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Systematically-designed mixtures outperform single fibers for gut microbiota support

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ABSTRACT

Dietary fiber interventions to modulate the gut microbiota have largely relied on isolated fibers or specific fiber sources. We hypothesized that fibers systematically blended could promote more health-related bacterial groups. Initially, pooled *in vitro* fecal fermentations were used to design dietary fiber mixtures to support complementary microbial groups related to health. Then, microbial responses were compared for the designed mixtures versus their single fiber components *in vitro* using fecal samples from a separate cohort of 10 healthy adults. The designed fiber mixtures outperformed individual fibers in supporting bacterial taxa across donors resulting in superior alpha diversity and unexpected higher SCFA production. Moreover, unique shifts in community structure and specific taxa were observed for fiber mixtures that were not observed for single fibers, suggesting a synergistic effect when certain fibers are put together. Fiber mixture responses were remarkably more consistent than individual fibers across donors in promoting several taxa, especially butyrate producers from the *Clostridium* cluster XIVa. This is the first demonstration of synergistic fiber interactions for superior support of a diverse group of important beneficial microbes consistent across people, and unexpectedly high SCFA production. Overall, harnessing the synergistic potential of designed fiber mixtures represents a promising and more efficacious avenue for future prebiotic development.

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Introduction

The human gut microbiota is a complex ecosystem composed of trillions of microorganisms that inhabit and interact with each other and the host throughout the gastrointestinal tract.[1] This microbial community plays an important role in maintaining overall health and well-being by participating in a range of physiological processes, such as nutrient metabolism, immune modulation, and defense against pathogens.[1–3] The presence and quantity of different beneficial microbes, each with specific functions, are key to maintaining a balanced ecosystem within the gut.[4–6] In this sense, dietary fibers, as the main energy source for gut microbes, play a pivotal role in shaping the composition and function of the gut microbiota, ultimately influencing human health outcomes.[7,8] Notably, microbes have different fiber degradation abilities and preferences,[9–11] and even small differences in dietary fiber structures can lead to marked shifts in bacterial outcomes.[12,13]

Dietary fiber refers mainly to a diverse group of plant-derived carbohydrates that resist digestion in the upper gastrointestinal tract and reach the large intestine intact.[14] Although humans lack the enzymes necessary to break down fiber, it serves as a valuable energy source for gut microbes, which possess the enzymatic machinery to ferment most of these complex carbohydrates.[15] Through fermentation, gut microbes convert fiber into various metabolites, including short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, which are relevant due to their health-promoting effects locally and systemically.[16,17]

It is important to note that the structures of dietary fibers align with bacterial utilization capabilities. Dietary fibers differ in structural features,

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such as the degree of branching, glycosidic linkages, and solubility, which influence their fermentability by gut bacteria.[11] While previous studies have explored the impact of individual dietary fiber types on gut microbiota modulation and SCFA production, few studies have focused on the collective effects of fiber mixtures. Evidence mapping of 141 publications revealed that most studies examined single fiber types, with only 7% investigating fiber combinations.[18] We have previously reported that fiber mixtures may delay the fermentation rate in the large intestine,[19] in agreement with previous clinical data showing that a mixture of fibers typical of a normal diet promotes more prolonged short chain fatty acid production compared to individual fiber sources.[20] While some clinical benefits of fiber mixtures have been observed,[21–26] no studies have investigated how mixtures can be made at a mechanistic level (i.e., how specific outcomes can be achieved through fiber mixtures). It, thus, remains unclear how human gut microbiota outcomes compare between mechanistically designed mixtures and their individual components. In synthetic communities, we have demonstrated that bacterial taxa employ various prioritization strategies for fiber utilization when present in a mixture, allowing them to coexist even in highly competitive environments, such as the gut microbiota.[27] Moreover, given that individuals respond differently to different fiber types,[28,29] it is likely that offering a broad range of fibers, as opposed to a single type of fiber, would be a more effective strategy to elicit consistent responses across different individuals. Still, most dietary interventions and investigations on gut microbial communities have focused on isolated fibers or specific fiber sources,[8,30–33] overlooking the intricate interactions and possible synergistic effects that arise when multiple fiber types are consumed together. Thus, there is an important knowledge gap regarding how the gut microbiota responds to dietary fiber mixtures and the subsequent implications for SCFA production.

Previously, we designed a fiber mixture that promoted different microbial groups and showed localized and systemic benefits to subjects with Parkinson's disease related to improvements in the gut community structure.[21] Our hypothesis continues to be that systematically designed fiber mixtures provide a plethora of structures that can accommodate the metabolic requirements and preferences of a wide range of gut bacteria, thereby supporting a more balanced and diverse gut microbiota than any single fiber. We also hypothesized that fiber mixtures can render more consistent responses across people than single fibers. In this study, we aimed to elucidate the effects of systematically designed fiber mixtures compared to their single fiber components on human gut microbiota composition and SCFA production *in vitro*.

Materials and methods

Materials

Individual fibers were tentatively selected to promote bacterial groups previously related to health benefits such as *Bacteroides*, *Ruminococcus*, *Clostridium* cluster XIVa, and *Bifidobacteria*, as well as to support production of different SCFAs, with special emphasis on propionate and butyrate. In total, 16 different fibers were screened for their ability to promote different bacterial groups and metabolites related to health (Supplementary Table 1). Based on studies from our laboratory and others, *Bacteroides* and propionate were proposed to be promoted by arabinoxylan (AX), arabinoxylan-oligosaccharides (AXOS), pectic galactan (PG), wheat bran (WB), and arabinan (A)[34–37] (Supplementary Table 1). *Ruminococcus* is known for starch degradation, with several butyrate producers from the *Clostridium* cluster XIVa benefiting from cross-feeding in starch.[38–40] Thus, type II resistant starches from green banana (BRS) and high-amylose corn (CRS), type IV resistant starch from wheat (WRS), and a resistant α-glucan (isomaltodextrin) (RAG) were tested to support Ruminococcus + *Clostridium* cluster XIVa and butyrate production (Supplementary Table 1). Chitin-glucan (CG), beta-glucan (BG), and konjac glucomannan (KG) were also tested for support of *Clostridium* cluster XIVa and butyrate production[41,42] (Table 1). Pectin (PEC) was specifically tested for promotion of *Faecalibacterium prausnitzii*, which is also a member of the *Clostridium* cluster XIVa with special relevance to intestinal health.[43] Finally, galactooligosaccharides (GOS), xylooligosaccharides (XOS), and

fructooligosaccharides (FOS) were tested for bifidobacteria support.[44] All material sources are summarized in Supplementary Table 1.

First set of in vitro fecal fermentations (pooled fecal sample)

In vitro fermentations of the 16 dietary fibers (described above and in Supp. Table 1) and the blank (no fiber added) were performed in triplicate according to the methodology described by Cantu-Jungles et al.[45] using a pooled fecal inoculum from 3 healthy donors (2 males and 1 female; age between 24 and 40; average age = 33). Briefly, carbonate-phosphate buffer was prepared and sterilized by autoclaving at 121°C for 20 min. The buffer was then cooled to room temperature, oxygen was removed by bubbling with carbon dioxide, and cysteine hydrochloride (0.25 g/liter of buffer) was added as a reducing agent. The prepared buffer was then placed into the anaerobic chamber the day before experimentation to complete buffer reduction. On the day of the experiment, freshly collected fecal samples from the 3 donors were pooled (in equal amounts) and homogenized with carbonate-phosphate buffer (1:3 [wt/vol]), followed by filtration through four layers of cheesecloth. Then, 1 ml of this fecal inoculum was added to Balch tubes (Chemglass Life Sciences, Vineland, NJ) containing 50 mg of the dietary fiber substrate and 4 ml of the carbonate-phosphate buffer. Tubes were closed with butyl rubber stoppers (Chemglass Life Sciences), sealed with aluminum seals (Chemglass Life Sciences), and incubated at 37°C in a shaker incubator (150 rpm; MaxQ 6000; Thermo Fisher, Waltham, MA) for 24 h. Aliquots of the baseline sample and samples after 24-h fermentation were prepared and stored at − 80°C until further use for SCFA analysis (0.5 ml) and DNA sequencing (1 ml). All sample manipulation was conducted under an anaerobic atmosphere (85% N2, 5% CO2, and 10% H2). Human stool collection and use were approved by the Institutional Review Board at Purdue University (IRB protocol no. 1510016635).

