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

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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 four in fermentation 2 to assess reproducibility. The supplier of one 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.

Quantitative PCR

146 The pooled triplicate DNA samples from the *in vitro* fermentations 1 and 2 were analysed by
147 quantitative real time PCR as described previously (Fuller *et al.*, 2007; Ramirez-Farias *et al.*, 2009)
148 with the following modifications. Reactions were performed in duplicate with iTaq^{TM} Universal
149 SYBR® Green Supermix (Bio-Rad, Hemel Hempstead, UK) in a total volume of 10 µl with primers
150 at 500 nM and 5 ng of DNA in optical-grade 384-well plates sealed with optical sealing tape in the
151 presence of 1 µg/ml herring sperm DNA (Promega, Madison, WI, USA). Amplification was performed
152 with a CFX384^{TM} Real-time System (Bio-Rad, Hemel Hempstead, UK) with the following protocol:
153 one cycle of 95 ºC for 3 min, 40 cycles of 95 ºC for 5 s and annealing temperature as per Table S3 for
154 30 s, 1 cycle of 95 ºC for 10 s and a stepwise increase of the temperature from 65 ºC to 95 ºC (at 5 s
155 per 0.5 ºC) to obtain melt curve data. Primers used for the quantification of the specific bacterial groups
156 are given in Table S3. Standard curves consisted of dilution series of amplified bacterial 16S rRNA
157 genes from reference strains. The abundance of 16S rRNA gene was determined from standard curves
158 and bacterial groups were either expressed as a percentage of total bacteria determined by universal
159 primers or as 16S rRNA gene copies per ml culture. The detection limit was determined with negative
160 controls containing only herring sperm DNA.
161
162 *454 sequencing*
163 Amplicon sequencing of the V1-V3 region of the 16S rRNA genes was performed on GS FLX 454
164 platform by the Centre of Genomic Research of the University of Liverpool and Bioinformatics were
165 conducted in-house using Mothur v. 1.34.4. software platform (Schloss *et al.*, 2009) on the University
166 of Aberdeen's HPC cluster (Maxwell). Full details are given in supplementary methods. Reads per
167 sample varied from 426 to 82 791 (average 9069.1 ± 11044.5). Good's coverage was over 95% for all
168 but one sample (Table S2B) and rarefaction and collectors curves (Fig. S1) showed that a good
169 coverage was achieved for all samples. Exclusion of samples with Good's coverage of less than 97%
170 resulted in very similar results (Table 2) and subsampling to 426 sequence reads per sample revealed

171 that the relative abundance of the top 50 OTUs (representing >88% of all reads) was very similar to
172 the full dataset (Fig. S2). It was therefore decided to work with the full dataset to preserve as much of
173 the data as possible. OTUs were generated at ≥97% sequence identity, which resulted in 1552 OTUs
174 (Table S2C), and the relative abundance was calculated. OTUs with an overall abundance of >100
175 reads (201 OTUs, 95.6-99.8% of sequence reads per sample) were analysed using the BLAST
176 algorithm (Altschul *et al.*, 1990) and compared to the taxonomy from the SILVA database (Quast *et*
177 *al.*, 2013). OTUs were then assigned to their corresponding qPCR assays if possible as detailed in
178 supplementary methods and Table S2A.
179
180 *Polysaccharide analysis*
181 All analyses were performed in duplicate. To evaluate authenticity and purity of the NDCs used in this
182 study, their monosaccharide composition was analysed by HPAEC-PAD after acid hydrolysis as
183 described previously (Wefers and Bunzel, 2015). Details of the hydrolysis conditions for the different
184 NDCs are given in supplementary methods.
185
186 *Statistical analysis*
187 Data from each fermentation study were analysed by ANOVA with random effect for Donor and fixed
188 effects for NDC, pH, and their interaction, followed by post-hoc t-test. Principal Components Analysis
189 and Partial Least Squares were used to investigate associations between bacteria and SCFA production.
190 Associations of interest were quantified by random effects regression with Donor as random effect and
191 with pH, microbial abundance and their interaction as fixed effects. The agreement between the 454
192 sequencing and qPCR methods between corresponding bacterial groups (expressed as percentage of
193 total bacteria) was investigated by linear regression. Full details are given in Supplementary methods.

For the regression analyses $P<0.05$ 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 $P<0.001$.

Results

SCFA from in vitro batch culture incubations

Anaerobic incubations were conducted with 15 different NDCs as energy sources (Table 1; 0.2% w/v) in the presence of human faecal slurries from three healthy donors (fermentation 1) at initial pH values of 5.5 and 6.5. For four NDCs, another fermentation was carried out approximately one year later to assess reproducibility of the responses (fermentation 2, two of the three donors were the same as in fermentation 1). Profiles of net SCFA production after 24 hours of incubation differed between NDCs, especially for those from different NDC classes. Analysis of the chemical composition of NDCs used (Table S4) showed that their composition was consistent with their description. For NDCs used in both fermentations the SCFA profiles were very reproducible (Fig. 1).

