Title

Microbiota responses to different prebiotics are conserved within individuals and associated with habitual fiber intake

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Abstract

Background: Short-chain fatty acids (SCFAs) derived from gut bacteria are associated with protective roles in diseases ranging from obesity to colorectal cancers. Intake of microbially accessible dietary fibers (prebiotics) lead to varying effects on SCFA production in human studies, and gut microbial responses to nutritional interventions vary by individual. It is therefore possible that prebiotic therapies will require customizing to individuals.

Results: Here, we explored prebiotic personalization by conducting a three-way crossover study of three prebiotic treatments in healthy adults. We found that within individuals, metabolic responses were correlated across the three prebiotics. Individual identity, rather than prebiotic choice, was also the major determinant of SCFA response. Across individuals, prebiotic response was inversely related to basal fecal SCFA concentration, which, in turn, was associated with habitual fiber intake. Experimental measures of gut microbial SCFA production for each participant also negatively correlated with fiber consumption, supporting a model in which individuals' gut microbiota are limited in their overall capacity to produce fecal SCFAs from fiber.

Conclusions: Our findings support developing personalized prebiotic regimens that focus on selecting individuals who stand to benefit, and that such individuals are likely to be deficient in fiber intake.

Keywords

Microbiome; prebiotic; short-chain fatty acids; diet; personalized nutrition; fiber

57 **Background**

58 A key beneficial role of the human gut microbiota is producing the short-chain fatty acids
59 (SCFAs) acetate, propionate, and butyrate, which are metabolic end products of anaerobic
60 fermentation (1, 2). These SCFA, most notably among them, butyrate, have been shown to
61 promote gastrointestinal (GI) barrier integrity, lower inflammation, increase resistance to
62 pathogen invasion, and contribute positively to enteroendocrine signaling and energy
63 homeostasis (3-9). Augmenting these functions may explain the protective role of SCFAs in
64 diseases ranging from obesity to inflammatory bowel diseases (3, 10-20).

65 The substrates for SCFA production in the human gut are predominately fibers, which are
66 dietary carbohydrates inaccessible to human enzymes and transporters (21). On average,
67 American adults consume only 21 to 38% of the fiber that is recommended by the USDA (22).
68 This lack of fiber is implicated in the etiology of Western diseases (15, 23, 24), which may in
69 part be tied to the effects of low fiber consumption on microbial SCFA production and SCFA
70 signaling (15, 25-29). To address fiber deficiencies, dietary supplements known as prebiotics
71 have been formulated using specific dietary fibers that are microbially fermentable but host-
72 inaccessible. Prebiotics can increase production of SCFAs by the gut microbiota (30-33), and
73 have emerged as a promising microbiota-targeting approach for promoting health.

74 A key challenge of using prebiotics to promote SCFA production is heterogeneity in
75 treatment response. Many distinct prebiotic formulations exist (34, 35), and different prebiotics
76 have been associated with diverging patterns of SCFA production (30, 32). Prebiotic trials have
77 also often revealed an increase in SCFA at the population level following treatment, but
78 inconsistent results at the level of individual (30-33). Still, prebiotic trials tend not to examine
79 how responses to different prebiotics vary within individuals, and have to date often profiled

80 single prebiotics (31, 33, 36, 37), or tested different prebiotics in separate cohorts (30, 32, 38).
81 More rare crossover studies have taken place, but have either not investigated individual
82 variation in prebiotic response (39), or solely utilized *in vitro* assays of prebiotic response (40,
83 41). If consistent evidence does emerge that responses to different prebiotics vary within people,
84 then effective prebiotic therapies will face the challenging task of personalizing prebiotic choices
85 for each recipient. Alternatively, if responses to different prebiotics are conserved within
86 individuals, effective prebiotic therapy could primarily focus on selecting individuals for
87 treatment. Evidence supporting this latter scenario include studies showing that individual
88 identity is a stronger predictor of SCFA concentrations than dietary intervention (42), and that
89 individual characteristics like habitual food choice can predict changes to microbiota community
90 composition following prebiotic supplementation (37).
91 To directly investigate the interaction of prebiotic and individual variation in prebiotic
92 response, we report here the results of a randomized, fully balanced, crossover trial where a
93 single set of individuals was treated with multiple prebiotics. Our primary lines of inquiry were
94 to assess the relative contribution of individual and prebiotic differences to SCFA responses to
95 supplementation, with an emphasis on butyrate production. Secondary analyses included
96 determining what host and microbiological factors shaped variation in prebiotic response.
97
98 **Methods**
99 Participant recruitment:
100 This study was conducted at Duke University in Durham, NC, USA, and was registered
101 with ClinicalTrials.gov (Identifier: NCT03595306). All recruitment and study procedures
102 involving human subjects were approved by the Duke University Institutional Review Board

103 (Pro00087214). Study participants were recruited by flyers, email list-servs, and word-of-mouth
104 at Duke University Campus. Individuals with a self-reported history of irritable bowel syndrome,
105 inflammatory bowel disease, type-2 diabetes, kidney disease, intestinal obstruction, or colorectal
106 cancer were excluded. Participants were also excluded if they had taken oral antibiotics within
107 the past 6 months, had any known food allergies, reported dietary intolerances of any kind, or
108 were pregnant or breastfeeding. If individuals identified as female, their urine was subjected to a
109 pregnancy test (Quidel QuickView One-Step hCG Urine Test) to minimize the risk that prebiotic
110 treatment would impact newborn or infant health. Participants who tested positive were deemed
111 ineligible for participation in the study. Inclusion criteria were that participants were between
112 the ages of 18 and 70, and able to provide stool samples at no risk to themselves. Fiber
113 consumption, prebiotic/probiotic usage, and dietary habits were not used as inclusion/exclusion
114 criteria, but participants were asked to report via surveys as part of the study requirements.
115 During this period, 81 individuals set up a phone interview to determine eligibility and interest to
116 participate in the study. Of these, 41 completed electronic informed consent to the study and
117 were subsequently enrolled. Ultimately, 8 subjects were lost to follow-up, 2 withdrew, and 3
118 were omitted from some or all analyses due to inability to collect sufficient usable sample,
119 resulting in 28 subjects included in all final data analyses.
120
121 Study design:
122 The three-period crossover longitudinal study was conducted from May of 2018 to March
123 of 2019. The crossover design allowed investigation of three prebiotic interventions while also
124 allowing participants to serve as their own controls (Figure 1). There were six arms of the study,
125 to cover all possible orders of prebiotic consumption and to eliminate the need to account for

126 uneven carry-over effects. The study consisted of an initial study visit, followed by a 6-week
127 study period with alternating weeks of baseline (no prebiotic consumption) and intervention
128 (prebiotic consumption). Participants were added sequentially to an arm group as they enrolled;
129 meaning the first participant was enrolled in Arm 1, the second to Arm 2 and so on. Participants
130 were blinded to the prebiotics that they were consuming until the end of the study. The prebiotics
131 were taken twice a day, Monday to Friday during the intervention weeks. Monday was a half-
132 dose, to help allow participants to adjust to the increase in fiber. Following consent, participants
133 had an initial study visit, to have their baseline physiology assessed by height, weight, and blood
134 pressure measurement, as well as provide a urine sample and receive the study kit and materials.
135 Participants were asked to self-collect stool at home three times a week each week during the
136 study period. Participants also completed 4 diet surveys throughout the study; one instance of the
137 Diet History Questionnaire Version 3 (DHQ3) (43), covering the one month period prior to the
138 study, and three instances of the Automated Self-Administered 24-hour (ASA24) Dietary
139 Assessment Tool, version 2017, developed by the National Cancer Institute, Bethesda, MD (44).
140 Two ASA24's were completed during the week, and one on a weekend. The DHQ3 was
141 completed prior to the 6-week study period. Additionally, participants completed online-surveys
142 about their experiences with the prebiotics following the intervention weeks.
143
144 Prebiotic supplementation:
145 Three prebiotics were tested in this study: inulin (manufactured by Now Foods,
146 Bloomingdale, IL), wheat dextrin (Benefiber, manufactured by GSK Consumer Healthcare,
147 Warren, NJ), and galactooligosaccharides (GOS) (Bimuno, manufactured by Clasado Limited,
148 Shinfield, UK). In a clean non-laboratory space, prebiotics were weighed into individual doses