SCFA analysis (pooled fecal sample)

Samples for SCFA analyses were prepared as previously described (10) and analyzed using a gas chromatograph (GC-FID 7890 A; Agilent Technologies Inc.) on a fused silica capillary column (Nukon Supelco no. 40369-03A; Bellefonte, PA) under the following conditions: injector temperature at 230°C, initial oven temperature at 100°C, and temperature increase of 8°C/min to 200°C with a hold for 3 min at final temperature. Helium was used as a carrier gas at 0.75 mL/min. Quantification was performed based on relative peak areas using external standards of acetate (A38S), propionate (A258), and butyrate (AC108111000) and an internal standard of 4-methylvaleric acid (AAA1540506) from Fisher Scientific (Hampton, NH).

DNA extraction and 16S rRNA gene amplicon sequencing (pooled fecal sample)

Stored samples for DNA extraction were thawed and centrifuged (13,000 rpm for 15 min), and supernatants were discarded. Automated DNA extraction of the precipitates was performed using the QIAcube Connect instrument (Qiagen, Germantown, MD) with the QIAamp PowerFecal Pro DNA kit (Qiagen) per manufacturer's instructions. The V4 region of the 16S rRNA gene was amplified using primers 515F (5′-GTGCCAGCMGCCGCGGTAA) and 806 R (5′-GGACTACHVHHHTWTCTAAT). The primers contained 5′ common sequence tags (known as common sequence 1 and 2 [CS1 and CS2]). First-stage PCR amplifications were performed in 10-µl reaction mixtures in 96-well plates, using MyTaq HS 2× master mix (Bioline, Memphis, TN). PCR conditions were 95°C for 5 min, followed by 28 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 60 s. Amplicons were generated using a two-stage PCR amplification protocol as described previously (37). The primers contained 5′ common sequence tags (known as common sequence 1 and 2 [CS1 and CS2]) as described previously (38). Subsequently, a second PCR amplification was performed in 10-µl reaction mixtures in 96-well plates. A master mix for the entire plate was made using MyTaq HS 2× master mix. Each well received a separate primer pair with a unique 10-base barcode, obtained from the Access Array Barcode Library for Illumina (Fluidigm, South San Francisco, CA; catalog no. 100-–4876). These Access Array primers contained the CS1 and CS2 linkers at the 3′ ends of the

oligonucleotides. Cycling conditions were 95°C for 5 min, followed by 8 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Samples were then pooled in equal volume using an EpMotion5075 liquid handling robot (Eppendorf, Hamburg, Germany). The pooled library was purified using an AMPure XP cleanup protocol (0.6×, vol/vol; Agencourt, Beckman-Coulter, Indianapolis, IN) to remove fragments smaller than 300 bp. The pooled libraries, with a 20% phiX spike-in, were loaded onto an Illumina MiniSeq midoutput flow cell (2 × 153 paired-end reads). Based on the distribution of reads per barcode, the amplicons (before purification) were repooled to generate a more balanced distribution of reads. The repooled library was purified using AMPure XP cleanup, as described above. The repooled libraries, with a 20% phiX spike-in, were loaded onto a MiniSeq flow cell and sequenced (2 × 153 paired-end reads). Fluidigm sequencing primers, targeting the CS1 and CS2 linker regions, were used to initiate sequencing. Demultiplexing of reads was performed on instrument. Library preparation, pooling, and sequencing were performed at the University of Illinois at Chicago Genome Research Core (GRC) within the Research Resources Center (RRC).

Second set of in vitro fecal fermentations (individual fecal samples)

Fecal material for the experiment was collected from 10 donors with good intestinal health (5 males and 5 females; age between 17 and 64; average age = 46). The fecal samples were collected only once and anonymously and are therefore considered not subjected to the WMO in The Netherlands. To collect the sample, the subjects used the FecesCatcher, a specimen collection device consisting of biodegradable paper to be placed under the toilet seat (fecesvanger.nl). Fecal material was collected with a sterile plastic spoon and placed in a tube that was, in turn, placed in an anaerobic jar (materials provided by TNO) with an AnaeroGen Sachet (Thermo Fisher Diagnostics GMBH). The jar was kept at 4°C until delivery at the laboratory (within 24 h from collection). There, the material was introduced in an anaerobic chamber, diluted 1:3 with phosphate-buffered saline, and homogenized. Finally, 20% glycerol was added before storing the material at − 80°C. Individual fermentations were performed with fecal material incubated anaerobically in the i-screen (intestinal screening) system.[46] First, the fecal samples were precultured overnight in modified standard ileal efflux medium (SIEM) in anaerobic conditions, at 37°C, and with shaking at 300 rpm.[47] The microbiota was then transferred to microtiter plates, and both individual fibers and fiber mixtures were added at a concentration of 4 mg/mL (fiber mixtures were composed of five components in equal proportions, 20% of each fiber). The i-screen incubation started with a fecal bacterial load of approximately 10^9 CFU/mL. The microbiota was cultured in SIEM with pH adjusted to 5.8. All compounds were tested in triplicate. After 24 h of anaerobic fermentation, the incubation material was sampled for DNA isolation and metabolite analysis.

SCFA analysis (individual fecal samples)

Supernatant samples were diluted 20× with 75% methanol. 50 µL of internal standard solution (d3-acetic acid, d3-propionic acid, d3-butyric acid, and d9-valeric acid) were added to 50 µL of diluted fecal material. This was followed by 50 µL of 50 mm 3-Nitrophenylhydrazine solution (75% methanol in water), 50 µL of 50 mm 1-Ethyl-3-(3-dimethylamino-propyl) carbodiimide solution (75% methanol in water), and 50 µL of pyridine (7.5% in 75% methanol). Samples were incubated for 30 min at 600 rpm at room temperature. Then 250 µL of 2% formic acid was added and mixed. The samples were stored at −80°C until analysis. The derivatized SCFA were analyzed by LC-MS using a high-resolution mass spectrometer (Q-Exactive, Thermo, USA) equipped with an electrospray source (HESI). The mass spectrometer was operated in positive ion mode at a resolution of 17,500. Data was acquired by scanning from m/z 100 to 700. Separation of the derivatized SCFA was done with an Acquity H-Class UPLC system (Waters) fitted with an Acquity BEH-C18 column (Waters, 150 × 2.1 mm, 1.7 µm). Mobile phase A was 0.1% formic acid in water, and mobile phase B was 100% acetonitrile. The gradient used was 16% B (0 min), 25% B (min), 40% B (9 min), followed by column wash-out at 95%B and equilibration at 16% B, at

a flow rate of 0.35 mL/min and a column temperature of 40°C. The injection volume was 2.0 µL.

SCFA concentrations were obtained by the analysis of calibration standards in 75% methanol in water (in total 7 concentrations for each SCFA). Concentration ranges were 0 to 100 µM (acetic acid) and 0 to 50 µM (propionic and butyric acid). The calibration standards were 100× diluted prior to adding internal standard solution and derivatization.

DNA extraction and 16S rRNA gene amplicon sequencing (individual fecal samples)

Automated DNA extraction of the precipitates was performed using the QIAcube Connect instrument (Qiagen, Germantown, MD) with the Dneasy 96 Powersoil Pro QIAcube HT kit (Qiagen) per manufacturer's instructions. Changes in the microbiota composition were analyzed by using 16S rDNA amplicon sequencing. The V4 hypervariable region was targeted. A total of 100 pg of DNA was amplified as described by Kozich et al.,[48] with the exception that 30 cycles were used instead of 35, applying F515/R806 primers.[49] Primers included Illumina adapters and a unique 8 nt sample index sequence key.[48] The amplicon libraries were pooled in equimolar amounts and purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Amplicon quality and size were analyzed on a Fragment Analyzer (Advanced Analytical Technologies, Inc., Heidelberg, Germany). Paired-end sequencing of amplicons (approximately 400 base pairs) was conducted on the Illumina MiSeq platform (Illumina, Eindhoven, The Netherlands).