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

When individual NDCs were compared to the no-NDC control, starch, β-glucan and inulin/oligofructose gave rise to significantly ($P<0.001$) increased butyrate concentrations when the initial pH was 5.5. At an initial pH of 6.5, this butyrogenic effect was seen for inulin/oligofructose but

not for the starches (Fig. 1). Pyrodextrin, laminarin, rhamnose, rhamnogalacturonan and the two galactomannans all gave rise to increased propionate at initial pH 5.5. These same NDCs increased propionate when the initial pH was 6.5, but in addition pullulan, β-glucan and some of the inulin-type NDCs also promoted propionate significantly at the higher pH (all $P<0.001$). The NDC that stands out with regard to propionate production however is rhamnose. The absolute amount produced was between 1.5- and 5.5-fold higher than in the presence of the other NDCs, and the molar ratio (40-42% of total SCFA) was similar to the percentage of acetate in both fermentations and independent of the pH (Fig. 1, Table S5).

Microbial population changes detected by qPCR

The microbial composition of the *in vitro* incubations from both fermentation experiments was analysed using qPCR against 21 different bacterial species and groups, in addition to total bacteria and methanogenic Archaea (Table S3). Analysis of the inocula revealed high inter-individual 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 ($P<0.05$) positive correlation of *Bacteroides* spp. and propionate production and of *A. hadrus*, *F. prausnitzii* and *Roseburia* group, respectively, and butyrate production (Fig. S3).

Relationship between microbiota composition and SCFA formation

In addition to qPCR, the influence of the different NDCs on the microbiota of fermentation 1 was analysed using 454 sequencing. Like qPCR, operational taxonomic unit (OTU) analysis revealed large inter-individual differences in the inocula. In the sample of donor 1 and donor 3 we detected 116 and 114 different OTUs, respectively, with an abundance of >100 reads, whereas the inoculum of donor 2 contained 162. OTU 1 (*Blautia obeum*) was the most abundant OTU in donors 2 and 3 and the second most abundant in donor 1 after OTU 8 (*A. hadrus*) (Table S2). Statistical analysis of all OTUs that were detected in at least two thirds of all samples after 24 h of incubation revealed that 17 OTUs,

293 covering a range of different Bacteroidetes and Firmicutes species, were significantly ($P<0.001$)
294 increased compared to the inoculum on at least one NDC (Table 2).

295 In order to compare the qPCR results with sequencing results, the OTUs were assigned to groups
296 that would be targeted by the qPCR primers used (Fig. S4, Table S2). This led to an assignment rate
297 of 29.2-83.2% per sample (average 59.1%) of all sequences. The results in Fig. S4 show that the
298 changes detected by 454 sequencing agree well with those detected by qPCR. This applies for example
299 to the increase in *Blautia* spp. with rhamnose, in *R. bromii* with RS and in *A. hadrus* with fructans.
300 Correlations were calculated between relative data from qPCR and the sum of OTUs assigned to the
301 corresponding primer set. Significant ($P<0.05$) correlations were found for all OTU groups which
302 could be assigned to corresponding qPCR primers, except for the *Oscillibacter* group (Fig. S5).
303 Weaker correlations likely reflect technical differences arising from either qPCR or sequencing
304 methodology, or a limited understanding of the groups under study, which may affect the accuracy of
305 assigning sequence OTUs to the corresponding qPCR group. *Bifidobacteria* were not included in this
306 comparison as they are underestimated by 454 sequencing with the primers used here (Walker *et al.*,
307 2015).

308 Heat map analysis of relative OTU abundance revealed high inter-individual variation (Fig. S6, 100
309 most abundant OTUs). Propionate- and butyrate-producing status was assigned to all classified OTUs
310 (Table S2A; 39-87% of sequence data per sample assigned to fermentation product formation based
311 on at least 97% sequence identity to known species) and heat maps for propionate- and butyrate-
312 producing OTUs, respectively, were generated (Fig. 4). This shows that different OTUs contribute to
313 SCFA production in different donors. However, regression analyses of the sum of all propionate- or
314 butyrate-producing bacteria (as percentage of total sequences) to percentage propionate or butyrate
315 produced over 24 h of incubation showed a strong correlation (Fig. 5, $P<0.001$ for both SCFAs). For
316 propionate the initial pH of the incubations had no effect on this relationship, but for butyrate a strong
317 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. S7A). Rhamnose incubations in particular clustered separately and were associated with propionate production, *Blautia* spp. and *E. hallii* (Fig. S7B&D). Long-chain inulin also tended to result in a bigger difference to no-NDC control than the other NDCs examined (Fig. S7B). 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. S7C&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

