identify NDCs that stimulate metabolite production, they were compared against the no-NDC control for each of the two pH levels, based on post-hoc t-tests with the appropriate standard error of the difference and residual degrees of freedom derived from the ANOVA output.

Bacterial 16S rRNA gene copies/ml culture obtained from qPCR were expressed relative to the no-NDC control for each donor and pH for each fermentation study. To achieve normality and constant variance, these ratios were log transformed, and were then analysed by ANOVA as follows. For fermentation 1, the data from pH 5.5 and 6.5 were analysed separately (as for this fermentation the 24 h sample for the no-NDC control for donor 2 at pH 5.5 was not available, therefore this donor was omitted for analysis of pH 5.5 data), with donor as a random effect and NDC as fixed effect. For fermentation 2, donor was taken as random effect and NDC, pH and their interaction as fixed effects. The mean log ratios and their corresponding 95% confidence intervals were back-transformed to allow for presentation of these findings in a meaningful manner (geometric means). To identify NDCs

that significantly altered the bacterial abundance compared to that of the no-NDC control (i.e. is the log-transformed ratio significantly different from 0), post-hoc t-tests were performed with the appropriate SEM and residual degrees of freedom derived from the ANOVA output. Statistical analysis of the qPCR data was performed only on those bacterial groups that were present in all three donors for each fermentation study (Table S1). Furthermore, for those substrates that were tested in both fermentations, the qPCR and SCFA data were summarised by Principal Components Analysis (PCA). To explore relationships between the bacterial abundance obtained from qPCR and SCFA production, the SCFA production was regressed on log bacterial 16S rRNA gene copies/ml culture observed at 24 h using a mixed effects model, combining data from both fermentations. Variation between donors and variation between years within donor were incorporated as random effects, and with fermentation, pH, log bacterial 16S rRNA gene copies/ml culture, and their interactions as fixed effects.

For fermentation 1, percentage bacterial composition data based on 454 sequencing were analysed by ANOVA with donor as a random effect and with NDC, pH and their interaction as fixed effects. To identify NDCs that significantly altered the percentage of bacteria compared to the inoculum, the percentage data were expressed as differences with respect to the inoculum and then analysed by ANOVA (as described above), followed by post-hoc t-tests with the appropriate SEM and residual degrees of freedom derived from the ANOVA output. To investigate associations between OTU and SCFA production, OTU which are known to produce butyrate were aggregated into 'known butyrate producers' and the observed butyrate production was regressed on these combined OTU, with variation between donors incorporated as random effect and with % known butyrate producers, pH, and their interaction as fixed effects. This was repeated in a similar fashion for propionate. Furthermore, Partial Least Squares, which is an exploratory multivariate analysis, was

employed to investigate if any OTU whose role in SCFA production is unknown, were flagged up as being associated with butyrate, propionate, or acetate production.

The agreement between the 454 sequencing and qPCR methods between corresponding bacterial groups (expressed as percentage of total bacteria) was investigated by linear regression.

All analyses are based on 24 h samples only. Analyses were performed in R (R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org). The R library nlme was used for random effects regression.

For the mixed effects regression and simple linear regression $P<0.05$ was regarded significant. For the ANOVA analyses and subsequent post-hoc comparisons, however, to reduce the reporting of false positives due to the large number of comparisons, an effect was considered significant only when $P<0.001$.

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## Supplementary Figures

**Fig. S1**: Rarefaction curves of 454 sequencing data. The inset shows collectors curves of observed OTUs for the two samples with the lowest number of sequences.

**Fig. S2**: Relative abundance of 50 most abundant OTUs after 24 h incubation of human faecal samples from fermentation 1 with different NDC from full sequence dataset (A) compared to corresponding OTUs after subsampling to 426 sequence reads (B).

**Fig. S3**: Total ion current chromatograms of laminarin purchased from Sigma (A) and Shaanxi Pioneer Biotech (B). Marked partially methylated alditol acetates were identified by their mass spectra and semiquantitatively determined by GC-FID**.**

**Fig. S4**: Relationship between log bacterial 16S rRNA gene copies/ml culture and propionate or butyrate production. Combined qPCR data from fermentations f1 and f2 for *Bacteroides* spp. (A), *A. hadrus* (B), *F. prausnitzii* (C) and the *Roseburia* groups (D). SCFA production was regressed on log bacterial 16S rRNA gene copies/ml culture observed at 24 h using a mixed effects model with donor and year within donor regarded as random effects, and with fermentation, pH, log bacterial 16S rRNA gene copies/ml culture, and their interactions as fixed effects. Significant effects ($P<0.05$) are listed above each plot. pH 5.5: blue, pH 6.5: red, fermentation 1: circles, fermentation 2: +. Lines correspond to the fit for each year and pH combination (solid lines: fermentation 1, dashed lines: fermentation 2).

**Fig. S5**: Relative abundance of OTUs after 24 h incubation of human faecal samples with different NDCs. Average data from three donors from fermentation 1 analysed by 454 sequencing. OTUs are grouped together per corresponding qPCR assay. Assignment of individual OTUs to corresponding qPCR groups are given in Table S2. This figure also provides a comparison of the microbiota composition between the inoculum and no-NDC (24 h incubation) control. Certain groups, notably *Blautia* spp., *Roseburia* and *E. hallii*, appear to have decreased in relative abundance from the inoculum to the 24 h incubation (no-NDC) at both pH values. Possibly these groups may be less able to replicate and/or become more prone to cell lysis in the absence of an added carbohydrate energy source; this effect may therefore have amplified some of the changes shown in Fig. 2, which compares 24 h incubations with and without added NDCs.

**Fig. S6**: Linear regression analysis between microbial composition as determined by qPCR and 454 sequencing. The analysis was performed for bacterial groups that were detected by both methods (*C. eutactus* was only found in D3 by qPCR, corresponding OTU-137 was found in 6 and 3 of 31 samples, respectively, in D1 and D2, see Table S2). Blue, donor 1; green, donor 2; purple, donor 3; lighter colours pH 5.5; darker colours pH 6.5.