149 and packaged into pre-labeled food-safe sealable plastic bags. Each participant received all three
150 prebiotics throughout the study; inulin at 9g / day, wheat dextrin at 9g / day, and
151 galactooligosaccharides (GOS) at 3.6g / day. These doses correspond to the recommended daily
152 intake on the product label. In order to further minimize the possibility of discomfort associated
153 with consuming prebiotics, the first day of dosing for each prebiotic was a half dose to allow
154 acclimation (4.5g for inulin and dextrin, 1.8g for GOS). Participants consumed one of the three
155 prebiotics at a time, twice a day, for a duration of 5 days, followed by a week of washout. The
156 order was determined by their assigned study arm. Participants were instructed to consume the
157 prebiotics by mixing the powders in any kind of beverage (such as water) or food (such as
158 applesauce), so long as it was cold or room temperature.
159
160 Stool collection:
161 At the pre-study visit, participants were provided with a standard urine specimen collection cup
162 and shown to a bathroom to collect the sample. Urine samples were also sent to LabCorp in a BD
163 Vacutainer UA tube to measure metabolic variables, including routine urinalysis, sodium,
164 potassium, urea nitrogen, and creatinine. Consented participants were asked to self-collect stool
165 three times a week at home using sampling kits provided to them. Participants were asked to
166 immediately store samples in their personal freezers (-20C). Participants were asked to bring
167 stored samples to the David Lab freezers on a weekly basis. Stool samples were transferred and
168 stored at -80C until thawed for DNA extraction and other sample processing.
169
170 *In vitro* fermentation:

171 *In vitro* fermentation of stool microbiota was conducted following protocols previously
172 published (40). In brief, a 10% w/v fecal slurry was made using thawed week-1 stool and
173 anaerobic 1X PBS (pH 7.0 ± 0.1). This slurry was filtered through 0.33mm pore size mesh, then
174 mixed 1:1 with a 1% w/v solution of prebiotic and incubated in duplicate in a 24-well cell culture
175 plate for 24hr. Three prebiotic treatments were used, corresponding to the three prebiotics used
176 in the *in vivo* portion of this study: inulin (Now Foods Inulin Powder, part #2944),
177 galactooligosaccharides (GOS; Bimuno Powder), and wheat dextrin (Benefiber Original). The
178 resulting fermentation conditions where therefore 5% fecal slurry with 0.5% prebiotic (w/v) and
179 occurred at 37°C for 24 hours. Following fermentation, a 1mL aliquot was taken from each well
180 for SCFA quantification.
181
182 Quantification of SCFA:
183 Quantification of SCFA by gas chromatograph flame ionization detection (GCFID) was
184 performed following protocols previously published (40). In brief, a 1mL aliquot of either 10%
185 fecal slurry in PBS or the fermentation vessel contents was obtained. To this, 50 µL of 6N HCl
186 was added to acidify the solution to a pH below 3. The mixture was vortexed, centrifuged at
187 14,000rcf for 5 minutes at 4°C to remove particles. Avoiding the pellet, 750 µL of this supernatant
188 was passed through a 0.22µm spin column filter. The resulting filtrate was then transferred to a
189 glass autosampler vial (VWR part #66009-882).
190 Filtrates were analyzed on an Agilent 7890b gas chromatograph (GC) equipped with a
191 flame-ionization detector (FID) and an Agilent HP-FFAP free fatty-acid column (25m x .2mm id
192 x .3µm film). Acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate were identified
193 and quantified in each sample by comparing to an 8-point standard curve that encompassed the

194 sample concentration range. Standards contained 0.1mM, 0.2mM, 0.5mM, 1mM, 2mM, 4mM,
195 8mM, and 16mM concentrations of each SCFA.
196
197 DNA extraction, PCR amplification, and sequencing:
198 We performed 16S rRNA gene amplicon sequencing on human stool samples to determine
199 microbiota community composition following protocols previously published (40). DNA was
200 extracted from frozen fecal samples with the Qiagen DNeasy PowerSoil DNA extraction kit (ID
201 12888-100). Amplicon sequencing was performed using custom barcoded primers targeting the
202 V4 region of the 16S gene (45), using published protocols (45-47). The sequencing library was
203 diluted to a 5pM concentration and sequenced using an Illumina MiniSeq and a MiniSeq Mid
204 Output Kit (FC420-1004) with paired-end 150bp reads.
205
206 Identifying sequence variant and taxonomy assignment:
207 We used an analysis pipeline with DADA2 (48) to identify and quantify sequence
208 variants, as previously published (40, 49). To prepare data for denoising with DADA2, 16S
209 rRNA primer sequences were trimmed from paired sequencing reads using Trimmomatic v0.36
210 without quality filtering (50). Barcodes corresponding to reads that were dropped during
211 trimming were removed using a custom python script. Reads were demultiplexed without quality
212 filtering using python scripts provided with Qiime v1.9 (51). Bases between positions 10 and 150
213 were retained for the forward reads and between positions 0 and 140 were retained for the
214 reverse reads. This trimming, as well as minimal quality filtering of the demultiplexed reads was
215 performed using the function fastqPairedFilter provided with the DADA2 R package (v1.8.0).
216 Sequence variants were inferred by DADA2 independently for the forward and reverse reads of

217 each of the two sequencing runs using error profiles learned from all 20 samples. Forward and
218 reverse reads were merged. Bimeras were removed using the function removeBimeraDenovo
219 with default settings. Taxonomy was assigned using the function assignTaxonomy from
220 DADA2, trained using version 123 of the Silva database.
221
222 **Results**
223 **Prebiotic supplementation affected SCFA production at the cohort-level.** We enrolled
224 participants into a six-week, three-period prebiotic intervention study (Figure 1). We used a
225 crossover design to compare multiple prebiotics across a subject pool. We selected inulin, wheat
226 dextrin, and galactooligosaccharides (GOS) as our prebiotics, as these are commonly consumed
227 (52), commercially available dietary supplements, and are monomerically distinct. Our crossover
228 was standardized in series and period, with each prebiotic occurring the same number of times
229 within each series (once) and study period (twice). The design was also balanced with respect to
230 first-order carry-over effects, meaning we shuffled the order in which prebiotics were
231 administered so as to minimize the interactive effects between any two pairs of consecutive
232 prebiotics. Each study period consisted of an intervention-free baseline week and a prebiotic
233 intervention week. Stool samples were collected on Wednesday, Thursday, and Friday of each of
234 the six study weeks (baselines and intervention). Of the 41 participants who initially enrolled, 2
235 withdrew, 3 were unable to collect at least one usable sample (i.e., insufficient volume or
236 inadequate labeling) per week, and 8 were lost to follow-up. We focused our subsequent analyses
237 on the remaining 28 participants who completed the study. These participants in total collected
238 413 stool samples (mean=2.46 stool samples per person per week).