343 substrates (Walker *et al.*, 2005; Duncan *et al.*, 2003; Chung *et al.*, 2016). By contrast in the present
344 study, this dominance of *Bacteroides* spp. was curtailed by the lower pH conditions, and probably also
345 by the reduction in the peptide content of the medium (to 0.1% casitone and 0.1% yeast extract). This
346 has helped to reveal the response of Firmicutes and Actinobacteria to different carbohydrates.
347 Lowering of gut pH due to increased fermentation may also contribute to the reduced abundance of
348 *Bacteroides* species often observed in human dietary trials with NDC (Martinez *et al.*, 2013; Duncan
349 *et al.* 2009).

350 An overview of the major microbiota responses on the different carbohydrate classes and
351 corresponding pathways for SCFA formation is provided in Fig. 6. The NDC that promoted by far the
352 highest SCFA proportion of propionate was rhamnose. This can be explained by the fact that rhamnose
353 is fermented via the propanediol pathway in some anaerobic bacteria, yielding propionate and
354 sometimes also propanol (Reichardt *et al.*, 2014; Scott *et al.*, 2006; Louis and Flint, 2017). The
355 distribution of the propanediol pathway of propionate formation from deoxy sugars is however quite
356 limited, being found so far in *Roseburia inulinivorans* and in *Blautia* spp. (Reichardt *et al.*, 2014). This
357 fits very well with the observed highly specific enrichment of *Blautia* spp. on rhamnose that was
358 detected both by 16S rRNA-based qPCR and sequence analysis. In other bacteria that can utilise deoxy
359 sugars, such as *Bacteroides* spp. (Rodionova *et al.*, 2013) and *Anaerostipes rhamnosivorans* (Bui *et*
360 *al.*, 2014), propionate is not formed and 1,2-propanediol is an end product. *E. hallii* also showed a high
361 qPCR ratio for rhamnose relative to the no-NDC control. Existing *E. hallii* strains are not known to
362 grow on rhamnose (Holdeman and Moore, 1974), but its stimulation is likely to be indirect, due to
363 cross-feeding of 1,2-propanediol formed from rhamnose by *Bacteroides* spp. and *A. rhamnosivorans*,
364 since a recent study demonstrated the ability of *E. hallii* to metabolise 1,2-propanediol (Engels *et al.*,
365 2016). *E. hallii* also has the ability to utilize lactate (Duncan *et al.*, 2004), which is a major fermentation
366 product of *Blautia faecis* (Park *et al.*, 2013), the *Blautia* species that was most strongly stimulated by
367 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. S3). 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

393 *R. bromii*, 94% identity) responded strongly to resistant starch in donor one, whereas it was low in
394 donor 3 and OTU 9 (*R. bromii*, 99%) responded strongly to resistant starch in this donor (Fig. S6).
395 When looking specifically at propionate- or butyrate-producing bacteria, it becomes clear that they
396 show a heterogeneous response to different carbohydrates (Fig. 4), but their combined response
397 correlates very strongly with the corresponding SCFA output (Fig. 5), revealing functional redundancy
398 in the microbiota. The activities of each microbiota member will be dependent not only on their genetic
399 potential to degrade certain NDCs and produce certain SCFA, but also on their interaction with other
400 microbes and their competitive fitness. This likely underlies the different response seen for some OTUs
401 in different donors (for example, OTU 8, *A. hadrus* (100%) showing an increase on arabinoxylan in
402 donor 1 and 3, but not donor 2). The poor response of *R. inulinivorans* on inulin and rhamnose likely
403 also reflects its poor ability to compete effectively in the complete microbiota, despite the fact that it
404 can grow on those NDCs in pure culture (Reichardt *et al.*, 2014; Scott *et al.*, 2014; Duncan *et al.*,
405 2006). This agrees with a human intervention study, which also failed to see an increase in this species
406 after inulin supplementation in subjects with high baseline levels of this species (Louis *et al.*, 2010).
407 Our data also reveal that while the % butyrate among SCFA products was related to the proportion
408 of butyrate-producing bacteria, the initial pH altered this relationship considerably (Fig. 5). It is known
409 that species such as *F. prausnitzii* and *Roseburia* spp. that use the butyryl-CoA:acetate CoA-transferase
410 route for butyrate formation, exhibit a shift in fermentation stoichiometry in pure culture at lower pH
411 (5.5) in favour of greater butyrate production and greater acetate consumption per mol of carbohydrate
412 consumed (Louis and Flint, 2017; Kettle *et al.*, 2015). The relationships seen in Fig. 5 indicate that
413 this shift in stoichiometry applies also to butyrate production by the mixed community, while
414 propionate production was simply related to the % propionate-producing bacteria regardless of the
415 initial pH.
416 In conclusion, the work presented here is one of few *in vitro* studies that compares the impact of a
417 large variety of NDCs on the composition and metabolic activity of the human faecal microbiota. Some