Bioinformatics

Samples from both the first set (pooled fecal fermentations) and the second set (individual fecal fermentations) of *in vitro* fecal fermentations were processed in a similar manner initially. Sequence reads were supplied as paired-end FASTQ sequence files and imported into QIIME 2 (q2) version 2--2021.11 for analysis.[50] Amplicon sequence variants (ASVs) were generated using DADA2[51] with sequences trimmed at 153 bp, and taxonomic assignment was carried out using the q2-feature-classifier plugin against the Silva reference database classifier with 99% similarity, specific for the V4 16S region (v. 138). Sequence alignment and construction of a phylogeny tree were obtained using the Qiime2 pipeline align-to-tree-mafft-fasttree. Non-rarefied ASVs were collapsed at the species level and relative abundances used for downstream analysis and visualization. Comparative analysis of taxa responses to the different fibers between the first and second sets of *in vitro* fecal fermentations was performed at the genus and species levels using hierarchical clustering with the Ward algorithm. The results were visualized through a heatmap of z-scores representing relative abundances. Heatmaps were created using R Stats software version 3.6.3 (R Core Team, Vienna, Austria) and SCFA data were visualized using GraphPad Prism (v. 9.5.1).

Additional analyses were performed for the second set of *in vitro* fecal fermentations to evaluate differences in community structure of single fibers *versus* the designed fiber mixtures. To minimize the effects of sequencing depth on diversity measurements, the number of reads from each sample was rarefied to 7,600, and alpha and beta diversities were calculated using the q2-diversity plugin, which included Shannon Index for alpha diversity and weighted UniFrac for beta diversity. Statistical differences in alpha diversity were calculated using q2-alpha-group-significance plugin. Detection of taxa significantly different from the blank at all taxonomic levels was obtained using the Wilcoxon Rank Sum test corrected for multiple comparisons and visualized through differential phylogenetic trees using METACODE. Differentially abundant genera of each fiber substrate versus the blank were also evaluated using a stricter linear mixed-effects model implemented in ANCOM-II (Analysis of Composition of Microbiomes-II, R-code https://github.com/FrederickHuangLin/ANCOM), accounting for the donor as the random effect and adjusting for time (before versus after substrate fermentation). Each fiber treatment was individually compared to the control group (blank) in ANCOM-II, and a genus was determined to be significant if it surpassed the 0.6 threshold for statistical significance. We have also used the variancePartition method to quantify the variation in gut microbial taxa attributable to donor and fiber type, when fibers were used individually or as a mixture.[52] PCoA plots, differential phylogenetic trees, and violin plots of genera differentially abundant detected through ANCOM in

response to fiber treatments, and from variancePartition analysis, were created using R Stats software version 3.6.3 (R Core Team, Vienna, Austria)

Results

Fiber mixture design

From collected know-how,[11] a range of 16 dietary fibers was selected for their ability to promote different groups of microbes related to health, namely *Bacteroides*, *Ruminococcus* (from both Clostridium Clusters IV and XIVa), other Clostridium cluster XIVa members, *Faecalibacterium prausnitzii* (Clostridium cluster IV), and *Bifidobacteria*. After 24 h of *in vitro* fecal fermentation using a pooled fecal sample from an American cohort of healthy adults, most abundant community members were clustered using Euclidean distances (Figure 1a). Four main bacterial clusters were identified, representing groups of bacterial species that exhibit similar patterns of abundance that are dependent on the type of substrate present (labeled and colored from 1 to 4 on the left side of the heatmap, Figure 1a). RAG, CG, BB, and KG were most promotive of Cluster 1 (in red), resistant starches BRS, CRS and WRS were most promotive of Cluster 2 (in green), oligosaccharides GOS, XOS and FOS were most promotive of Cluster 3 (in purple), and AX, AXOS, PG, WB, A and PEC were most promotive of Cluster 4 (in blue) (Figure 1a). Moreover, specific short chain fatty acid profiles were related to the fiber type, with several fibers promotive of Cluster 1 being also related to propionate production, and fibers related to the promotion of Cluster 2 related to butyrate production (Figure 1b).

Thus, individual fibers were selected to make mixtures different in composition that would promote all four complementary bacterial clusters as well as different SCFAs in an attempt to support community diversity. Fiber mixture 1 was composed of AX, PEC, CRS, KG and FOS in equal amounts (Supp. Figure S1). Fiber mixture 2 was composed of AXOS, BRS, CG, KG, and GOS in equal amounts (supplementary Figure S1). Finally, fiber mixture 3 was composed of equal amounts of fiber mixtures 1 and 2.

Designed mixtures better support microbial diversity and SCFA production than single fibers

In the second set of experiments, *in vitro* fecal fermentations were conducted with each fiber mixture (where each of the 5 component fibers represents 20% of the mixture) and the individual fibers comprising each mixture separately. This was performed using fecal inocula from 10 distinct donors from a cohort from healthy adults from The Netherlands. Promotion of the clusters (defined above from the pooled fecal fermentation set of experiments) was then evaluated in each donor separately, and a high interindividual response variability was observed (Figure 2a-c). Also, no *Butyrivibrio* or *Catenibacterium* taxa were observed (Figure 2a,b) due to differences in microbiota composition from the two experimental sets. Fiber responses were not always consistent with the first experimental set as noted for several taxa like *Ruminococcus* torques group (not promoted by any of the fibers tested), *Subdoligranulum* (better supported by PEC than FOS in mixture 1), and *Bacteroides* (better supported by KG than AX in mixture 1), among others. These observations, in alignment with prior reports, indicate that fiber responses can vary across donors[29,53,54] and even more so between donors from different regions of the world, which will likely have different gut microbial communities,[55] as was the case in this study. Still, several fiber responses observed were congruent with expectations based on the initial set of experiments. For instance, *Parabacteroides*, *Lachnospiraceae*, and *Roseburia* in cluster 1 (outlined in red) were well promoted by KG, a component of both mixtures (Figure 2a,b). In cluster 2 (in green), CRS most effectively promoted *Ruminococcaceae* (CAG-352) in mixture 1, and BRS was the most effective promoter of *Agathobacter* in mixture 2 (Figure 2a,b). For cluster 3 (in pink), FOS and GOS from mixture 1 and 2, respectively, were the most effective at promoting *Anaerostipes* and *Bifidobacterium* (Figure 2a,b). In cluster 4 (in blue), PEC most effectively promoted *Faecalibacterium* in mixture 1, and AXOS was the most effective promoter of *Bacteroides* in mixture 2 (Figure 2a,b)

In both experimental sets, no single individual fiber was particularly effective at supporting all