239 We first sought to examine differences in microbiome response during each of the
240 different prebiotic treatments. Analyses of gut microbial community composition did not reveal
241 associations with inulin, GOS, or wheat dextrin treatment ($p > 0.05$, PERMANOVA using
242 unweighted UniFrac distance at species level). In addition, no species were statistically
243 differentially abundant during any prebiotic intervention and its corresponding prebiotic-free
244 baseline ($p > 0.05$, Benjamini-Hochberg corrected Wilcoxon Rank Sum tests using the R
245 package ALDEx2). While previous studies have observed changes in community composition
246 following prebiotic administration, such effects have been observed in a subset of tested
247 prebiotics (30, 32), and with different dosing schedules (30-32, 37).
248 Still, in contrast to the lack of a coherent 16S rRNA shifts among participants, we found
249 the three prebiotics varied according to resulting fecal SCFA profiles relative to baseline. GOS
250 was associated with decreased total stool SCFA concentrations (Table 1, Figure S1C, effect size
251 = -0.071, $p = 0.038$, GLMM), an outcome that has been previously observed (53). Differences
252 within individual SCFAs were also apparent when analyzing absolute fecal SCFA concentrations
253 (Table 1). Dextrin increased propionate concentration (effect size = 0.074, $p = 0.038$, GLMM),
254 and GOS decreased both propionate and butyrate concentrations (effect size = -0.087, $p = 0.015$;
255 effect size = -0.14, $p = 0.002$, GLMMs, respectively). Still, changes in total and individual SCFA
256 concentrations may be caused by effects of stool hydration, which can vary up to three-fold and
257 directly impacts measures of SCFA concentration in feces (54, 55). To assess the relative
258 production of each SCFA, we therefore normalized levels of individual SCFAs to account for
259 differences in stool hydration. Our resulting analysis found that inulin supplementation increased
260 proportional butyrate concentrations (butyrate concentration / total SCFA concentration) in
261 participants' stool (effect size = 0.082, $p = 0.007$, GLMM; Figure 2, Table 1). These findings are

262 consistent with prior reports that inulin supplementation may increase butyrogenesis (56-58).
263 Our analyses also suggested GOS and wheat dextrin decreased proportional butyrate
264 concentration in participants' stool (GOS; effect size = -0.074, $p = 0.012$. Dextrin; effect size = -
265 0.071, $p = 0.015$, GLMM; Figure 2, Table 1). These findings align with prior trials that found
266 both GOS (53, 59, 60) and dextrin (61, 62) increases acetate production over more reduced
267 SCFAs, including butyrate and propionate, potentially by shunting nutrients away from butyrate
268 production. No prebiotic altered proportional acetate or proportional propionate concentration at
269 the cohort level ($p > 0.05$, GLMM; Figures S1A and S1B, Table 1).
270
271 **Butyrogenic responses are more strongly predicted by individual identity than by prebiotic.**
272 We next investigated how prebiotic responses varied among individuals. We focused our
273 analysis on prebiotic impacts on butyrate production because it was the sole SCFA that was
274 altered at the cohort-level, and it is also the microbially derived SCFA most commonly
275 implicated in enteric health (2, 6-8, 63-66). We also focused on proportional butyrate
276 concentrations to avoid the effects of stool hydration on our analysis and to be consistent with
277 previous individual-level analyses of fecal SCFAs (32, 42). In line with prior studies of prebiotic
278 response, (40) statistical modeling yielded evidence for individual-specific butyrogenic
279 responses to each of the prebiotics (that is, an interaction between individual and prebiotic terms
280 in a GLMM). Nonetheless, further analyses suggested that the strongest determinant of
281 proportional butyrate responses was shaped by individual identity: individual-specific effects
282 (pseudo-$R^2 = 0.39$, $p < 0.0001$; GLM F-test) were much stronger than prebiotic-specific effects
283 (pseudo-$R^2 = 0.05$, $p = 0.001$; GLM F-test). Additionally, individuals who exhibited the largest
284 response in proportional stool butyrate concentration on a given prebiotic were more likely to

285 exhibit larger responses on the other two prebiotics ($\rho = 0.49$, 0.37, and 0.28, $p = 0.008$, 0.05,
286 and 0.15, respectively for inulin vs dextrin, dextrin vs GOS, and inulin vs GOS, Spearman
287 correlations; Figure 3). This pattern was apparent in spite of correcting for baseline differences in
288 SCFA production between individuals, which were also consistent over time and between
289 baseline and treatment weeks.
290
291 **Individuals consistently varied according to gut metabolic state, microbiota community**
292 **composition, and metabolic potential**. Why might individual-specific effects be stronger than
293 prebiotic-specific effects in our cohort? We observed that individuals were idiosyncratic in their
294 SCFA profiles over time. Both proportional butyrate concentration and butyrate concentration
295 were correlated within individuals between the first and last baseline weeks ($\rho = 0.49$, $p < 0.007$;
296 and $\rho = 0.63$, $p = 0.0003$, respectively, Spearman correlations; Figure S2). Variation between
297 individuals' SCFA profiles could also be linked to inter-individual microbiota variation, a well-
298 known factor in prebiotic response (42). When we divided participants into 'high' (> median)
299 and 'low' (<= median) fecal butyrate groups, we observed a significant effect of this grouping on
300 microbiota community composition ($R^2 = 0.075$, $p = 0.0022$, PERMANOVA using unweighted
301 UniFrac distance; Figure S3). We identified three specific genera that were differentially
302 abundant between the groups: *Anaerofilum* and *Ruminiclostridium_5* were decreased in the high
303 butyrate group, and *Lachnospira* was increased in the high butyrate group ($p = 0.009$, .043, and
304 0.030, respectively; Benjamini-Hochberg corrected Wilcoxon Rank Sum tests; Figure S3). We
305 additionally observed a relationship between baseline microbiota community structure and
306 average response to all three prebiotics (Δ%butyrate). After dividing participants into
307 'responders' and 'non-responders', based on whether average Δ%butyrate was positive or

308 negative, we found a significant effect of this grouping on microbiota community composition
309 ($R^2 = 0.059$, $p = 0.028$, PERMANOVA using unweighted UniFrac distance; Figure S4). Thus,
310 individuals' starting microbiome composition was associated with prebiotic treatment outcome.
311
312 **Intake patterns of dietary fiber negatively correlated with response to prebiotic**
313 **intervention.**
314 Next, we sought to understand what host factors might influence individuals' microbiomes and
315 ultimately shape their butyrogenic responses across the tested prebiotics. We focused on fiber
316 intake, which has been shown to shape microbiota community composition and metabolic output
317 (67, 68). We measured fiber consumption using a dietary questionnaire spanning the month
318 preceding the study (43), and we compared fiber intakes to each individual's average response to
319 the three prebiotic interventions (average Δ%butyrate). Despite prior studies suggesting that
320 plant or fiber intake would augment gut microbial metabolic capacity (37, 68-70), our
321 comparison revealed that dietary fiber consumption was negatively correlated with average
322 Δ%butyrate ($\rho = -0.40$, $p = 0.046$; Spearman correlation; Figure 4A). Thus, higher habitual fiber
323 intake was associated with a lower average Δ%butyrate during the prebiotic intervention. To
324 corroborate these findings using an objective biomarker, we also compared average Δ%butyrate
325 to spot urinary potassium, a known marker for fruit and vegetable intake (71), as well as for
326 general healthy eating (72, 73). We validated that spot urinary potassium was correlated with
327 dietary fiber intake in our study ($\rho = 0.47$, $p = 0.031$; Spearman correlation; Figure S5A) and
328 found evidence for a negative correlation between spot urinary potassium and average
329 Δ%butyrate ($\rho = -0.38$, $p = 0.071$; Spearman correlation; Figure S5B).
330

331 **Observational evidence that baseline metabolic state and dietary history limits prebiotic**
332 **response.** To understand why habitual fiber intake would be negatively associated with prebiotic
333 responses, we considered recent ecological studies suggesting that nutrient limitation is a key
334 factor shaping the metabolism of mammalian gut microbiota (74, 75). Even if excess fiber is
335 available to polysaccharide utilizing microbes, fixed caps in the availability of other nutrients
336 involved in carbohydrate fermentation would ultimately constrain the total amount of SCFAs
337 that can be produced from dietary fibers. Individuals who naturally consume high levels of fiber
338 and already activate SCFA production pathways would therefore exhibit reduced capacity to
339 increase SCFA production following prebiotic treatment.