**Fig. S7**: Relative abundance of 100 most abundant OTUs after 24 h incubation of human faecal samples from fermentation 1 with different NDCs (white - black: 0 - 51%). Relative production of the corresponding SCFA is given at the top of each heat map (white - black: 0 - 47%). OTUs showing a significant increase ($P<0.001$) under certain conditions, compared to inoculum, are indicated by * (for details see Table 2). NDC abbreviations as per Fig. 4.

**Fig. S8**: Principal component analysis of relative SCFA and qPCR data of NDCs included in both fermentation years. A: scores plot colour-coded by donor and year. B: scores plot colour-coded by NDC. C: scores plot colour-coded by pH. NDC abbreviations as per Fig. 4; pH 6.5, italics. D: loading plot of variables. Ac, acetate; But, butyrate; Prop, propionate; Ahad, *A. hadrus*; Bact, *Bacteroides* spp; Bif, *Bifidobacterium* spp.; Blaut, *Blautia* spp.; Dorea, *Dorea* spp.; Ehal, *E. hallii*; Fprau, *F. prausnitzii*; Neg, Negativicutes; Osc, *Oscillibacter* group; Rbro, *R. bromii*; Rfla, *R. flavefaciens* group; Rinul, *R. inulinivorans*; Ros, *Roseburia* group.

Fig. S1

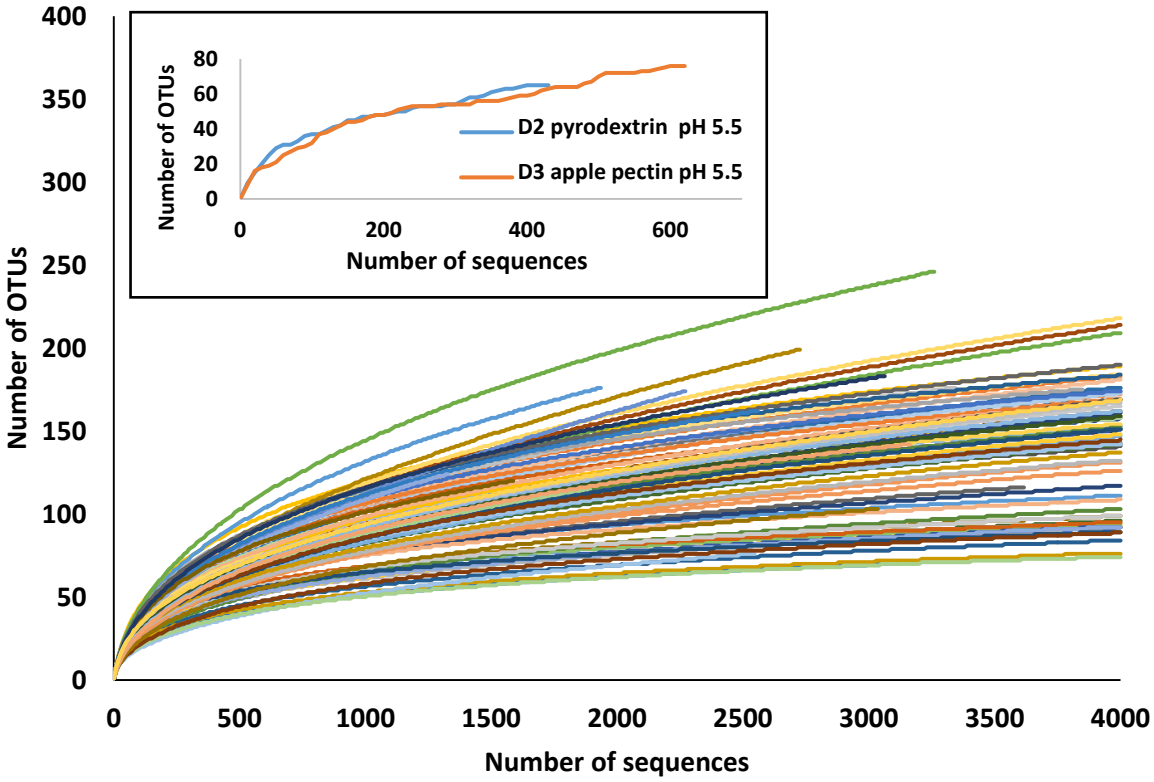
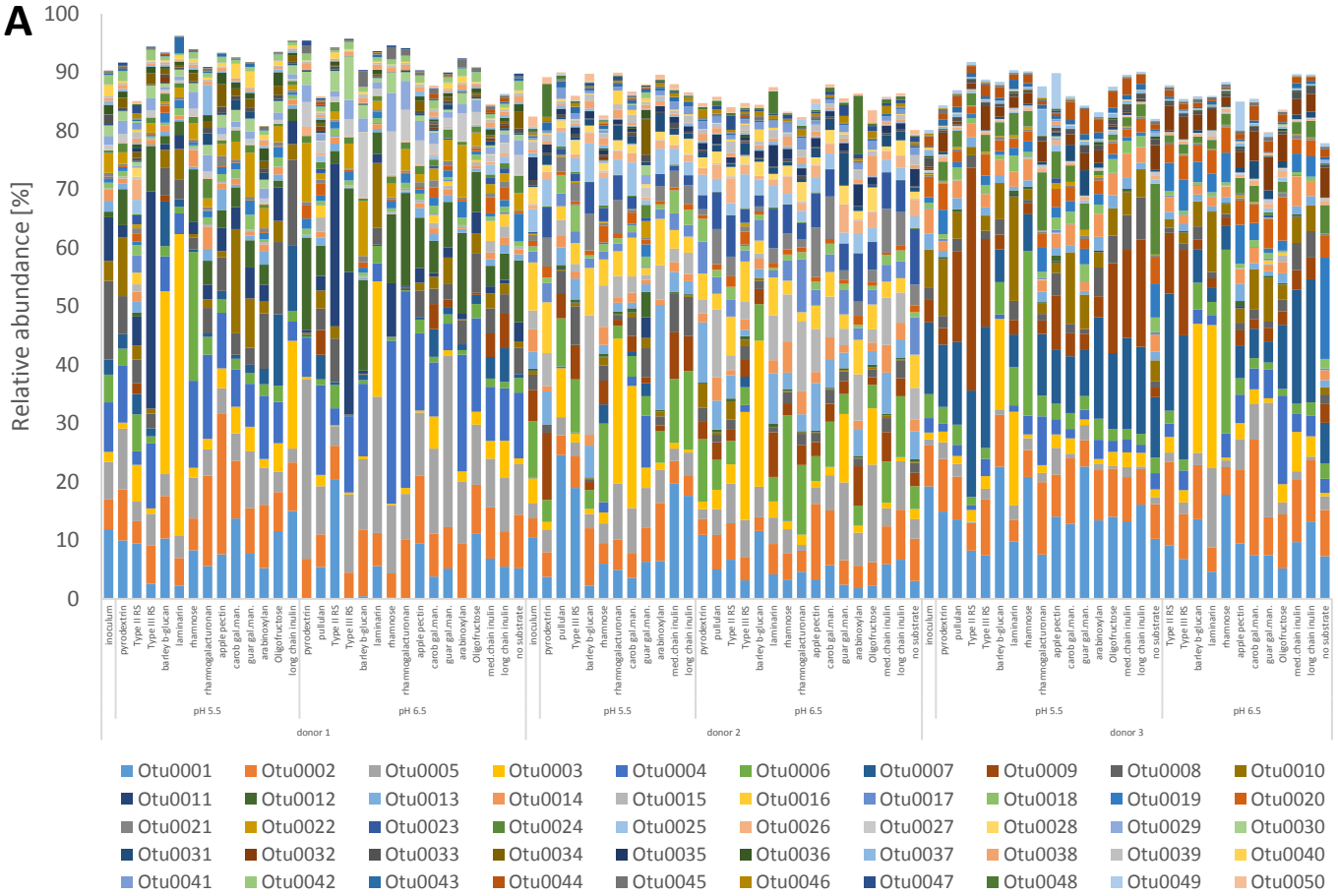