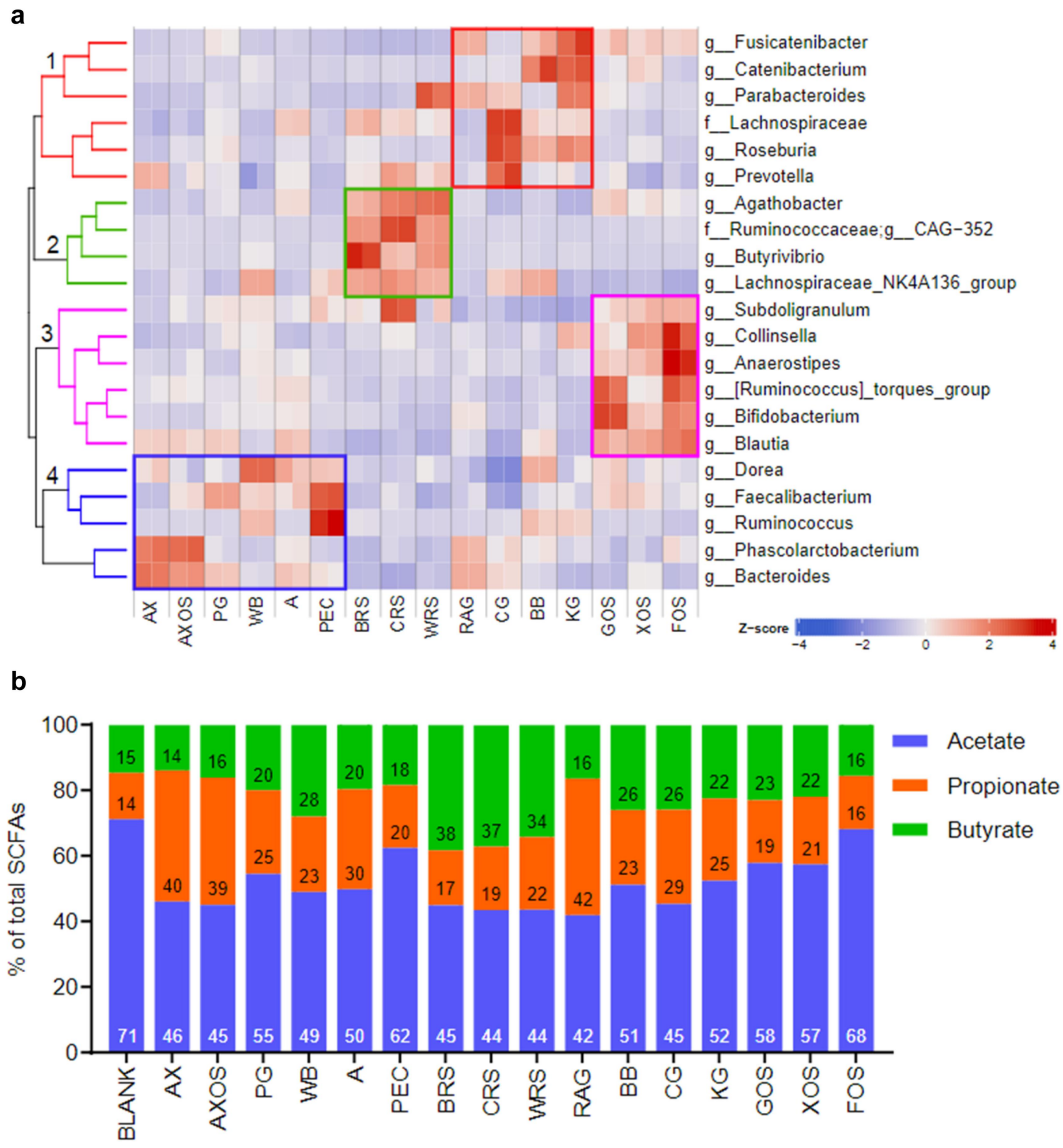

Figure 1. Different fibers promote specific bacterial groups and SCFA production in *in vitro* fecal fermentations using pooled fecal samples from 3 donors. a) heatmap of relative abundances (presented as Z-scores) of most abundant taxa after fiber fermentations. Hierarchical clustering of taxa was performed using Euclidean distances, and 4 main clusters of taxa (colored in red, green, purple and blue) were observed and associated with fiber types. b) proportions of produced butyrate, propionate and acetate relative to the total of SCFA produced. Results are presented for no added fibers (blank), arabinoxylan (AX), arabinoxylan-oligosaccharides (AXOS), pectic galactan (PG), wheat bran (WB), arabinan (A), green banana resistant starch (BRS), high-amylose corn resistant starch (CRS), wheat resistant starch (WRS), resistant α-glucan from enzymatic modification of isomaltodextrin (RAG), Chitin-glucan (CG), beta-glucan (BG), konjac glucomannan (KG) pectin (PEC), galactooligosaccharides (GOS), xylooligosaccharides (FOS) and fructooligosaccharides (FOS).

clusters of taxa (Figures 1a and 2a,b). However, when mixtures of the single fibers were composed, a broader support of different taxa was achieved (Figure 2a,b). Beyond that, for some taxa, like *Fusicatenibacter*, *Agathobacter*, *Roseburia*, *Blautia* and *Dorea*, the mixtures performed better than any of the single fibers tested (Figure 2a,b). Thus, the mixture of fibers targeted toward different bacterial taxa was overall better for broader community support. When comparing the three mixtures (Figure 2c), mixture 1 seemed to offer support to the broadest range of taxa. Importantly, blending mixtures 1 and 2 to produce mixture 3 did not provide additional support for new microbes, but rather an intermediate effect to those observed for fiber mixtures 1 and 2 was achieved (Figure 2c).

These findings were further substantiated by alpha diversity analysis, as measured by the

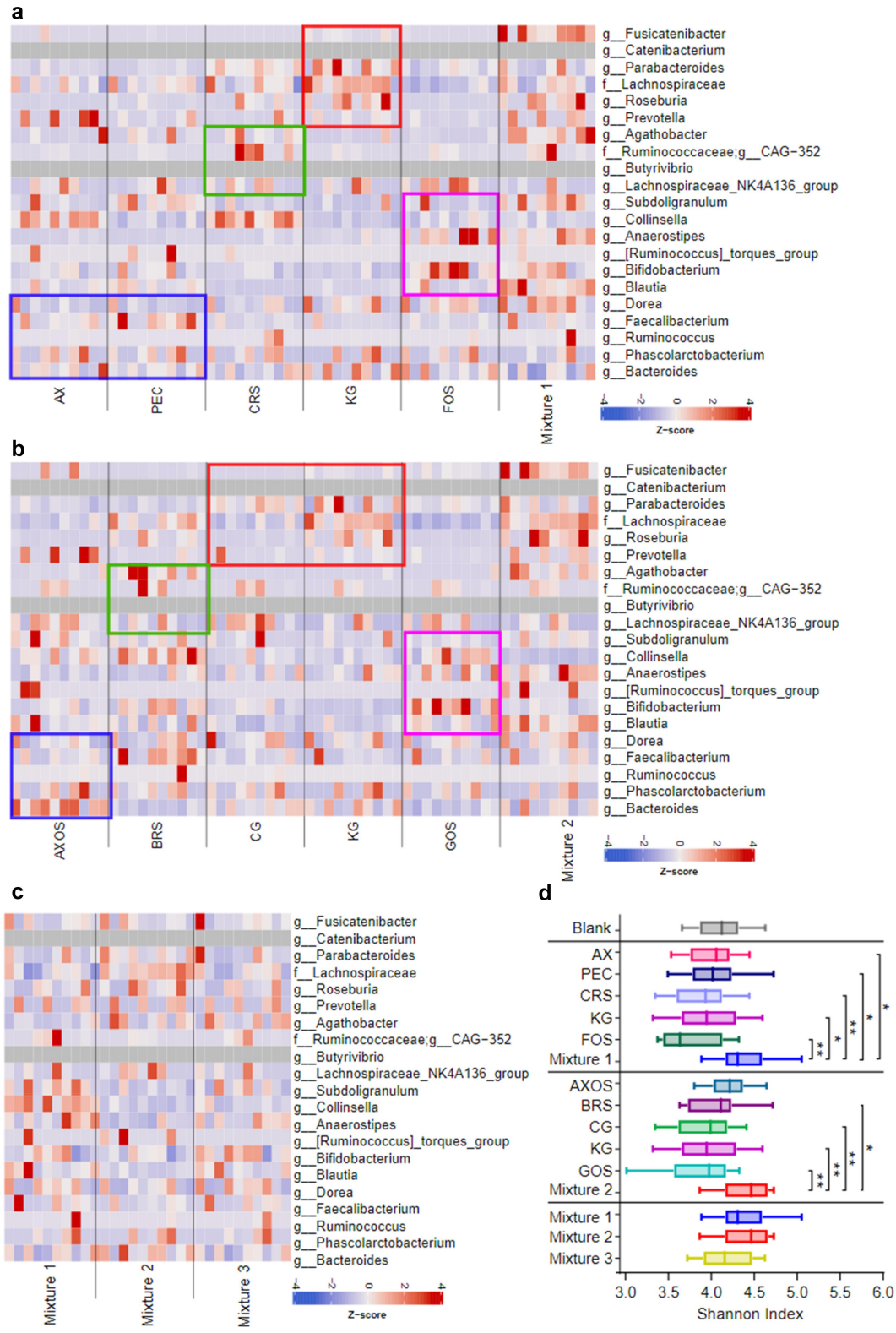

Figure 2. Fiber mixtures promote more bacterial taxa and higher diversity in *in vitro* fecal fermentations using fecal samples from 10 donors. Heatmaps of relative abundances (presented as Z-scores) of most abundant taxa after fiber fermentations are shown in a) for mixture 1 versus its individual fiber components b) for mixture 2 versus its individual fiber components, and c) for mixtures 1, 2 and 3. Colored boxes (in blue, green, red and purple) indicate taxa expected to be promoted by individual fibers based on the initial pooled *in vitro* fecal fermentation experiment. d) alpha diversity plots of fermented samples as measured by Shannon index. The asterisks denote significance (*, $p < 0.05$; **, $p < 0.01$). Results are presented for no added fibers (blank), arabinoxylan (AX), pectin (PEC), high-amylose corn resistant starch (CRS), konjac glucomannan (KG), fructooligosaccharides (FOS), arabinoxylan-oligosaccharides (AXOS), green banana resistant starch (BRS), chitin-glucan (CG), and galactooligosaccharides (GOS).