340 We identified three findings that were consistent with a threshold model of metabolic
341 capacity limitation. First, we observed that individuals with higher starting proportional
342 concentrations of butyrate exhibited the weakest butyrogenic prebiotic responses. ($p = 0.008$, ρ
343 $= -0.49$, Spearman correlation; Figure 4B). No participant ultimately exhibited butyrate
344 concentrations in excess of 29mM following prebiotic treatment.

345 A second finding linking background nutrient intake and metabolic limitation involved
346 baseline proportional butyrate concentrations and habitual diet. Dietary fiber consumption was
347 positively correlated with proportional butyrate concentration at baseline week 1, prior to any
348 prebiotic intervention (1g fiber/2000 kilocalorie diet led to an increase in proportional butyrate
349 concentration of 0.010, $p = 0.012$, beta regression; Figure S6) as has been previously suggested
350 (33, 36, 76). Furthermore, using an ecological technique for identifying relationships between
351 multivariate datasets known as co-inertia analysis (77), we found a significant interaction
352 between baseline proportional SCFA concentrations and food choices($p = 0.031$, coinertia
353 analysis). This analysis associated intake of several fiber-rich food groups with baseline

354 proportional butyrate concentrations including legumes, berry fruits, and dark-green vegetables
355 (Figure 5).
356
357 **Culture-based experimental evidence links fiber intake to limits on microbiome metabolic**
358 **response**. A third line of experimental evidence arose linking habitual nutrient intake and
359 microbial SCFA production capacity. We hypothesized that if baseline fiber consumption
360 reduced the capacity of gut microbiota to produce SCFAs, the metabolic activity of fecal
361 microbiota would exhibit a negative relationship with fiber intake. To test this hypothesis, we
362 applied a recently developed an *ex vivo* fermentation model (40) to baseline stool samples
363 collected from each study participant. This model experimentally tests the metabolic effects of
364 supplementing a microbial community using a given prebiotic as the sole additional carbon
365 source, independent of potentially confounding host factors like SCFA absorption. In support of
366 the model's capability to produce SCFAs following prebiotic administration, we observed an
367 increase in overall SCFA production relative to control ($p < 0.0001$, t-test; average of 4.41-fold
368 increase across participants, calculated using the average total SCFA concentration of 3 separate
369 prebiotic treatments) when the *in vitro* assays were supplemented with prebiotics as the sole
370 carbon source. When we compared SCFA production to fiber intake, we observed as expected a
371 negative correlation between fiber consumption and SCFA production ($p = 0.021$, $\rho = -0.46$,
372 Spearman correlation using average of three prebiotic treatments; Figure 4C). We also observed
373 a negative correlation between fiber consumption and SCFA production in prebiotic-free control
374 treatments ($p = 0.019$, $\rho = -0.47$, Spearman correlation; Figure S7. Thus, individuals who tended
375 to eat more fiber in our cohort also harbored gut microbiota that produced less SCFA when
376 exposed to prebiotics.

377

378 **Consistent growth of specific microbial genera supports individual-specific prebiotic**
379 **response.** Notably, our *ex vivo* fermentations also associated specific gut bacterial taxa with
380 prebiotic response. Following 24hr fermentation, microbial community composition was
381 assayed from each prebiotic-supplemented culture and compared to prebiotic-free control
382 cultures, for each of the 28 participants, using ALDEx2. This analysis revealed multiple genera
383 that were over or under-represented in each prebiotic treatment (Figure 6).
384 Additionally, most genera that were differentially abundant in one prebiotic treatment exhibited
385 similar differential abundance in the other prebiotic treatments. All genera exhibiting conserved
386 higher abundance in two or three prebiotic treatments compared to control (*Collinsella*,
387 *Faecalibacterium*, *Lachnospiraceae*, *Marvinbryantia*, and *Ruminiclostridium*) are known
388 degraders of common dietary fibers with broad specificity (78-80). By contrast, of the genera
389 exhibiting conserved lower abundance in two or three prebiotic treatments compared to control
390 (*Alistipes*, *Anaerofilum*, *Flavonifractor*, *Holdemania*, and *Parabacteroides*), we are not aware of
391 reports linking them to degradation of inulin, dextrin, or GOS, and only *Parabacteroides* has
392 been shown to degrade any dietary fiber (78, 80). Overall, the consistent response of select
393 bacterial taxa to prebiotic addition *in vitro* further supports the conclusion that responses across
394 a range of prebiotics will be linked to individual-specific factors like gut microbial community
395 composition.
396

397 **Discussion**

398 In this study, we administered the prebiotics inulin, GOS, and dextrin to a set of
399 individuals using a fully balanced crossover design. We found that these prebiotics varied in

400 their impact on fecal SCFA concentrations when data was agglomerated at the population scale,
401 with inulin increasing proportional butyrate and both GOS and wheat dextrin decreasing
402 proportional butyrate. Yet, our analysis also suggested that individual identity exerted a much
403 stronger effect on prebiotic response, and we observed that responses to each prebiotic were
404 correlated within individuals. Individual response variation was associated with differences in
405 baseline microbiological and dietary factors. In particular, we found that the general butyrogenic
406 response to prebiotics was anti-correlated with habitual fiber intake. This negative relationship
407 may be explained by limitations in the overall SCFA production capacity of human gut
408 microbiota.

409 From a microbial ecological perspective, our findings support a model where microbial
410 metabolic repertoires within individuals' microbiota have the capacity to utilize a diverse array
411 of chemically distinct fiber types. Evidence in favor of this model include *in vitro* prebiotic
412 screens suggesting that individuals uniformly harbor bacteria capable of degrading a wide variety
413 of carbohydrate species (81). Such metabolic plasticity may benefit gut microbiota by enhancing
414 community stability and invasion resistance (82-84), and could be enabled by substrate-
415 switching at the level of strains (84, 85) or cross-feeding interactions (86). Still, differences in
416 the levels of strains underlying these metabolic interactions may vary between individuals (81,
417 87), which could in turn lead to inter-individual variation in the capacity of individuals'
418 microbiota to ferment prebiotics.