Number of OTUs
400
350
300
250
200
150
100
50
0
0
500
1000
1500
2000
2500
3000
3500
4000
Number of sequences
Number of OTUs
80
60
40
20
0
0
200
400
600
Number of sequences
D2 pyrodextrin pH 5.5
D3 apple pectin pH 5.5

Fig. S2


A
Relative abundance [%]
0
10
20
30
40
50
60
70
80
90
100
inoculum
pyrodextrin
Type II RS
Type III RS
barley b-glucan
laminarin
rhamnose
rhamnogalacturonan
apple pectin
carob gal.man.
guar gal.man.
arabinoxylan
Oligofructose
long chain inulin
pullulan
med.chain inulin
no substrate
pH 5.5
pH 6.5
donor 1
donor 2
donor 3
Otu0001 Otu0002 Otu0005 Otu0003 Otu0004 Otu0006 Otu0007 Otu0009 Otu0008 Otu0010
Otu0011 Otu0012 Otu0013 Otu0014 Otu0015 Otu0016 Otu0017 Otu0018 Otu0019 Otu0020
Otu0021 Otu0022 Otu0023 Otu0024 Otu0025 Otu0026 Otu0027 Otu0028 Otu0029 Otu0030
Otu0031 Otu0032 Otu0033 Otu0034 Otu0035 Otu0036 Otu0037 Otu0038 Otu0039 Otu0040
Otu0041 Otu0042 Otu0043 Otu0044 Otu0045 Otu0046 Otu0047 Otu0048 Otu0049 Otu0050