Shannon index, of single fibers versus their corresponding mixtures (Figure 2d). Mixture 1 outperformed all of its individual fiber components in supporting alpha diversity, and mixture 2 was superior to most of its single fiber components (with the exception of AXOS) in supporting alpha diversity (Figure 2d). Additionally, combining mixtures 1 and 2 (both of which were designed for the support of similar clusters of taxa) into mixture 3 did not further enhance alpha diversity (Figure 2d). No significant differences were detected among the three mixtures tested (Figure 2d). Hence, the composition of mixtures with fibers of overlapping bacterial targets does not confer additional benefits to diversity and highlights the importance of mixtures composed of different target-specific single fibers.

Regarding SCFA production, mixtures 1, 2, and 3 were among the most promotive of acetate, butyrate, and propionate production, and higher than some of their single fiber components (Figure 3a-c). Also, the three mixtures performed similarly in increasing the production of the three SCFAs (Figure 3a-c). Accordingly, the production of total SCFAs was more pronounced for the mixtures than for several of their individual fiber components (Figure 3d). Forty percent of the single fibers composing mixture 1 and 80% of the single fibers composing mixture 2 had a lower total SCFA production than the mixtures. SCFA production of the mixtures was not merely a summation of SCFA production of each single fiber component, but was significantly higher in amount, suggesting a synergistic effect when fibers are combined together in mixtures, which is more advantageous for SCFA production.

Overall, systematically designed mixtures that support complementary groups of bacteria were better than single fibers at supporting a diverse range of gut microbes as well as the production of health-related metabolites, namely butyrate, propionate, and acetate.

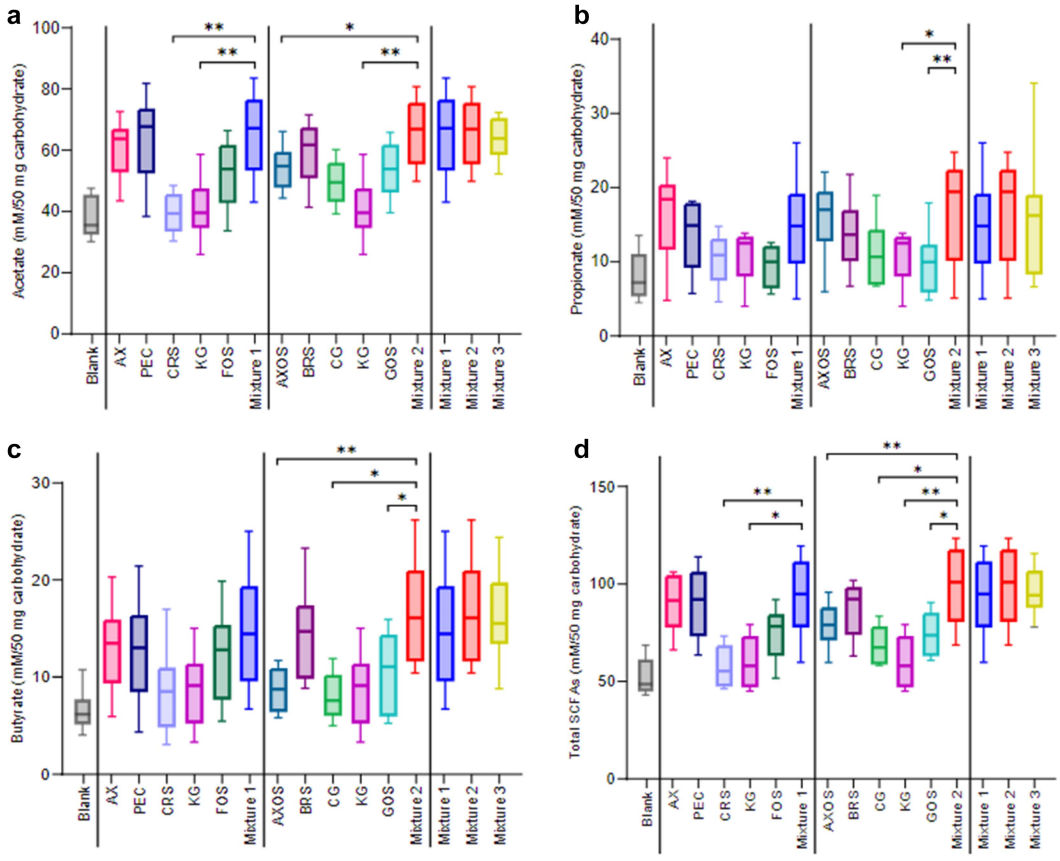

Figure 3. Fiber mixtures promote higher SCFA production in *in vitro* fecal fermentations using fecal samples from 10 donors. Short chain fatty acid (SCFA) production in response to fiber mixtures and their individual components are shown for a) acetate, b) propionate, c) butyrate and d) total SCFA production. Values are presented as mM/50 mg of carbohydrate. The asterisks denote significance (*, $p < 0.05$; **, $p < 0.01$). Results are presented for no added fibers (blank), arabinoxylan (AX), pectin (PEC), high-amylose corn resistant starch (CRS), konjac glucomannan (KG), fructooligosaccharides (FOS), arabinoxylan-oligosaccharides (AXOS), green banana resistant starch (BRS), chitin-glucan (CG), and galactooligosaccharides (GOS).