419 The nutritional implications of our work were, in our view, more surprising. A positive
420 relationship between dietary fiber consumption and microbiota community structure (88), and
421 metabolic function (68, 89-91) is well established, with higher fiber intake linked to greater
422 SCFA and, specifically, butyrate production. Thus, we expected that individuals habituated to a

423 high-fiber diet would have a greater capacity to utilize incoming novel fiber, not a lower capacity
424 as we observed. Still, prior studies reporting links between diet patterns and microbiota response
425 have specifically associated changes to microbial community composition with baseline fiber
426 intake (37). Associations between fiber consumption and SCFA production, by contrast, have
427 either not been tested for in prebiotic trials (92, 93) or not revealed a positive relationship (37).
428 Additionally, while our analyses confirmed an association between baseline proportional
429 butyrate levels and intake of plant foods groups linked to dietary fiber (e.g. legumes and dark
430 green vegetables), it also linked food groups typically associated with a Mediterranean diets (e.g.
431 seafood, dairy, fruit, and poultry (94)) with higher baseline levels of proportional acetate. Future
432 prebiotic trials are needed to determine the generality of our observed association between
433 habitual diet and SCFA production
434
435 **Conclusions**
436 Our study provides evidence that the responsiveness of an individual to prebiotic
437 treatment may be predictable from diet and baseline concentrations of SCFA in stool. We
438 emphasize that we do not believe these predictions should be used to modify the diets of
439 individuals who normally consume high levels of fiber or have higher concentrations of SCFA in
440 stool. Ample evidence supports the benefits of both a high fiber diet and adequate microbial
441 SCFA production (70, 95, 96). Rather, we argue our findings support strategies for personalizing
442 microbiota therapy that focus on individual selection. Ongoing prioritization efforts are being
443 used to identify patients most likely to respond to microbiota-directed and microbiota-dependent
444 disease therapies (97-100), and are also being explored to enhance prebiotic therapy (30, 69, 90,
445 91). Our study here suggests that such efforts to personalize prebiotic supplementation can rely

446 on dietary history and fecal metabolite levels as informative and non-invasive biomarkers of
447 treatment outcome.
448
449 Ethics approval and consent to participate
450 All recruitment and study procedures involving human subjects were approved by the
451 Duke University Institutional Review Board (Pro00087214). All subjects provided electronic
452 informed consent prior to enrollment.
453
454 Competing interests
455 The authors declare that they have no competing interests
456
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468 collection, analysis, or interpretation of the data nor in writing the manuscript.

469

470 Authors' contributions

471 ZH, MV, JS, LD, and HD conceptualized and designed the study. HD, ZH, SJ, and LD
472 coordinated recruitment and engaged with participants. ZH was blinded to participant identity
473 and study arm assignment where possible. ZH, SJ, ED, and HD processed samples, ran *in vitro*
474 fermentation assays, and analyzed samples via gas-chromatography and sequencing. PHL and
475 BP supported collection and analysis of nutrition data. ZH analyzed all data. ZH and LD wrote
476 the manuscript. All authors read and approved the manuscript.

477

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480 kitchen facilities for food-safe repackaging of prebiotics, and Jeffrey Letourneau, Rachael
481 Bloom, and Savita Gupta for assistance with study coordination and logistics.

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Figures

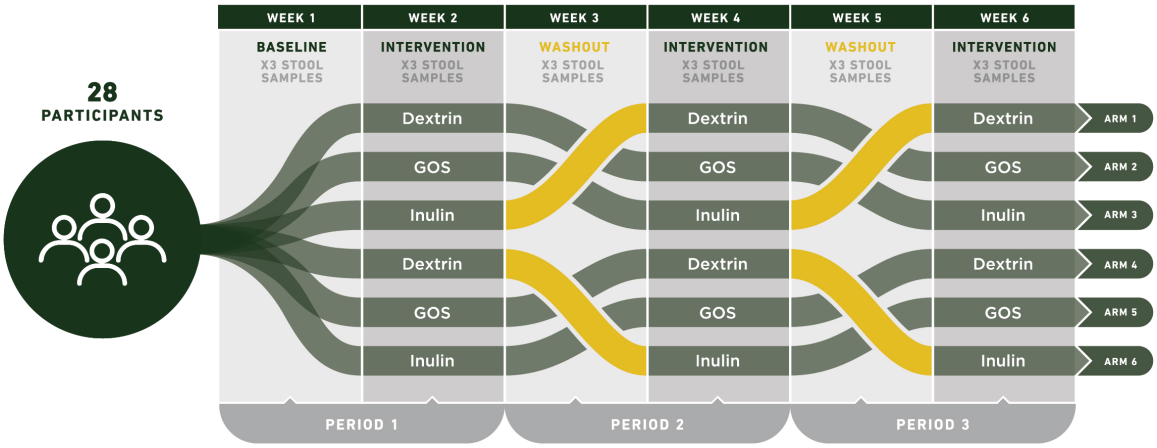


Figure 1: Prebiotic trial design. A three-period crossover design was standardized in series and period, with each prebiotic occurring the same number of times within each arm (once) and study period (twice). The design was also balanced with respect to first-order carry-over effects, meaning prebiotics were administered in all possible orders. Each of the three periods consisted of a prebiotic-free week followed by a prebiotic-supplemented week. Six study arms encompassed all possible orders of prebiotic supplementation, and subjects were assigned to arms sequentially by time of enrollment, resulting in approximately equal numbers of individuals in each arm. A total of 41 participants were enrolled and 28 were considered to have completed the study.