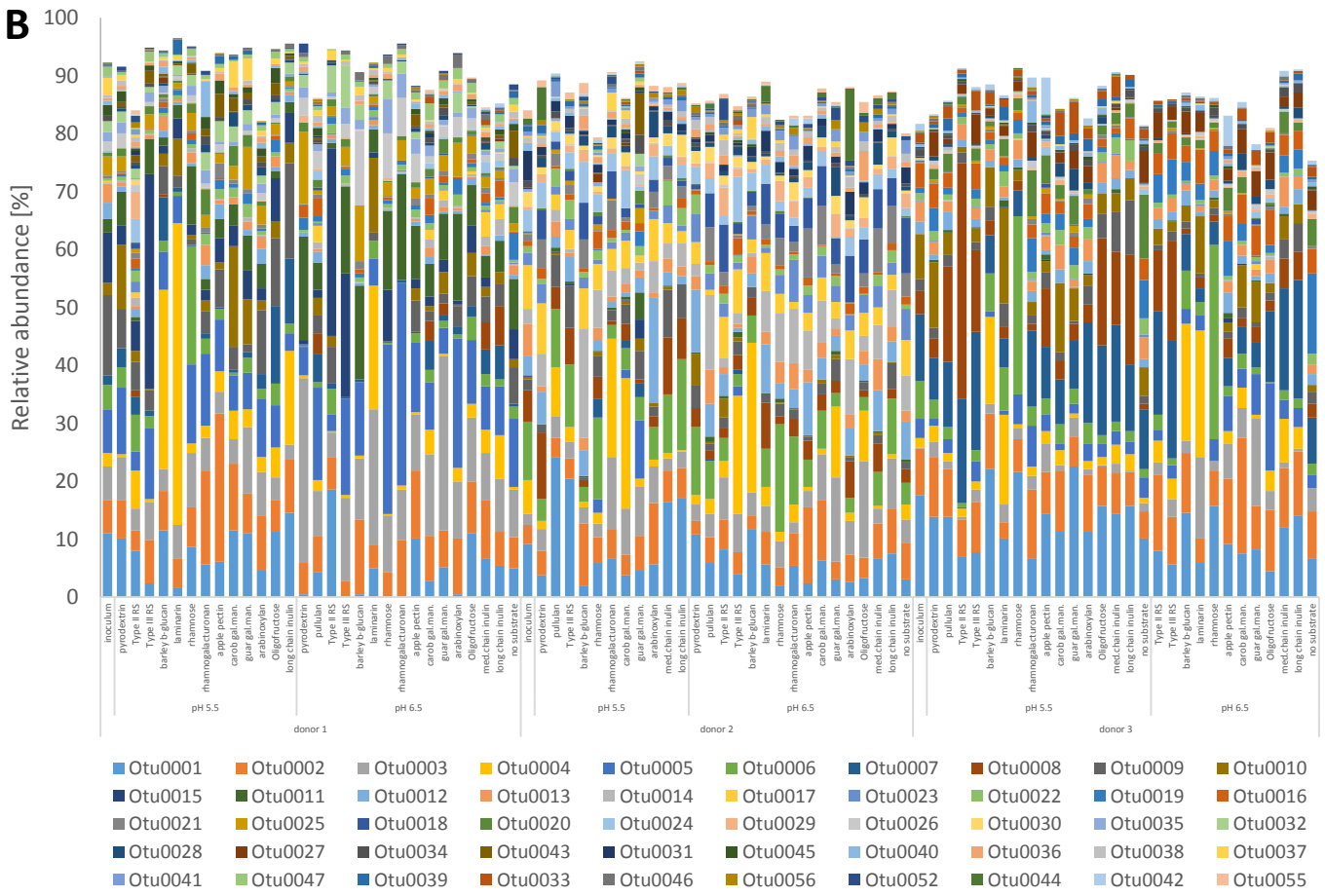

B
Relative abundance [%]
0
10
20
30
40
50
60
70
80
90
100
inoculum
pyrodextrin
Type II RS
Type III RS
barley b-glucan
laminarin
rhamnose
rhamnogalacturonan
apple pectin
carob gal.man.
guar gal.man.
arabinoxylan
Oligofructose
long chain inulin
pullulan
med.chain inulin
no substrate
pH 5.5
pH 6.5
donor 1
donor 2
donor 3
Otu0001 Otu0002 Otu0003 Otu0004 Otu0005 Otu0006 Otu0007 Otu0008 Otu0009 Otu0010
Otu0015 Otu0011 Otu0012 Otu0013 Otu0014 Otu0017 Otu0023 Otu0022 Otu0019 Otu0016
Otu0021 Otu0025 Otu0018 Otu0020 Otu0024 Otu0029 Otu0026 Otu0030 Otu0035 Otu0032
Otu0028 Otu0027 Otu0034 Otu0043 Otu0031 Otu0045 Otu0040 Otu0036 Otu0038 Otu0037
Otu0041 Otu0047 Otu0039 Otu0033 Otu0046 Otu0056 Otu0052 Otu0044 Otu0042 Otu0055

Fig. S3

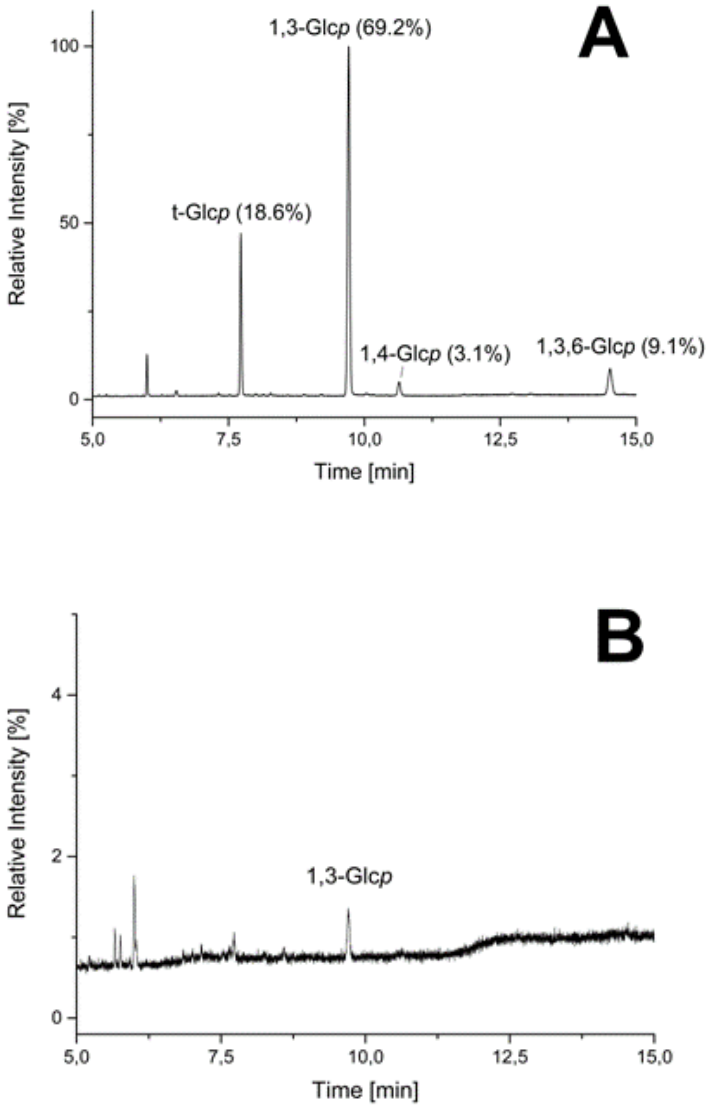

A
1,3-Glcp (69.2%)
t-Glcp (18.6%)
1,4-Glcp (3.1%)
1,3,6-Glcp (9.1%)
Relative Intensity [%]
100
50
0
5,0
7,5
10,0
12,5
15,0
Time [min]
B
1,3-Glcp
Relative Intensity [%]
4
2
0
5,0
7,5
10,0
12,5
15,0
Time [min]