418 of the NDCs investigated here are currently classed as prebiotics, but this study does not reveal a clear
419 distinction between those and NDCs currently classed as dietary fibre, in terms of a selective
420 stimulation of specific bacteria. Prebiotic NDCs are considered to have consequences for health mainly
421 through their impact upon the gut microbiota. These impacts can be ascribed to two types of
422 mechanism. First, as shown here, prebiotics can promote the growth of a limited number of bacterial
423 species, boosting their populations and their representation within the gut microbiota, although the
424 pattern of stimulation can vary between microbiota from different individuals. Some of these bacteria
425 may interact with the host's immune system, but we can expect that inter-individual variability in the
426 microbiota and the selective effects of different NDCs may result in wide variation in health
427 consequences. Second, we know that the metabolites produced by the microbial community have
428 important consequences for health. Here our results suggest, at least for short chain fatty acids, that
429 the consequences of NDC fermentation are likely to be more consistent between individuals. This is
430 explained by the large number of gut anaerobes capable of producing the major SCFA, resulting in
431 functional redundancy that tends to mask inter-individual variation at the species level. Thus we have
432 shown that, in spite of inter-individual differences in microbiota composition, SCFA profiles were
433 very similar for each individual and for a given NDC. We should also note however that for metabolites
434 whose production is limited to a smaller number of species, individual variability is likely to be
435 correspondingly greater. In addition, if keystone species are absent, the capacity of the microbiota to
436 ferment NDC can be greatly reduced, as shown for individuals lacking *R. bromii* on diets high in RS
437 (Walker *et al.*, 2011). There is currently much debate on the prebiotic concept, and the stipulation that
438 they have to selectively stimulate certain microbes is increasingly challenged (Louis *et al.*, 2016;
439 Bindels *et al.*, 2015; Steinert *et al.*, 2016). The data presented here are in support of a more general
440 definition with regard to the modulation of the gut microbiota in order to achieve a beneficial effect
441 on the host.
442

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586 **Figure legends**

587

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

594

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

606

607 **Figure 3:** Growth response of individual *Bifidobacterium* species on α-glucans, arabinoxylan and
608 fructans. Data shown are from individual faecal incubations (fermentation 1 donor 1, 2, 3; fermentation
609 2 donor 2b, 3b, 4) analysed by qPCR after 24 h incubation at pH 5.5. At 0 h, *Bifidobacterium* species
610 levels in all donors ranged from 6.1×10^4 to 9.7×10^6/ml (see Table S1).

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

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

Figure 6: Overview of known fermentation pathways for SCFA formation in human gut bacteria. 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: 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. S4: 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 S2A. 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. S5: 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. S6: 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. S7: 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 (mM) 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	
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