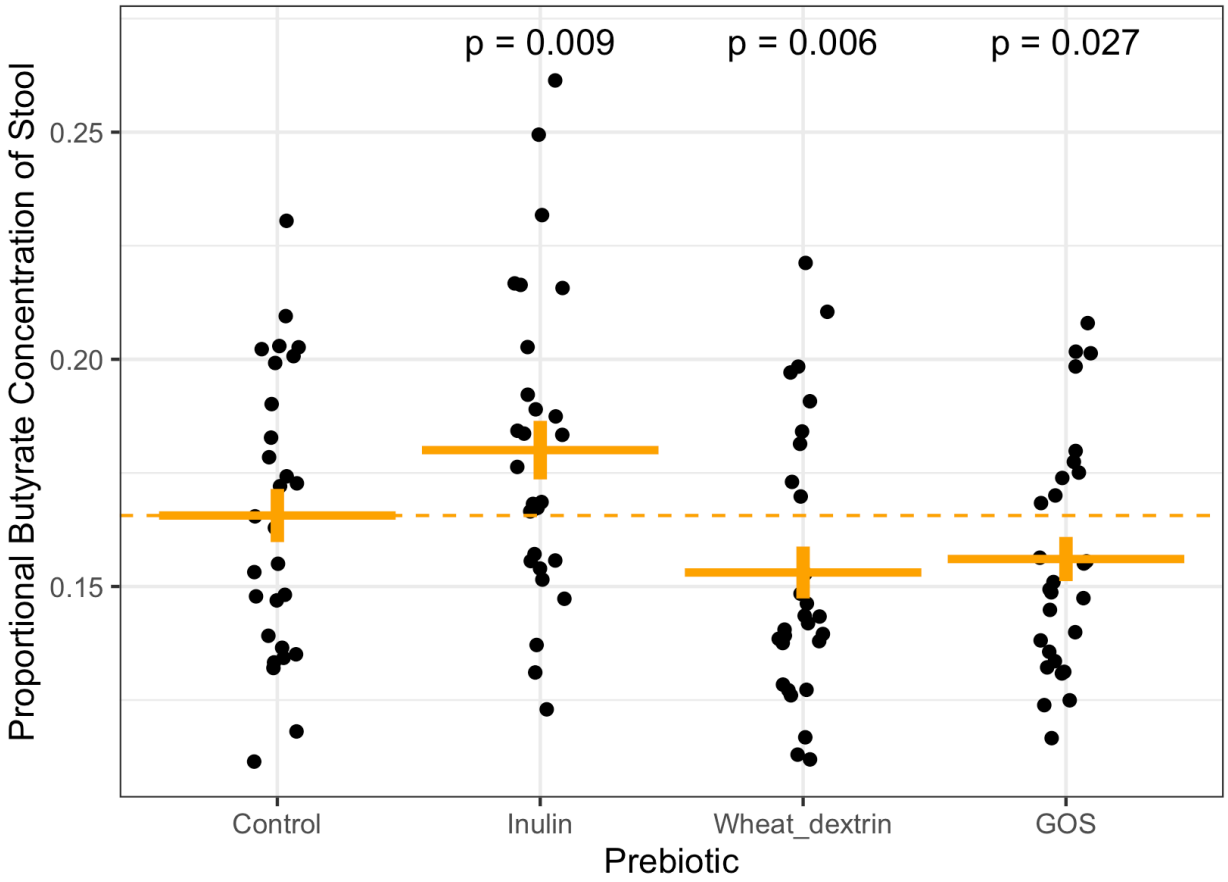


Figure 2: Effects of prebiotic supplementation on proportional butyrate concentration of stool. Plotted are the average proportional butyrate concentrations for each study participant during each prebiotic intervention period (up to 3 samples per person per week), and the average of all three baseline weeks (up to 9 samples per person). Inulin was associated with significantly increased proportional butyrate concentration of stool averaged across individuals, while both dextrin and GOS were associated with decreased proportional butyrate relative to control (dashed line) (generalized linear mixed model).

	Control	Inulin		GOS		Dextrin	
	value	value	p	value	p	value	p
CONCENTRATION							
Acetate (mM)	48.1	46.4	ns	47.8	ns	51.7	ns
Propionate (mM)	13.8	13.7	ns	13.3	**0.015**	15.7	**0.038**
Butyrate (mM)	12.4	13.7	ns	11.2	**0.002**	12.2	ns
Total SCFA (mM)	74.5	73.9	ns	72.4	**0.038**	79.6	ns
PROPORTION							
Acetate (%)	64.3	62.5	ns	65.3	ns	64.7	ns
Propionate (%)	19.2	19.5	ns	19.1	ns	20	ns
Butyrate (%)	16.5	18.0	**0.009**	15.6	**0.027**	15.3	**0.006**

512
513 **Table 1: Concentrations and proportions of SCFA in stool during prebiotic and control**
514 **weeks, averaged across individuals**. Significance was determined by generalized linear mixed
515 models.

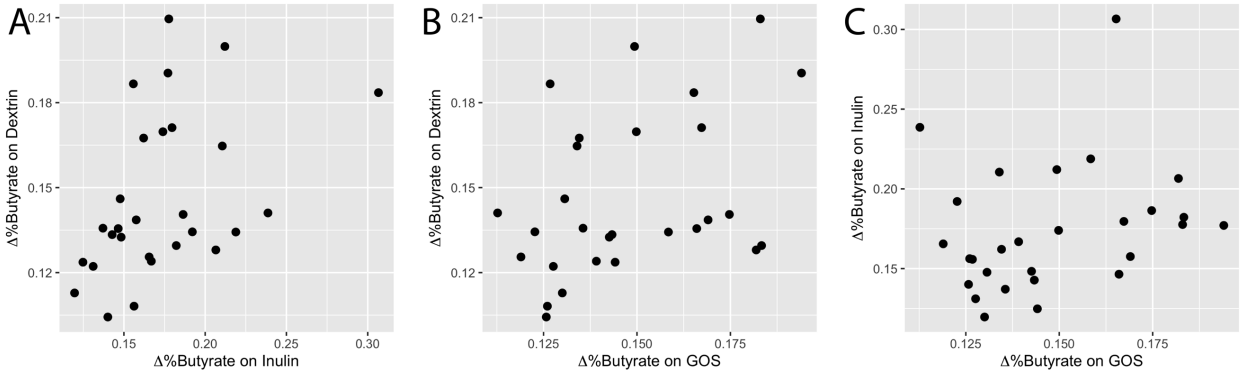


Figure 3: Individual changes in proportional butyrate concentration (Δ%butyrate) compared across multiple prebiotics. Changes in Δ%butyrate were positively correlated when comparing responses to dextrin and inulin ($\rho = 0.49$, $p = 0.008$; Spearman correlation), as well as responses to dextrin and GOS ($\rho = 0.37$, $p = 0.05$). The relationship between inulin and GOS response trended towards a positive association ($\rho = 0.28$, $p = 0.15$).

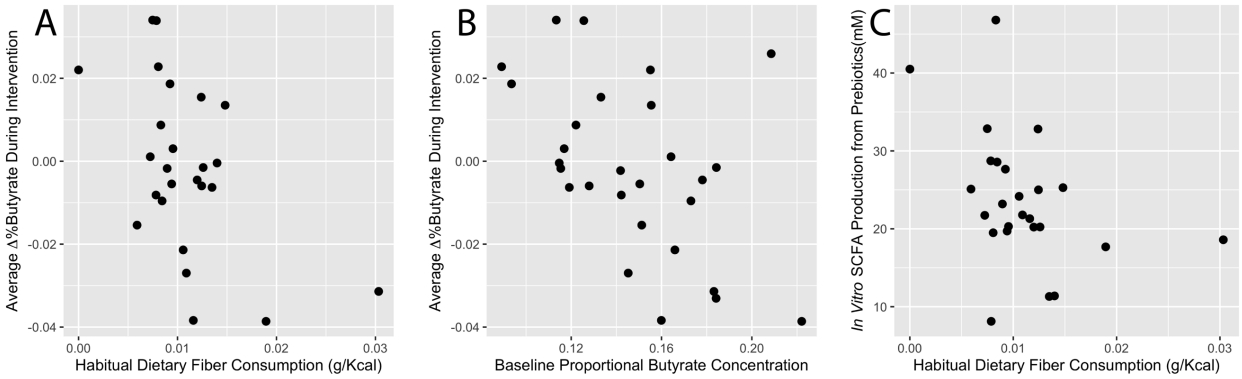
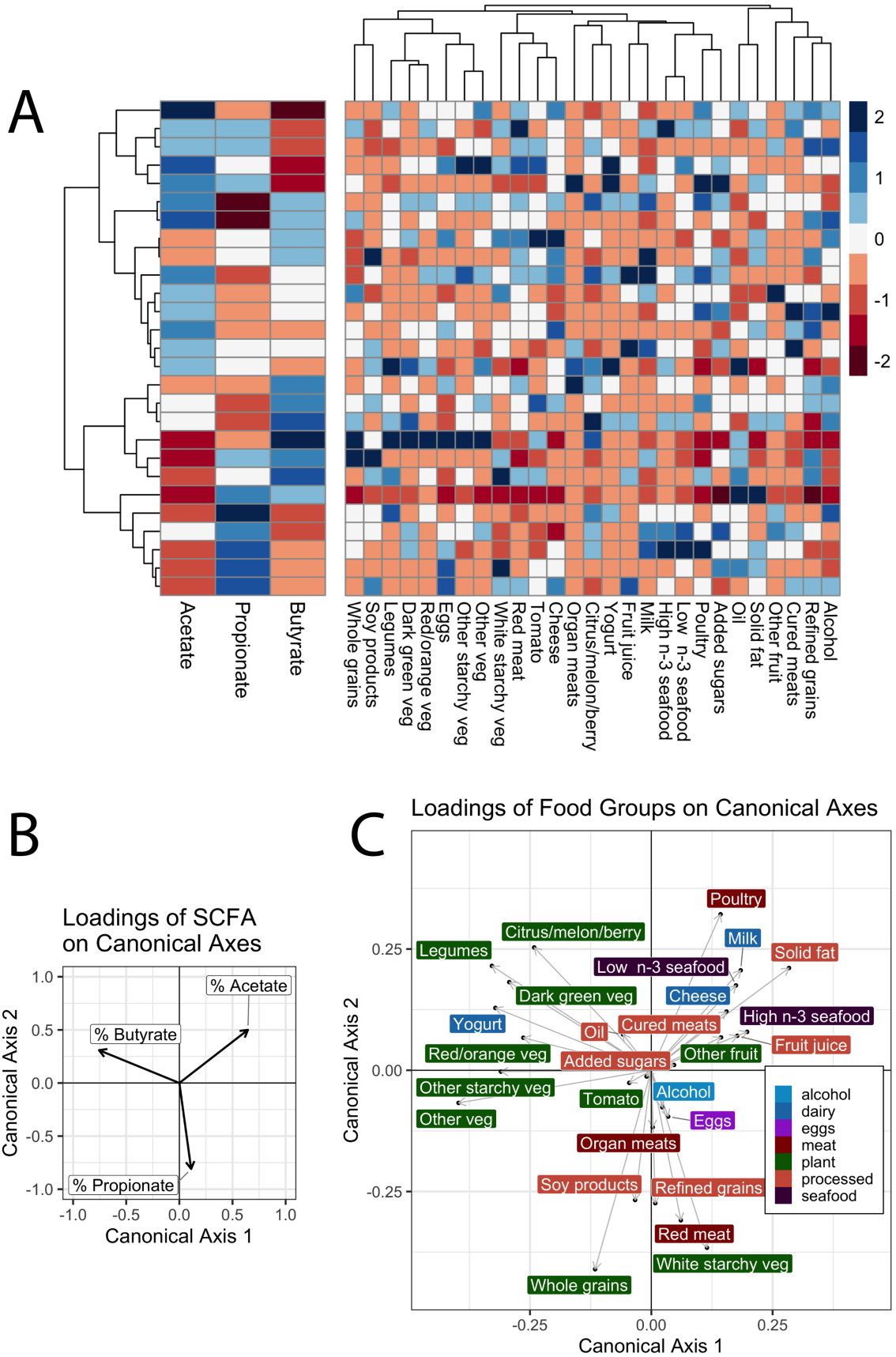