Fig. S4

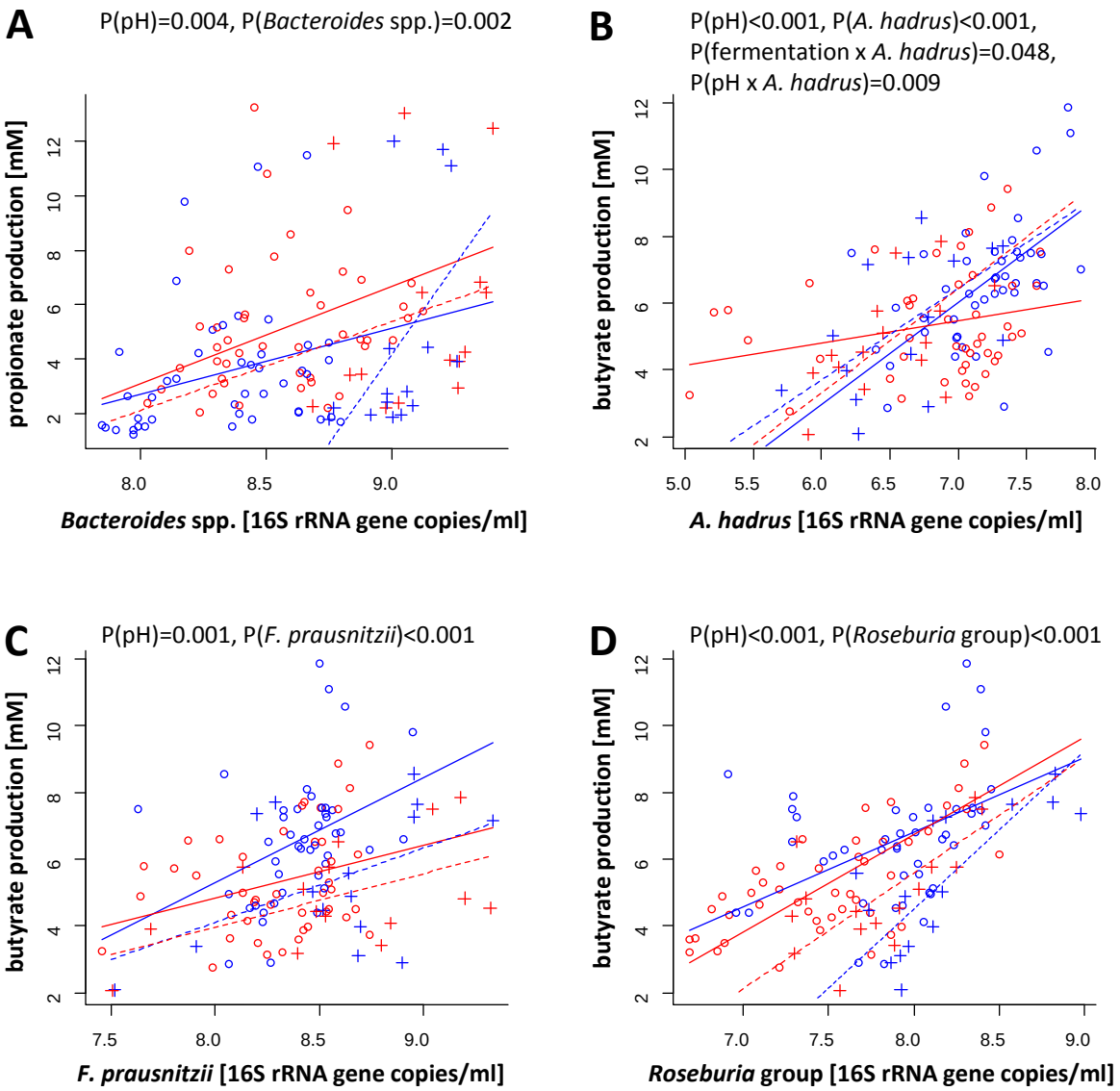

A
P(pH)=0.004, P(Bacteroides spp.)=0.002
propionate production [mM]
Bacteroides spp. [16S rRNA gene copies/ml]
B
P(pH)<0.001, P(A. hadrus)<0.001,
P(fermentation x A. hadrus)=0.048,
P(pH x A. hadrus)=0.009
butyrate production [mM]
A. hadrus [16S rRNA gene copies/ml]
C
P(pH)=0.001, P(F. prausnitzii)<0.001
butyrate production [mM]
F. prausnitzii [16S rRNA gene copies/ml]
D
P(pH)<0.001, P(Roseburia group)<0.001
butyrate production [mM]
Roseburia group [16S rRNA gene copies/ml]