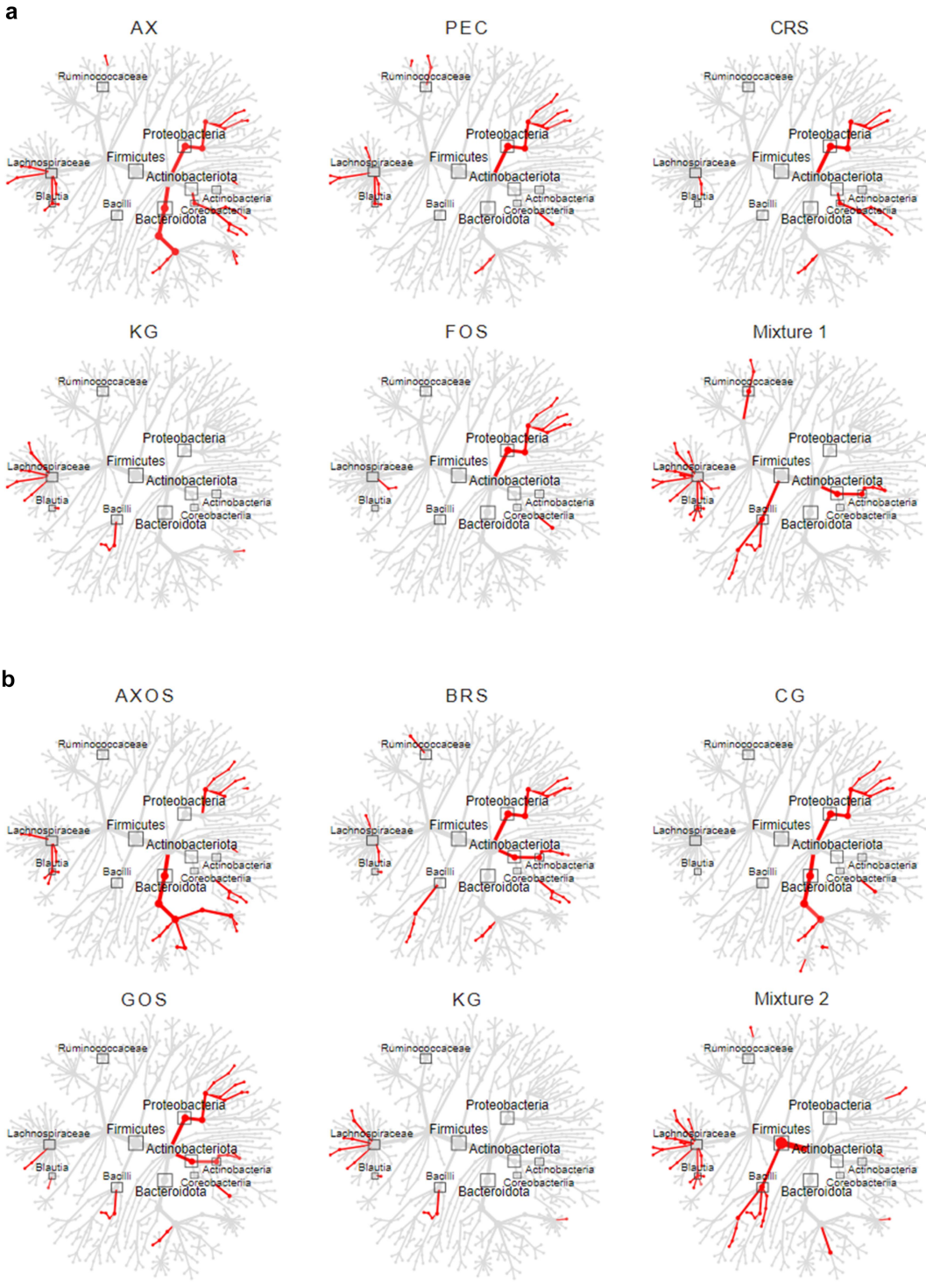

Figure 4. Combining fibers in mixtures leads to the promotion of unique bacterial taxa not promoted individual fibers. Differential phylogenetic trees depicting significant differences in taxon abundance between the blank and fiber-fermented samples for a) mixture 1 and its individual components, and b) mixture 2 and its individual components. Red colored nodes indicate a significantly higher ($p \leq 0.05$ after "fdr" correction) proportion of a taxon in fiber treated samples. Node sizes are proportional to the number of ASVs within each taxon. Names of taxon discussed in the text were added and its representative nodes marked with gray squares. Names of non-discussed taxon are depicted in supplementary figure 3. Results presented refer to the second *in vitro* fecal fermentation experiments using individual fecal samples from 10 people. Arabinoxylan (AX), pectin (PEC), high-amylose corn resistant starch (CRS), konjac glucomannan (KG), fructooligosaccharides (FOS), arabinoxylan-oligosaccharides (AXOS), green banana resistant starch (BRS), chitin-glucan (CG), and galactooligosaccharides (GOS).

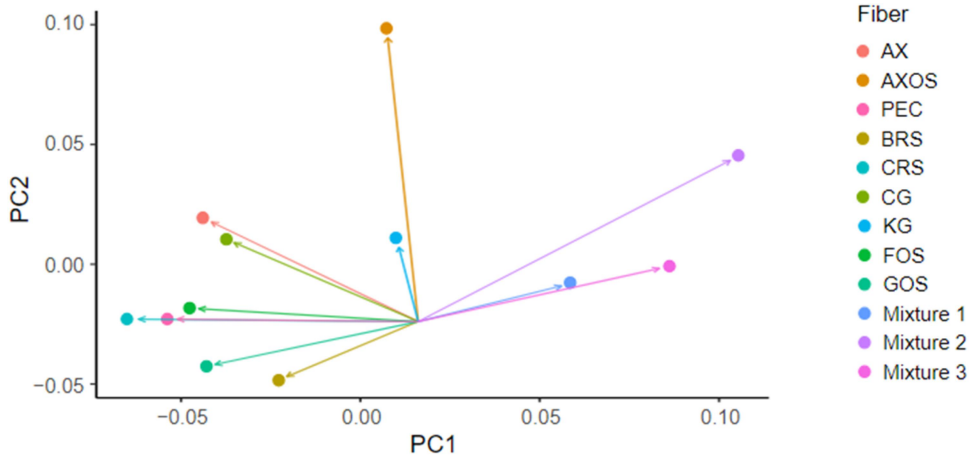

Figure 5. Fiber mixtures lead to different shifts in community structure if compared to its individual fiber components. PCoA plot of weighted UniFrac measure of beta diversity. Results presented refer to the second *in vitro* fecal fermentation experiments using individual fecal samples from 10 people. Arabinoxylan (AX), pectin (PEC), high-amylose corn resistant starch (CRS), konjac glucomannan (KG), fructooligosaccharides (FOS), arabinoxylan-oligosaccharides (AXOS), green banana resistant starch (BRS), chitin-glucan (CG), and galactooligosaccharides (GOS).

Synergistic effect of fibers in a mixture for unique promotion of bacterial taxa

Differential phylogenetic trees were used for the visualization of significant changes in microbial taxa abundance at multiple taxonomic levels for each fiber type compared to the blank, using data from the second set of *in vitro* fecal fermentations. In agreement with heatmaps and alpha diversity results discussed above, mixtures 1 and 2 promoted more bacterial taxa (represented by colored nodes in the heat tree) than any of their single fiber components (Figure 4a,b). This is clearly observed for several taxa, including those belonging to the *Lachnospiraceae* family (at the left side of the heat tree). *Lachnospiraceae* is an important family of bacteria related to health, which includes a variety of butyrate producers. Mixtures 1 and 2 promoted, respectively, 13 and 12 different tree tip nodes (representing different species) of *Lachnospiraceae*, while their summed single fiber components ranged from promoting only 1 in mixture 1 (FOS and CRS) and 0 in mixture 2 (CG), to a maximum of 5 (KG) *Lachnospiraceae* nodes in both mixtures. Surprisingly, not only were more taxa promoted by the mixtures, but several new taxa were found which were not promoted by their individual component fibers fermented separately. For instance, some *Blautia spp*. were uniquely promoted by the mixtures, but not by their individual fibers. Also, at the class level, Bacilli were only promoted by the mixtures, and not by their individual fibers. At the phylum level, Actinobacteria was significantly promoted in mixture 1 (and phylogenetically at the genus level, *Bifidobacterium* was also significantly promoted), but not by any of mixture 1's single fiber components. Similarly, the Firmicutes phylum was only promoted by mixture 2, but not by any of its single fiber components, compared to the blank. Interestingly, Proteobacteria, a phylum known to have several pathogens and usually related to a proinflammatory phenotype, presented increases in relative abundances when several of the single fibers were tested in comparison to the blank, but this was not observed when fibers were blended together. Mixture 3 had a similar, and perhaps intermediate effect, to fibers 1 and 2 (Supp. Figure S2), corroborating results previously presented.

To understand how the different fibers tested changed the overall community structure, shifts in beta diversity before and after fermentation were evaluated using weighted UniFrac samples. Interestingly, the direction of changes in PCoA plots, averaged by donor, were markedly different for single fibers compared to those obtained from fiber mixtures (Figure 5). While all single fibers trended in a direction toward the left side of the