Figure 4: Relationships between habitual diet, baseline proportional butyrate concentration, and metrics of SCFA production. (A) Average change in proportional butyrate concentration of stool during intervention (Δ%butyrate) was negatively correlated with habitual dietary fiber consumption ($\rho = -0.40$, $p = 0.046$; Spearman correlation). (B) Average Δ%butyrate was also negatively correlated with baseline proportional butyrate concentration ($\rho = -0.49$, $p = 0.008$; Spearman correlation). (C) This relationship between habitual fiber consumption and SCFA production from prebiotics was also observed *in vitro* ($\rho = -0.46$, $p = 0.021$; Spearman correlation).


A
Acetate
Propionate
Butyrate
Whole grains
Soy products
Legumes
Dark green veg
Red/orange veg
Eggs
Other starchy veg
Other veg
White starchy veg
Red meat
Tomato
Cheese
Organ meats
Citrus/melon/berry
Yogurt
Fruit juice
Milk
High n-3 seafood
Low n-3 seafood
Poultry
Added sugars
Oil
Solid fat
Other fruit
Cured meats
Refined grains
Alcohol
2
1
0
-1
-2
B
Loadings of SCFA on Canonical Axes
% Acetate
% Butyrate
% Propionate
Canonical Axis 2
Canonical Axis 1
1.0
0.5
0.0
-0.5
-1.0
C
Loadings of Food Groups on Canonical Axes
Poultry
Citrus/melon/berry
Milk
Legumes
Solid fat
Low n-3 seafood
Dark green veg
Cheese
High n-3 seafood
Yogurt
Oil
Cured meats
Fruit juice
Red/orange veg
Added sugars
Other fruit
Other starchy veg
Tomato
Alcohol
Other veg
Eggs
Organ meats
Soy products
Refined grains
Red meat
White starchy veg
Whole grains
alcohol
dairy
eggs
meat
plant
processed
seafood
Canonical Axis 2
Canonical Axis 1
0.25
0.00
-0.25

537 **Figure 5: Relationship between baseline SCFA profiles and habitual intake of food groups.**
538 (**A**) Baseline SCFA profiles and average food intakes among participants. Participants (rows)
539 clustered based on proportional SCFA profiles. Cells values represent z-scores within each
540 column. Loadings shown of proportional SCFA concentrations (**B**) and consumed food groups
541 (**C**) along canonical axes 1 and 2 resulting from co-inertia analysis of SCFA and diet data ($p =$
542 0.031). Each axis is represented identically in each plot, so that eigenvectors (direction of
543 arrows) correspond between the SCFA and food group plots and the magnitude of eigenvalues
544 (length of arrows) is related to the strength of that component in the axis loading.