Fig. S5

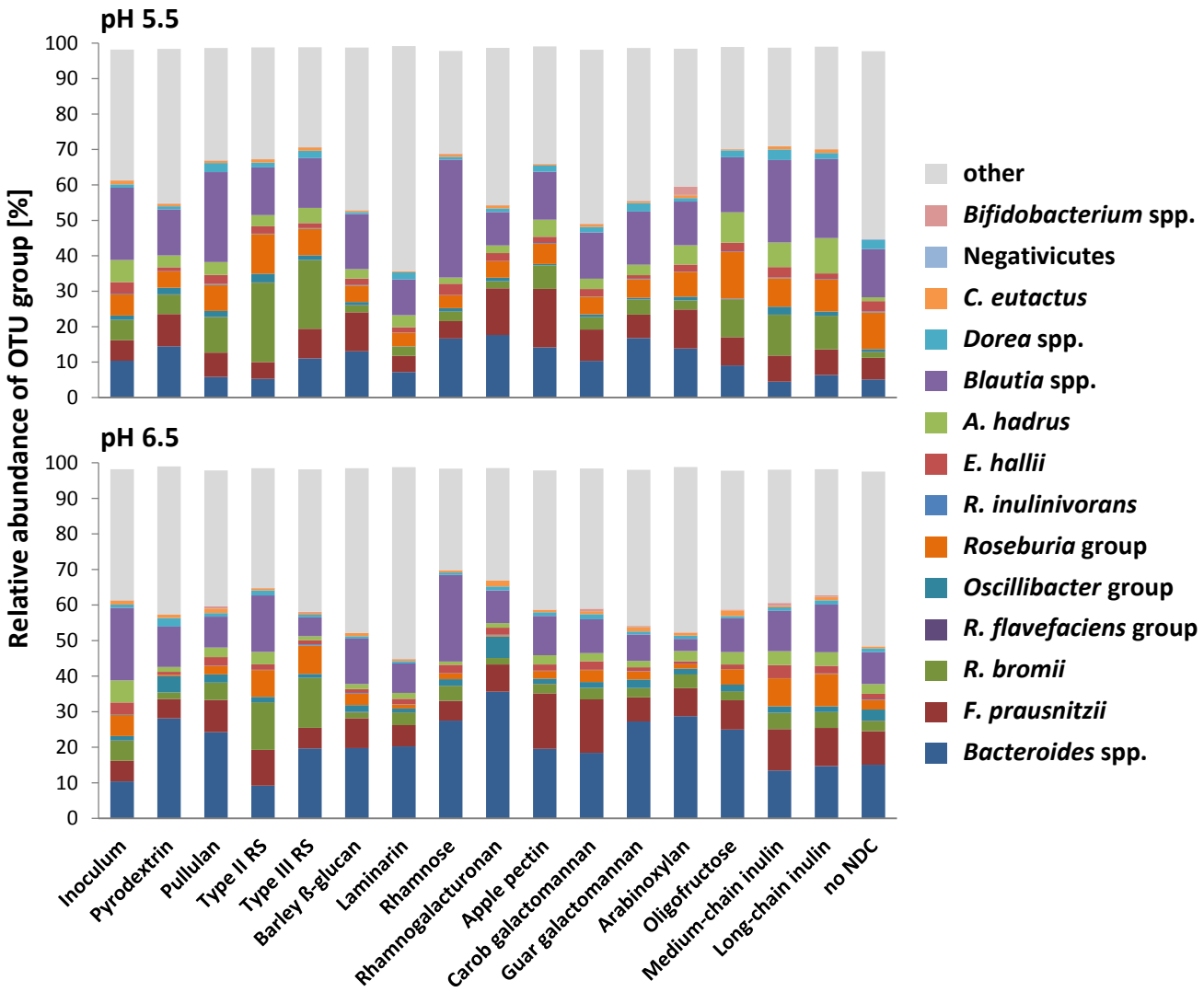

pH 5.5
pH 6.5
Relative abundance of OTU group [%]
100
90
80
70
60
50
40
30
20
10
0
Inoculum
Pyrodextrin
Pullulan
Type II RS
Type III RS
Barley ß-glucan
Laminarin
Rhamnose
Rhamnogalacturonan
Apple pectin
Carob galactomannan
Guar galactomannan
Arabinoxylan
Oligofructose
Medium-chain inulin
Long-chain inulin
no NDC
other
Bifidobacterium spp.
Negativicutes
C. eutactus
Dorea spp.
Blautia spp.
A. hadrus
E. hallii
R. inulinivorans
Roseburia group
Oscillibacter group
R. flavefaciens group
R. bromii
F. prausnitzii
Bacteroides spp.

Fig. S6

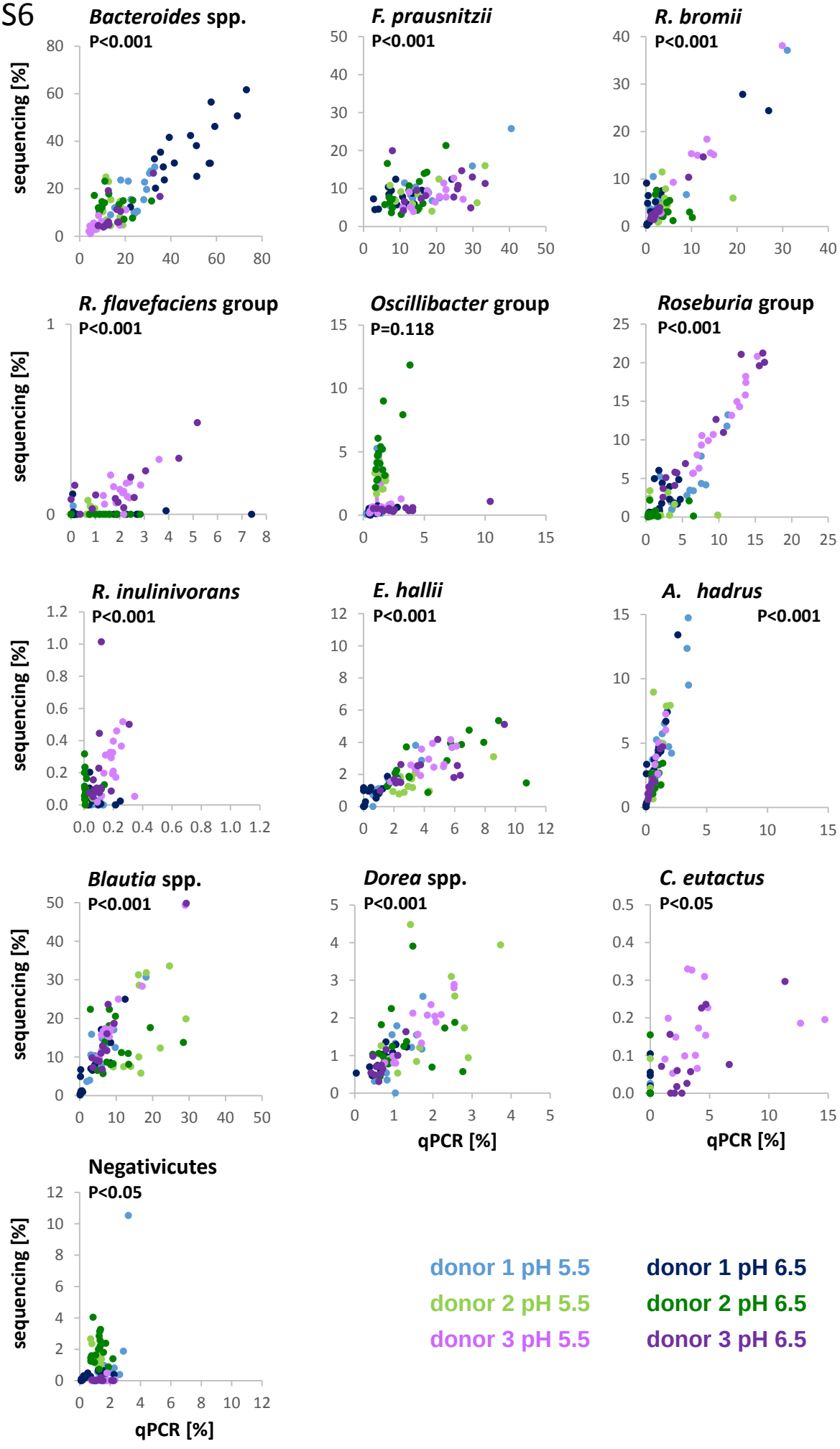

Bacteroides spp.
P<0.001
F. prausnitzii
P<0.001
R. bromii
P<0.001
R. flavefaciens group
P<0.001
Oscillibacter group
P=0.118
Roseburia group
P<0.001
R. inulinivorans
P<0.001
E. hallii
P<0.001
A. hadrus
P<0.001
Blautia spp.
P<0.001
Dorea spp.
P<0.001
C. eutactus
P<0.05
Negativicutes
P<0.05
sequencing [%]
qPCR [%]
donor 1 pH 5.5
donor 2 pH 5.5
donor 3 pH 5.5
donor 1 pH 6.5
donor 2 pH 6.5
donor 3 pH 6.5