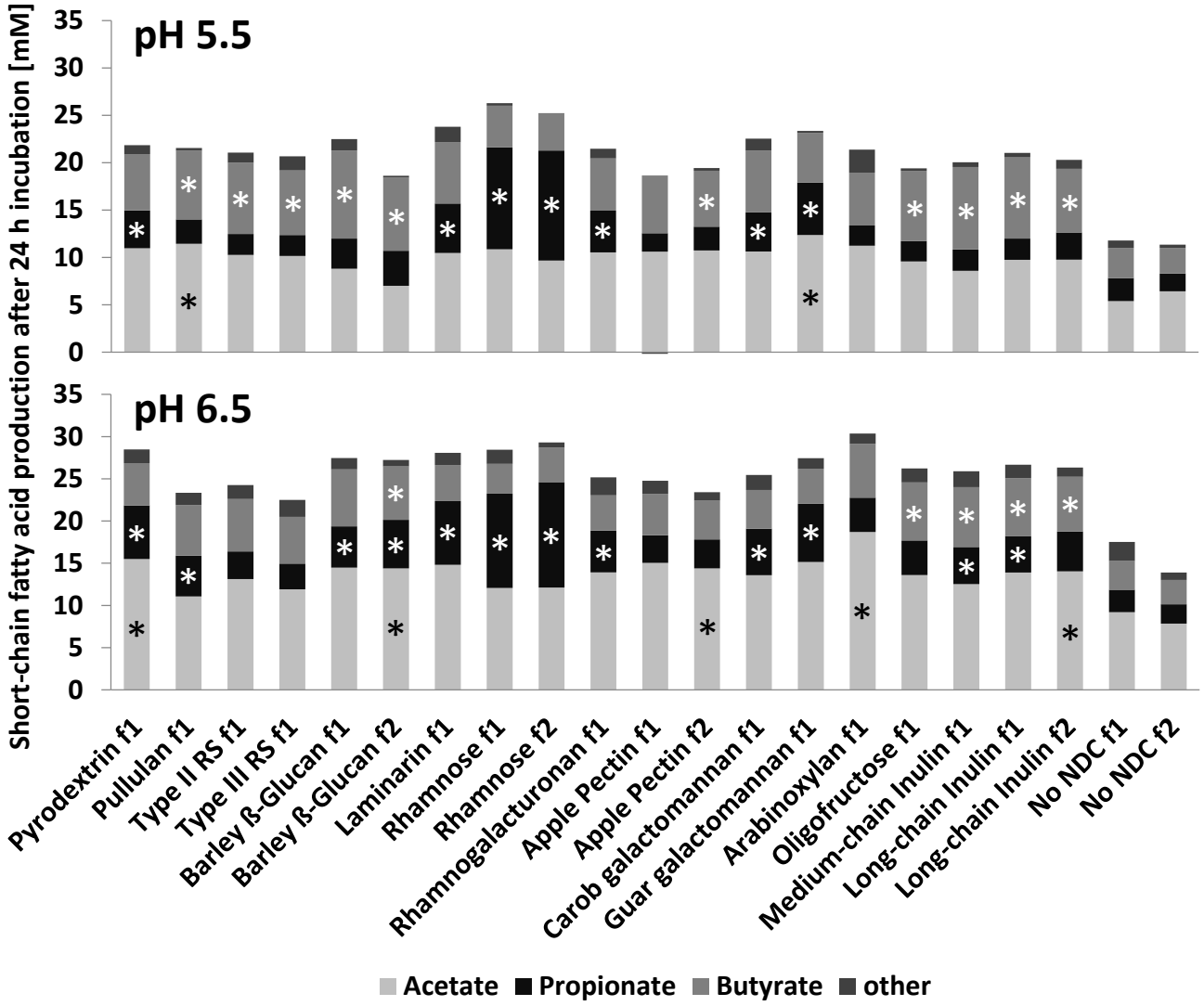
Table 2: Operational taxonomic units **(**OTUs) from 454 sequencing analysis of fermentation 1 exhibiting a significant increase on specific NDCs and pH values (P<0.001, see Table S2).