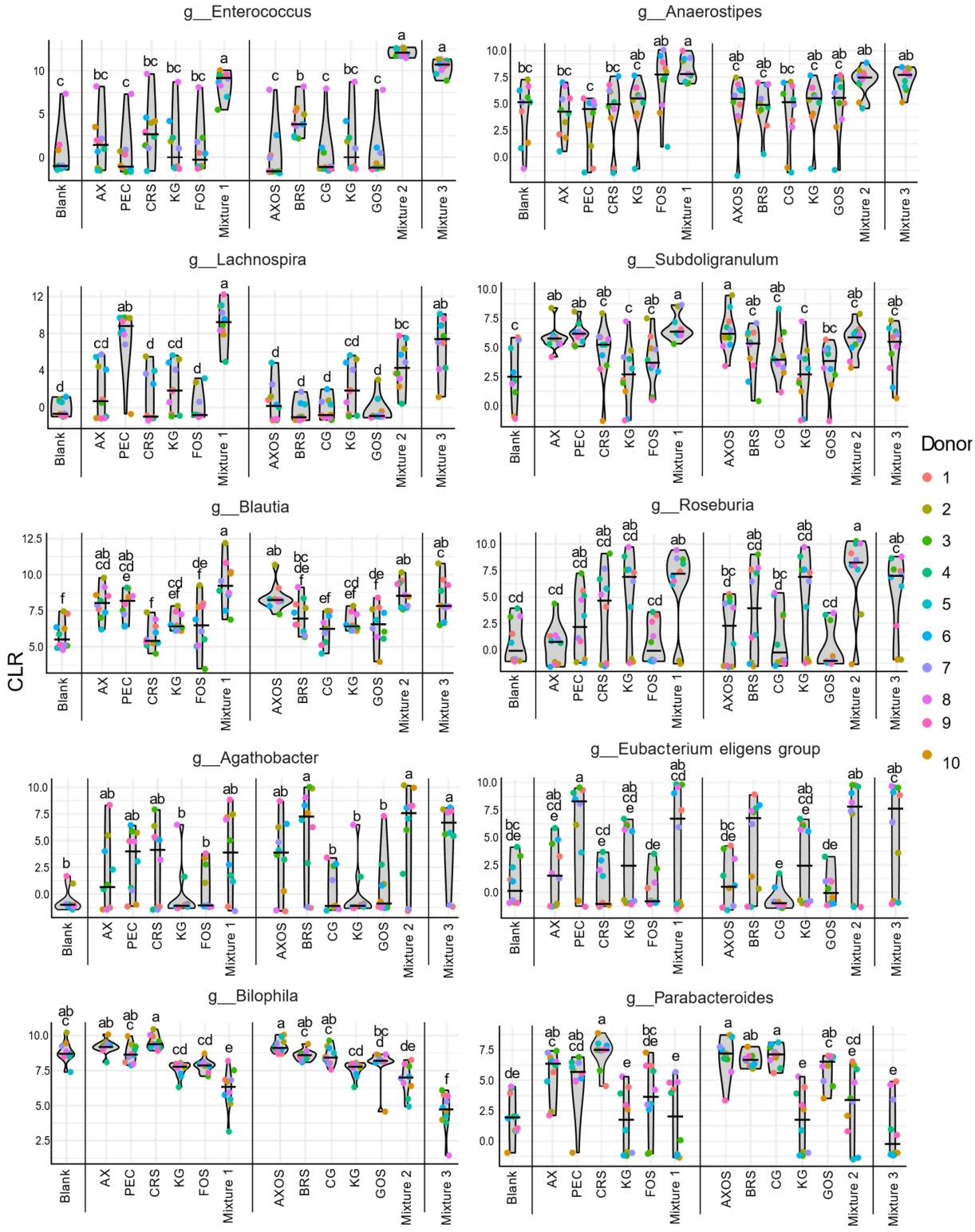

Figure 6. Mixtures improve fiber response similarity across donors for several taxa. Violin plots displaying the centered-log-ratio transformed relative abundances of genera identified as differentially abundant by ANCOM II across various fiber types. Results presented refer to the second *in vitro* fecal fermentation experiments using individual fecal samples from 10 people. The treatments are as follow: no added fibers (blank), arabinoxylan (AX), pectin (PEC), high-amylose corn resistant starch (CRS), konjac glucomannan (KG), fructooligosaccharides (FOS), arabinoxylan-oligosaccharides (AXOS), green banana resistant starch (BRS), chitin-glucan (CG), and galactooligosaccharides (GOS). Pairwise comparisons were made on ANCOM ii-detected taxa using TUKEY HSD post-hoc test. Different letters indicate significant differences at $p < 0.05$.

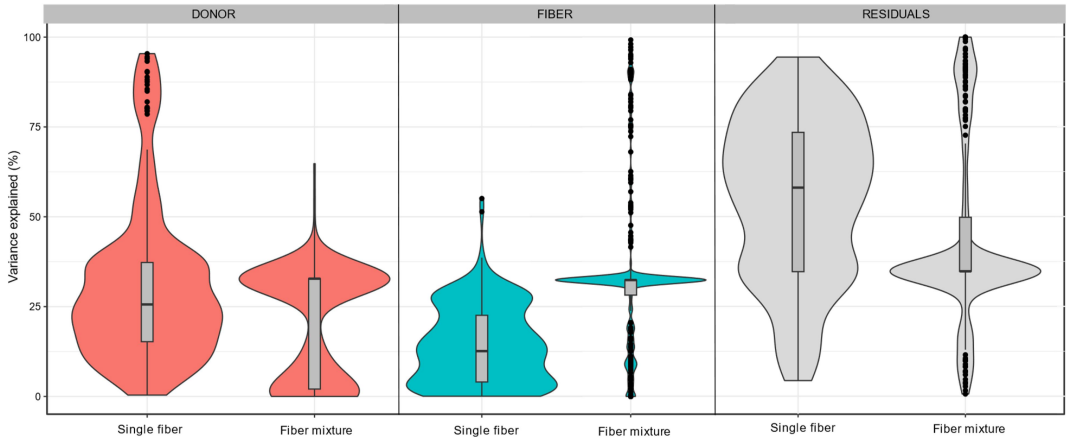

Figure 7. Violin plots showing the percent of variance of bacterial taxa explained after partitioning variance by fiber types and donors across samples. Single fiber group was designated for fermentations with single fibers (arabinoxylan, pectin, high-amylose corn resistant starch, konjac glucomannan, fructooligosaccharides, arabinoxylan-oligosaccharides, green banana resistant starch, chitin-glucan, and galactooligosaccharides) and fiber mixture group was designated for fermentations with the fiber mixtures (mixture 1, 2 and 3).

PCoA plot, all three fiber mixtures shifted toward the right side of the graph. Taken together, these results show that changes in community structure promoted by fiber mixtures are not merely a summation or average of the changes promoted by the individual fibers of which they are composed. Rather, a synergistic effect is observed when blending fibers together into mixtures, which culminates in unique changes in specific taxa as well as in the overall community structure that are not observed in the mixture components separately.

Mixtures are more consistent in changing the gut microbial community

The specific genera enhanced by single fibers and fiber mixtures, as compared to the blank, were identified using ANCOM II. Ten bacterial genera were identified as being promoted by one or more types of fiber. These included *Enterococcus* from the Bacilli phylum, promoted by all three mixtures, and several members from the *Lachnospiraceae* family, such as *Anaerostipes* (promoted by FOS and mixture 1), *Lachnospira* (promoted by mixtures 1 and 3), *Subdoligranulum* (promoted by mixture 1), *Blautia* (promoted by mixtures 1 and 3), *Roseburia* (promoted by mixture 1), *Agathobacter* (promoted by mixture 3), and the *Eubacterium eligens* group (promoted by mixture 2). *Parabacteroides* were supported by BRS and CG (Supp. Table 2).

Centered log ratios of genera detected by ANCOM were plotted for all fiber types in violin plots to evaluate the distribution of donors' responses (Figure 6). Notably, genera such as *Enterococcus*, *Anaerostipes*, and *Lachnospira* were promoted to some extent in all individuals by the three fiber mixtures. While some single fibers were capable of promoting these genera, no single fiber was effective in all donors (except for BRS in promoting *Enterococcus*). The fiber mixtures also generally promoted *Subdoligranulum* and *Blautia* in all subjects to some degree, with few individual fibers displaying such consistent effects across subjects. Another observation was that no single fiber or fiber mixture was universally successful in promoting *Roseburia*, although the mixtures did support this genus in a larger number of subjects compared to any individual fiber. In the case of genera such as *Agathobacter* and the *Eubacterium eligens* group, we noted variations in response across subjects for all tested fibers. Yet intriguingly, the responses to the mixtures closely mirrored those to the most promotive individual fibers, even though they comprised only a fraction of the mixture and other individual fibers in the mix showed little or no response. In fact, mixtures were equally or more promotive than single fibers for most taxa, and the only taxa detected through ANCOM to be better

supported by single fibers than the mixtures were *Parabacteroides* and *Bilophila*. In several instances, the mixtures were more promotive of taxa than any of their individual fiber components (i.e., *Enterococcus* in all mixtures, *Blautia* in mixture 1 and *Anaerostipes* and *Lachnospira* in mixture 2). It is also noteworthy that in several instances, donors with taxa that were not responsive to individual fibers became responsive when fibers were blended together. For instance, in the case of donor 5, neither *Enterococcus* nor *Anaerostipes* were promoted when exposed to the individual fibers that make up mixture 1. Yet, surprisingly, these bacteria were promoted when fermented with mixture 1. This was also observed with the same donor for *Anaerostipes* and mixture 2, with *Lachnospira* in donor 3 with mixture 2, among others.