Fig. S7

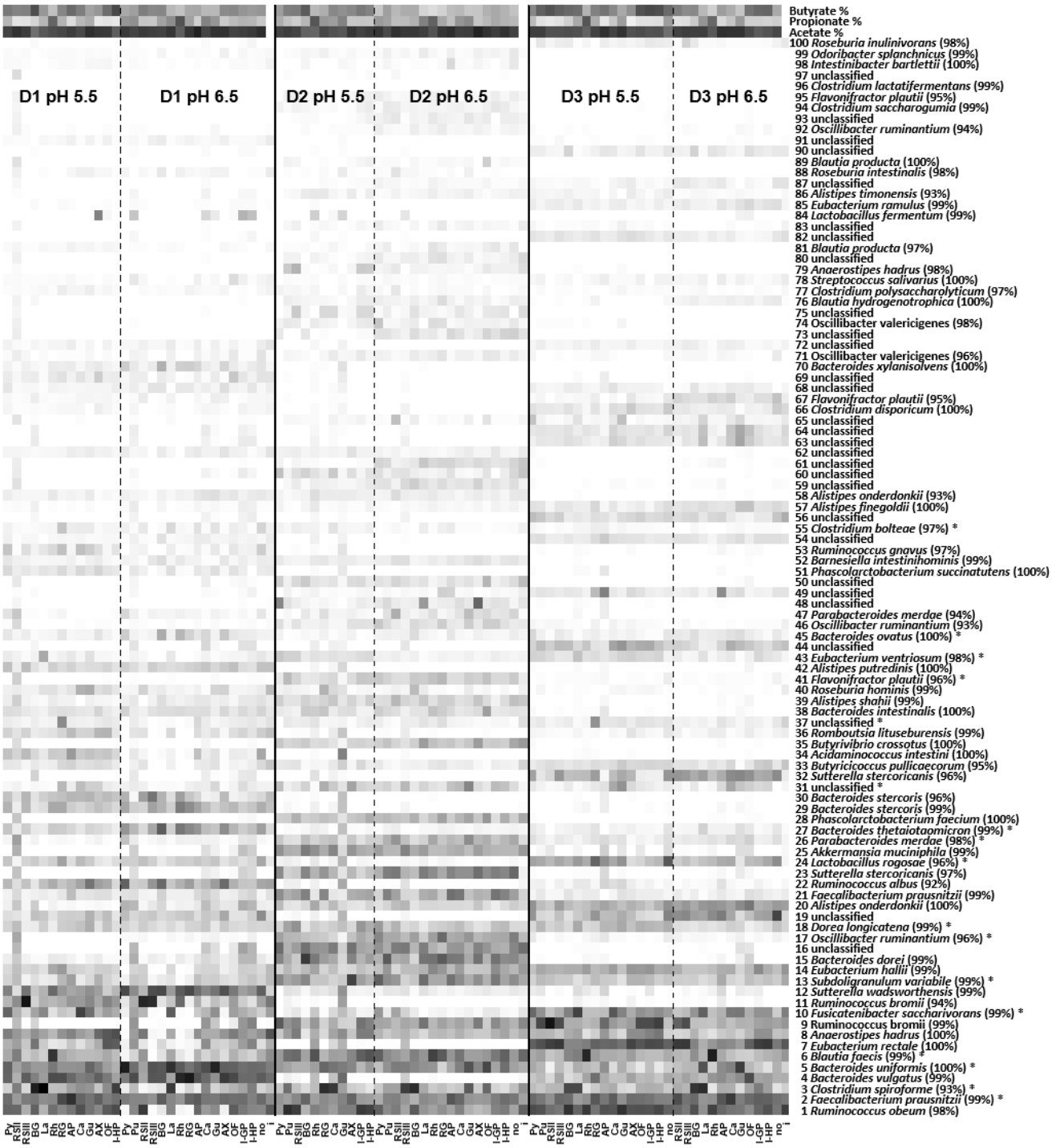
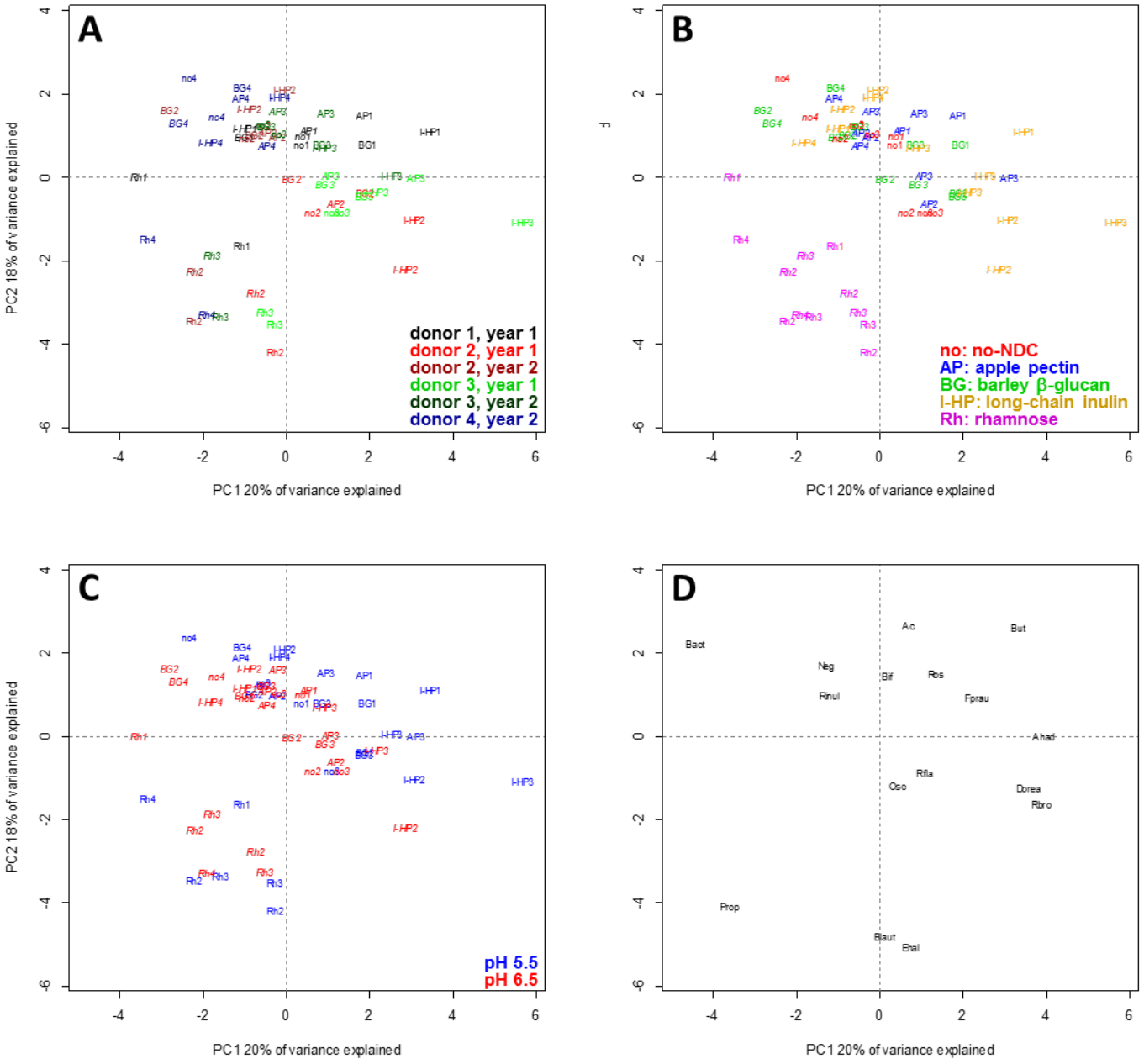