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

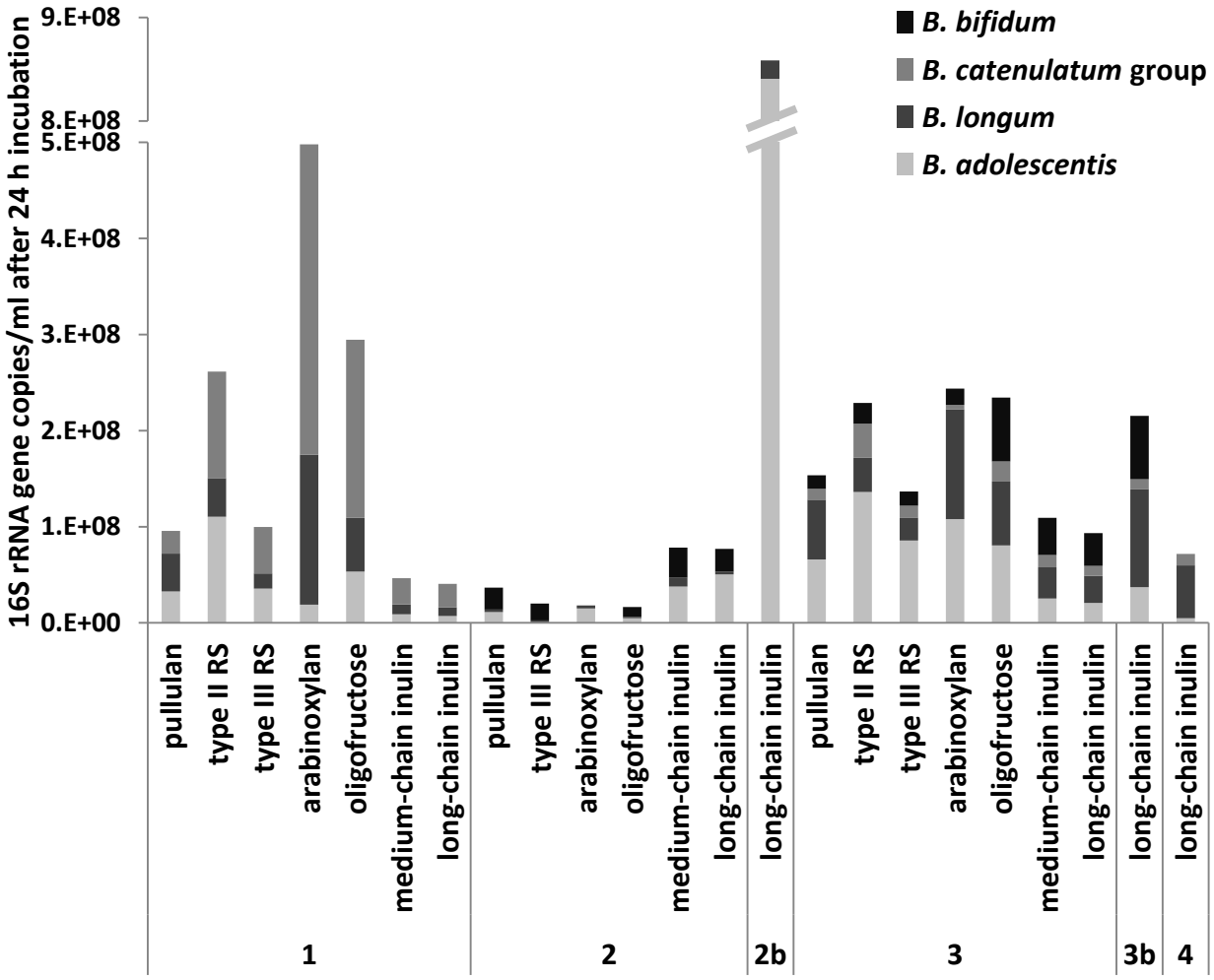

16S rRNA gene copies/ml after 24 h incubation
9.E+08
8.E+08
5.E+08
4.E+08
3.E+08
2.E+08
1.E+08
0.E+00
B. bifidum
B. catenulatum group
B. longum
B. adolescentis
pullulan
type II RS
type III RS
arabinoxylan
oligofructose
medium-chain inulin
long-chain inulin
1
2
2b
3
3b
4