The observed improved consistency in response of bacterial taxa promoted across donors could infer some degree of predictability of gut microbiota responses related to fiber. To compare the overall predictability of responses across people when using individual fibers versus fiber mixtures, we have performed a variance partitioning analysis which determined how much variation in the gut microbiota response was attributed to the donor, fiber treatment (single fibers versus fiber mixtures), or residuals (unexplained variation that cannot be accounted for by the fibers or donor of fecal sample). Results showed that more variance is explained by the treatment when fibers are presented as a mixture compared to individual fibers (Figure 7). The multimodal distribution for variance explained by donor, fiber, and residuals with single fibers also indicates that the response to single fibers tested here is not as straightforward and may depend on more precise or individual factors specific to each donor's microbiota. Similarly, the lack of explained variability for individual fibers was significantly higher than for mixtures, as shown by greater residuals (Figure 7). These results corroborate the above analysis and suggest that better consistency in responses across different donors is achieved by using mixtures rather than individual fibers.

Discussion

Several groups of microbes in the gut have been related to human health and also have been proposed to have preferential utilization of some dietary fiber types.[38,56–60] In this study, we have systematically designed three fiber mixtures composed of single fibers that together could stimulate all four distinct bacterial clusters observed in a first set of pooled *in vitro* fecal fermentation experiments, aiming to support different microbial groups related to health and produce a variety of different SCFAs. The following dietary fibers were selected: AX, CRS, KG and FOS (to compose mixture 1) and AXOS, BRS, KG, and GOS (to compose mixture 2) due to their abilities to promote similar targets including *Bacteroides*, *Ruminococcus* + *Clostridium* cluster XIVa, other *Clostridium* cluster XIVa, and *Bifidobacteria*, respectively. Moreover, PEC was included in mixture 1 for promotion of *Faecalibacterium prausnitzii* from *Clostridium* Cluster IV and CG was included in mixture 2 for the promotion of other *Clostridium* cluster XIVa bacteria. To understand if increasing the number of fiber types in a mixture that have similar/overlapping bacterial targets further improves the response, we have also designed mixture 3 composed of equal amounts of mixtures 1 and 2.

The three designed mixtures were tested against their individual components in a second set of *in vitro* fecal experiments performed individually for 10 different donors. Despite the differences observed compared to the first set of experiments, single fibers were still overall promotive of specific bacterial taxa, whereas the fiber mixtures, as expected, gave better support to a broader number of bacteria than any of the individual fibers. Unexpectedly, not only more of the targeted groups were promoted by the mixtures, but also, for several taxa, the support was as good or better than that of the summed individual fibers, despite each only representing 20% of the mixtures 1 and 2. Surprisingly, different and new taxa were promoted by the mixtures, but not by any of their single fiber components, indicating a synergistic effect of fibers when present together as a mixture. Similarly, the SCFA production from mixture fermented samples was not an average of

its composed fibers, but instead was produced in higher levels for all mixtures, despite several of its components leading to low-SCFA production individually.

These results indicate that there is a heretofore unreported synergistic advantage in using a range of substrates to support different bacterial taxa simultaneously. Moreover, only certain fiber combinations produced this effect. This notion resonates with the principles of resource hierarchical utilization, as observed in our own microbial study, where species reciprocally prioritize certain resources over others, ensuring that they do not directly compete for the exact same resource at the same time.[27] Such differentiation in fiber preferences can allow for stable coexistence of different species in complex microbial communities when using fiber mixtures, aligned with the higher diversity we observed compared to single fibers. Furthermore, the support of diverse bacterial species can lead to beneficial interactions among them that can include the production of growth-promoting substances, alterations to the local environment that are beneficial for growth, or the suppression of potential pathogens.[61–63] Metabolic cooperation can also be beneficial where some bacteria produce metabolic byproducts that other bacteria can utilize as substrates (i.e., cross-feeding) , which can support their growth even when they are not directly fed.[63] Whereas deciphering specific mechanisms is out of the scope of this study, probably several of these factors act together to produce the observed synergistic effects of fibers toward gut microbial promotion and diversity, where each bacterial species can better thrive, despite individual members not receiving a large amount of direct substrate.

Several genera were detected to be promoted by the mixtures through ANCOM II, all belonging to Firmicutes, and mostly to the *Lachnospiraceae* family from *Clostridium* cluster XIVa, such as *Lachnospira*, *Subdoligranulum*, *Blautia*, *Roseburia*, *Agathobacter*, and the *Eubacterium eligens* group. Whereas other groups were initially targeted by the mixtures, such as *Bacteroides* and bifidobacteria, taxa from *Clostridium* cluster XIVa were notably the main target for 40% of the fibers included in mixture 1 (CRS and KG), 60% of the components included in mixture 2 (BRS, KG and CG), and 50% of the components of mixture 3 (CRS, BRS, KG and CG). Notably, each component from mixtures 1 and 2 was tailored toward specific taxa in the *Clostridium* cluster XIVa group, so that there was little overlap in their fiber components regarding preferential support of taxa. The *Clostridium* cluster XIVa is a key group of gut bacteria that plays an essential role in maintaining gut health and homeostasis.[56] These mucosal bacteria are crucial in the production of short-chain fatty acids, particularly butyrate, which provides numerous health benefits including nourishing the gut lining, supporting the immune system, and potentially protecting against certain types of colorectal cancer.[56] Moreover, we envision that other fiber mixtures could be systematically put together to improve the support other bacterial groups related to health that were not the main target of this study.

Another observation is that blending fibers 1 and 2 together to make mixture 3 did not provide additional advantages regarding increasing the number of bacteria supported as observed from alpha diversity, heatmaps, differential phylogenetic trees, intensity and similarity of responses across donors as observed in violin plots, and SCFA production. Thus, mixtures with fibers of overlapping bacterial targets do not seem to provide further benefits and these results highlight the importance of systematic selection of fibers to compose mixtures that are target-specific. This could explain a previous report showing lack of synergistic interaction by using a different fiber blending strategy.[64]

Our findings on the synergistic effects of designed dietary fiber mixtures to promote a larger number of beneficial microbes and boost SCFAs align with previous observations. For instance, different studies indicate that a more diverse intake of fiber-rich foods (a natural fiber mixture analogue) is related to higher gut microbial diversity.[65–67] Moreover, in certain populational groups, a higher dietary diversity was shown to be related to higher SCFAs-producing bacteria.[67] Similarly, research in mice suggests that certain fiber mixtures are more effective than individual components in increasing cecal SCFA content.[68,69]

Interestingly, in our study, mixtures were also more consistent in the promotion of specific

genera across different donors than single fibers. In general, bacterial genera respond distinctively to fiber types, and blending fibers could increase chances that the appropriate fiber type for a specific genus is present for more donors. However, that does not seem to fully explain the observed results. Upon further evaluation of some specific donor responses, it was noted that for some taxa only the mixture, and not any of the single fibers making up the mixture, led to bacterial enhancement compared to the control group. It is possible that the factors previously discussed (i.e., changes in competitive pressures for substrate utilization and beneficial interactions among supported bacteria) could also contribute to more homogeneous responses across people for several selected taxa. In agreement, when variability predicted by the fiber treatment was evaluated, higher residuals were observed for individual fibers, highlighting the challenges in predicting and achieving consistent responses in different people with individual fibers. On the other hand, the greater amount of variance explained by the fiber treatment when presented as mixtures indicates a greater level of predictability and consistency in their impact on gut microbiota communities of different people.

Previously, we proposed and showed that single fibers of high specificity regarding their physicochemical features promote homogeneous *in vitro* fecal fermentation taxa responses across people, whereas those of low specificity do not.[53,70] Here, we show that another strategy to achieve some consistency in response of fibers of lower specificity could be through designed fiber mixtures. Overall, the systematically-designed fiber mixtures first reported here show promise of synergistically increasing the amount and diversity of beneficial gut microbes, supporting short chain fatty acid production beyond the summation of single fibers in a mixture, and achieving more consistency in fiber responses across people. Also, support of some bacterial taxa was only attained through certain fiber mixtures and not any of their individual fiber components. Thus, these findings underscore the importance of viewing prebiotic fiber responses as products of the gut microbiota's intricate ecological interactions, rather than merely the cumulative response of its single fiber constituents.

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Data availability statement

Raw reads are available in the National Center for Biotechnology Information Sequence Read Archive (NCBI; SRA), BioProject PRJNA1092637, and BioSamples SAMN40630087 to SAMN40630556.

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