D1 pH 5.5
D1 pH 6.5
D2 pH 5.5
D2 pH 6.5
D3 pH 5.5
D3 pH 6.5
Butyrate %
Propionate %
Acetate %
100 Roseburia inulinivorans (98%)
99 Odoribacter splanchnicus (99%)
98 Intestinibacter bartlettii (100%)
97 unclassified
96 Clostridium lactatifermentans (99%)
95 Flavonifractor plautii (95%)
94 Clostridium saccharogumia (99%)
93 unclassified
92 Oscillibacter ruminantium (94%)
91 unclassified
90 unclassified
89 Blautia producta (100%)
88 Roseburia intestinalis (98%)
87 unclassified
86 Alistipes timonensis (93%)
85 Eubacterium ramulus (99%)
84 Lactobacillus fermentum (99%)
83 unclassified
82 unclassified
81 Blautia producta (97%)
80 unclassified
79 Anaerostipes hadrus (98%)
78 Streptococcus salivarius (100%)
77 Clostridium polysaccharolyticum (97%)
76 Blautia hydrogenotrophica (100%)
75 unclassified
74 Oscillibacter valericigenes (98%)
73 unclassified
72 unclassified
71 Oscillibacter valericigenes (96%)
70 Bacteroides xylanisolvens (100%)
69 unclassified
68 unclassified
67 Flavonifractor plautii (95%)
66 Clostridium disporicum (100%)
65 unclassified
64 unclassified
63 unclassified
62 unclassified
61 unclassified
60 unclassified
59 unclassified
58 Alistipes onderdonkii (93%)
57 Alistipes finegoldii (100%)
56 unclassified
55 Clostridium bolteae (97%) *
54 unclassified
53 Ruminococcus gnavus (97%)
52 Barnesiella intestinihominis (99%)
51 Phascolarctobacterium succinatutens (100%)
50 unclassified
49 unclassified
48 unclassified
47 Parabacteroides merdae (94%)
46 Oscillibacter ruminantium (93%)
45 Bacteroides ovatus (100%) *
44 unclassified
43 Eubacterium ventriosum (98%) *
42 Alistipes putredinis (100%)
41 Flavonifractor plautii (96%) *
40 Roseburia hominis (99%)
39 Alistipes shahii (99%)
38 Bacteroides intestinalis (100%)
37 unclassified *
36 Romboutsia lituseburensis (99%)
35 Butyrivibrio crossotus (100%)
34 Acidaminococcus intestini (100%)
33 Butyricicoccus pullicaecorum (95%)
32 Sutterella stercoricanis (96%)
31 unclassified *
30 Bacteroides stercoris (96%)
29 Bacteroides stercoris (99%)
28 Phascolarctobacterium faecium (100%)
27 Bacteroides thetaiotaomicron (99%) *
26 Parabacteroides merdae (98%) *
25 Akkermansia muciniphila (99%)
24 Lactobacillus rogosae (96%) *
23 Sutterella stercoricanis (97%)
22 Ruminococcus albus (92%)
21 Faecalibacterium prausnitzii (99%)
20 Alistipes onderdonkii (100%)
19 unclassified
18 Dorea longicatena (99%) *
17 Oscillibacter ruminantium (96%) *
16 unclassified
15 Bacteroides dorei (99%)
14 Eubacterium hallii (99%)
13 Subdoligranulum variabile (99%) *
12 Sutterella wadsworthensis (99%)
11 Ruminococcus bromii (94%)
10 Fusicatenibacter saccharivorans (99%) *
9 Ruminococcus bromii (99%)
8 Anaerostipes hadrus (100%)
7 Eubacterium rectale (100%)
6 Blautia faecis (99%) *
5 Bacteroides uniformis (100%) *
4 Bacteroides vulgatus (99%)
3 Clostridium spiroforme (93%) *
2 Faecalibacterium prausnitzii (99%) *
1 Ruminococcus obeum (98%)
Py Pu RSII RSIII BG La Rh RG AP Ca Gu AX OF I-GP I-HP no i

Fig. S8


A
B
C
D
PC1 20% of variance explained
PC2 18% of variance explained
donor 1, year 1
donor 2, year 1
donor 2, year 2
donor 3, year 1
donor 3, year 2
donor 4, year 2
no: no-NDC
AP: apple pectin
BG: barley β-glucan
l-HP: long-chain inulin
Rh: rhamnose
pH 5.5
pH 6.5
Ac
But
Bact
Neg
Bif
Ros
Rinul
Fprau
Ahad
Rfla
Osc
Dorea
Rbro
Prop
Blaut
Ehal