Fig. 4

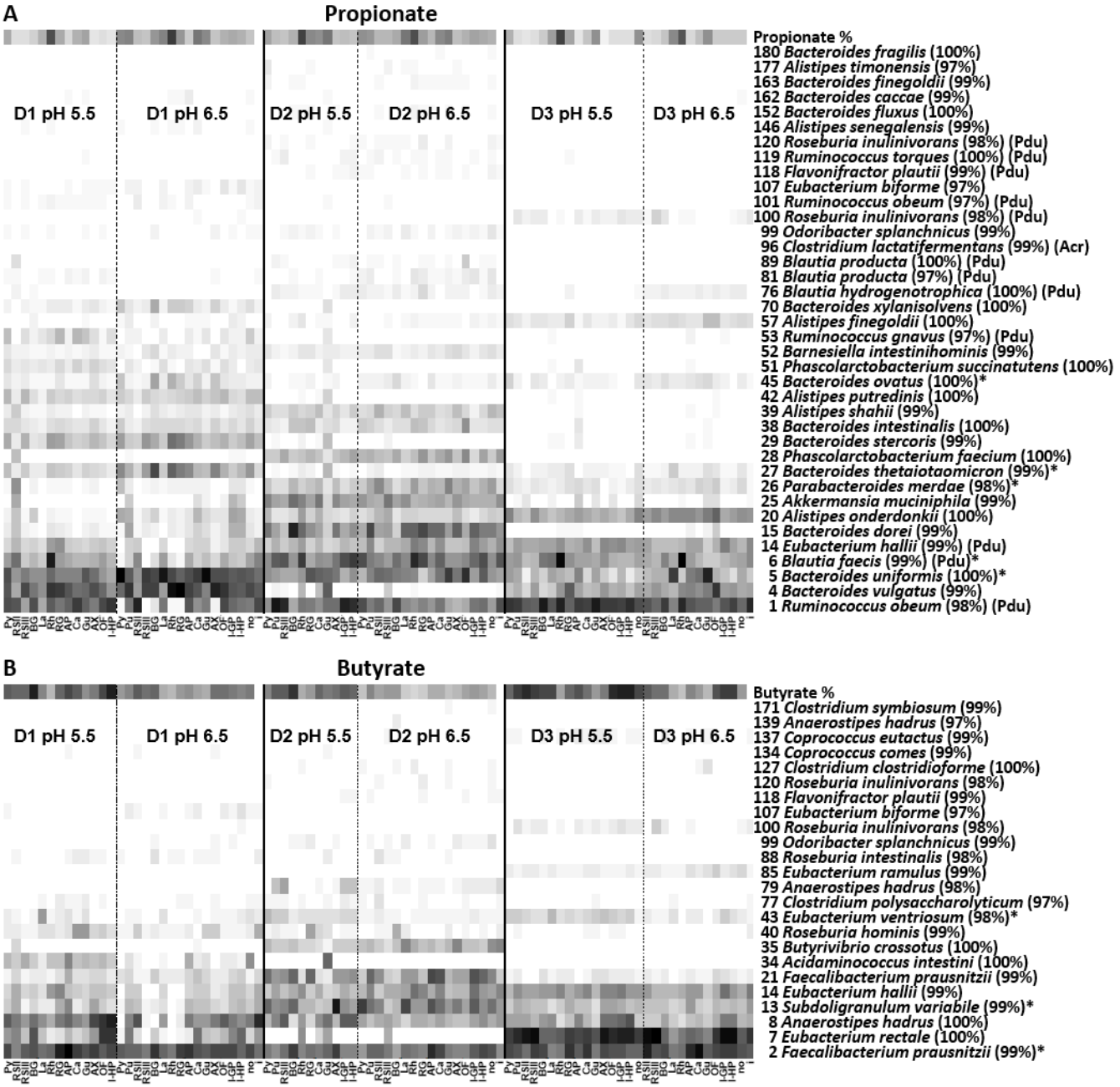
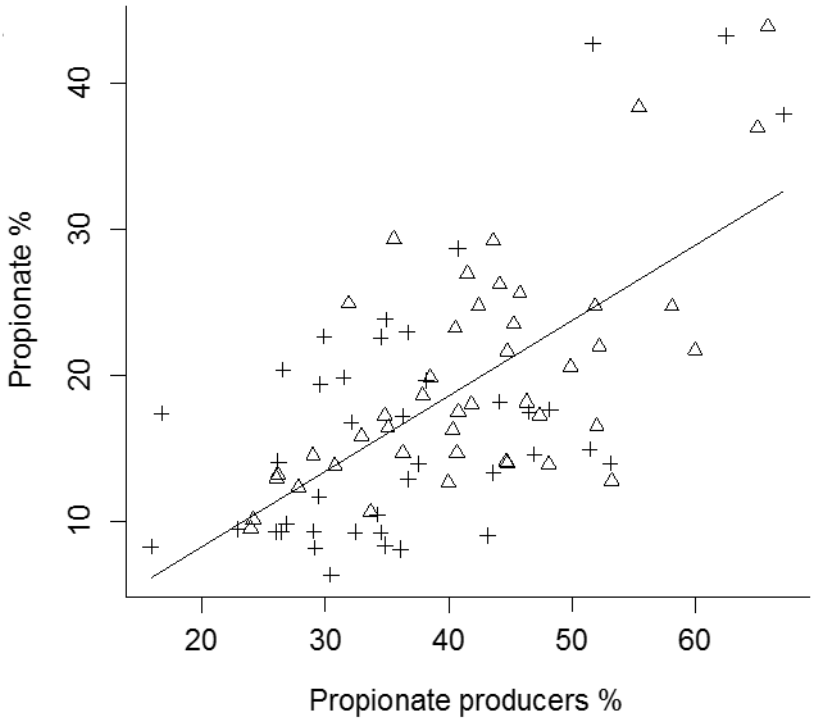
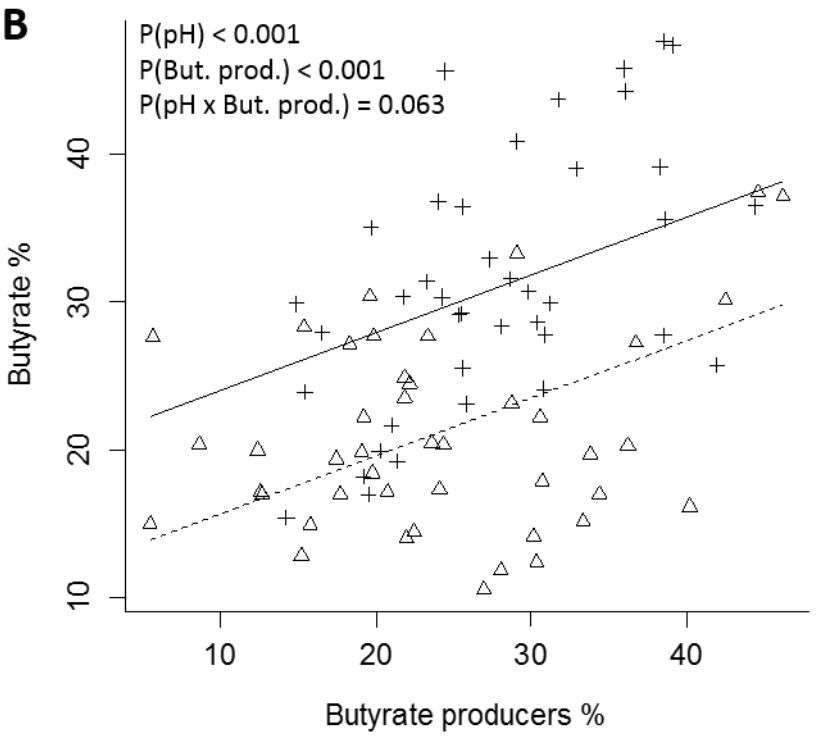
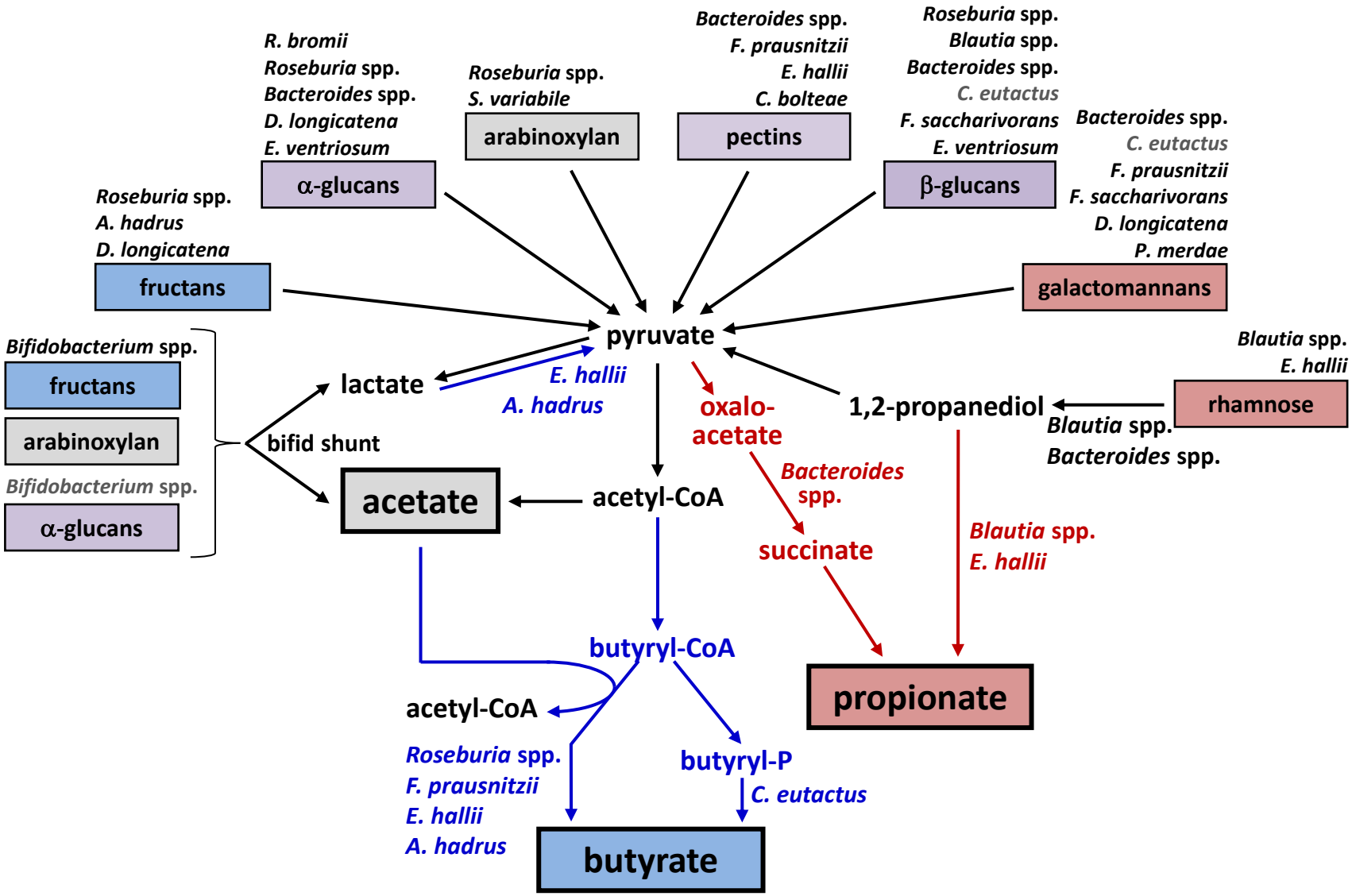

A
Propionate
D1 pH 5.5
D1 pH 6.5
D2 pH 5.5
D2 pH 6.5
D3 pH 5.5
D3 pH 6.5
Propionate %
180 Bacteroides fragilis (100%)
177 Alistipes timonensis (97%)
163 Bacteroides finegoldii (99%)
162 Bacteroides caccae (99%)
152 Bacteroides fluxus (100%)
146 Alistipes senegalensis (99%)
120 Roseburia inulinivorans (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