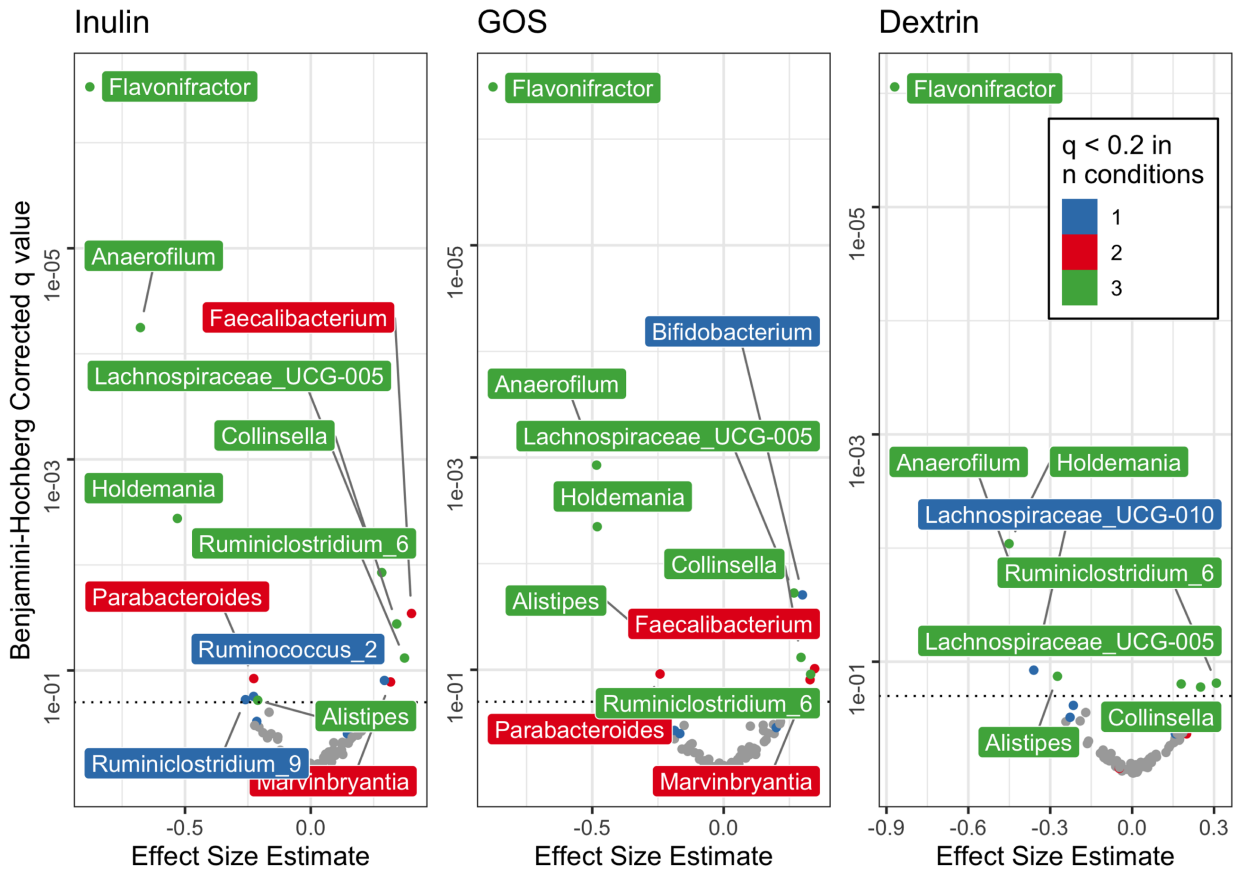


Figure 6: Gut bacterial taxa whose abundance changes during prebiotic-supplemented *ex vivo* fermentation. Bacterial abundances were compared to prebiotic-free control fermentation. Colors represent the number of prebiotic conditions in which a genus was differentially abundant (Benjamini-Hochberg corrected q value < 0.2, visualized with horizontal dashed line). Effect size estimate as reported by ALDEx2 is the median difference between groups (prebiotic and control) divided by the maximal difference within either group, in clr-transformed coordinates.

Supplementary figures and tables

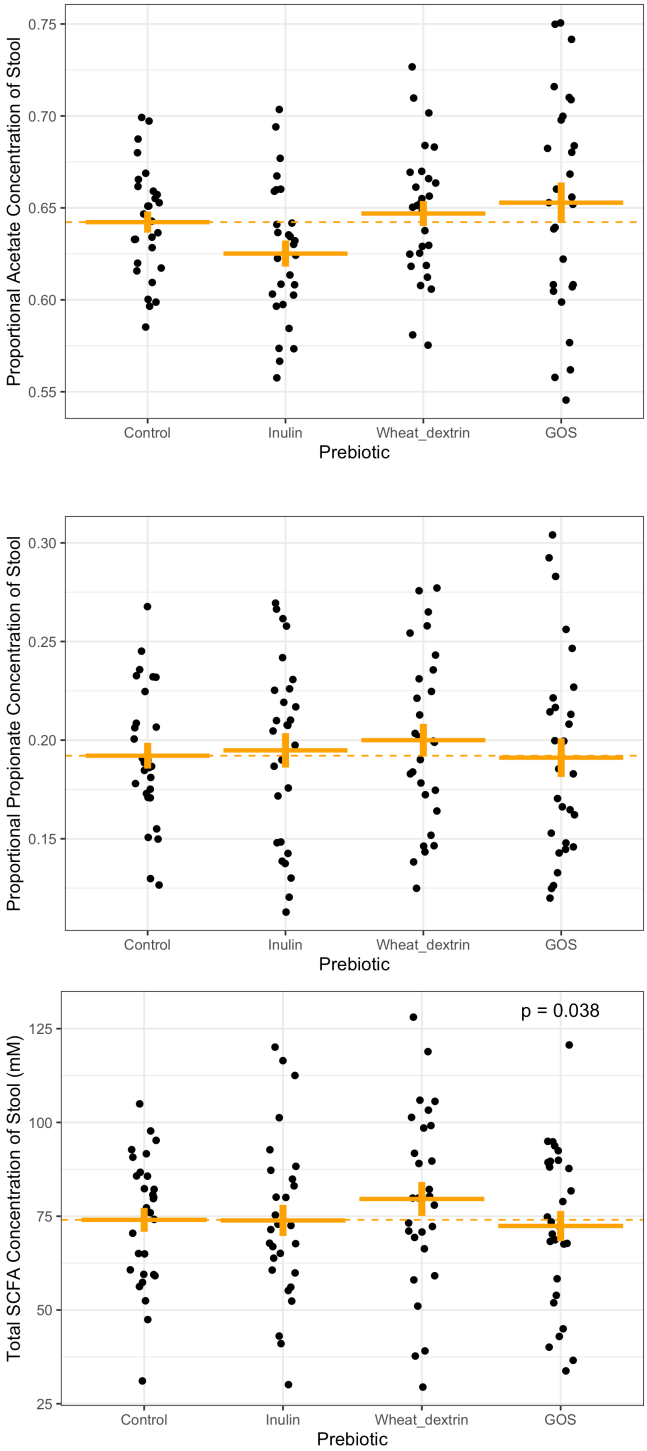


Figure S1: Proportional Acetate (A), proportional propionate (B), and total SCFA (C) concentrations during baseline and prebiotic interventions. All significant differences between prebiotic treatments and control are designated by displayed p values, calculated using a series of generalized linear mixed models.

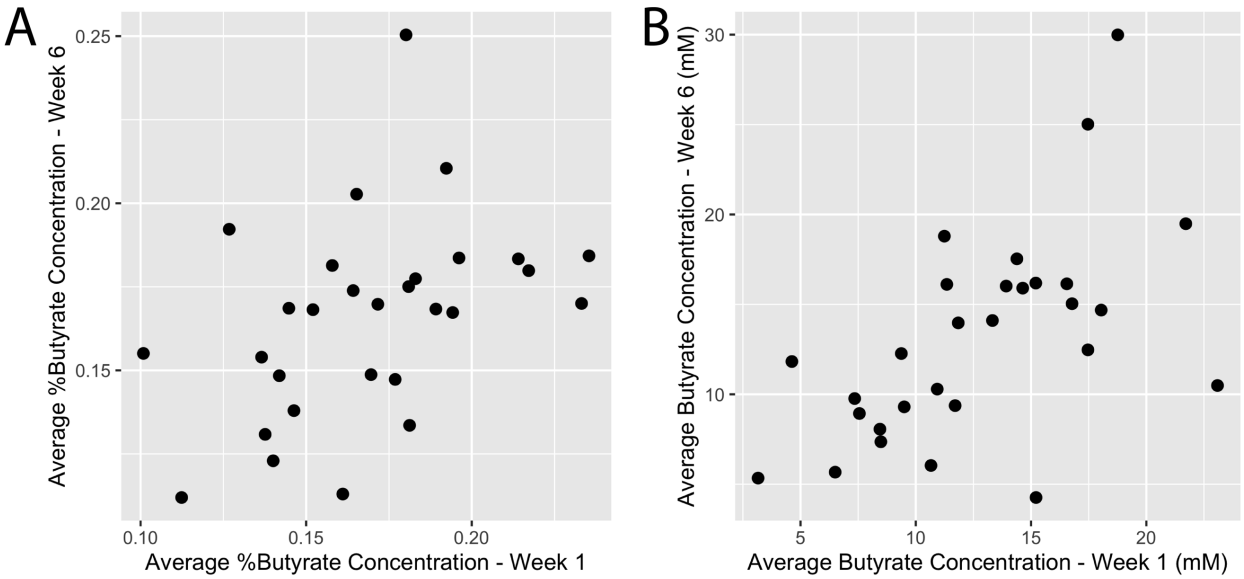


Figure S2: Average proportional butyrate concentration (A) and average butyrate concentration (B) in participant stool during the first and last week of the study. Both proportional concentration and raw concentration were correlated between the first and last weeks ($\rho = 0.49$, $p < 0.007$; and $\rho = 0.63$, $p = 0.0003$, respectively, Spearman correlations).

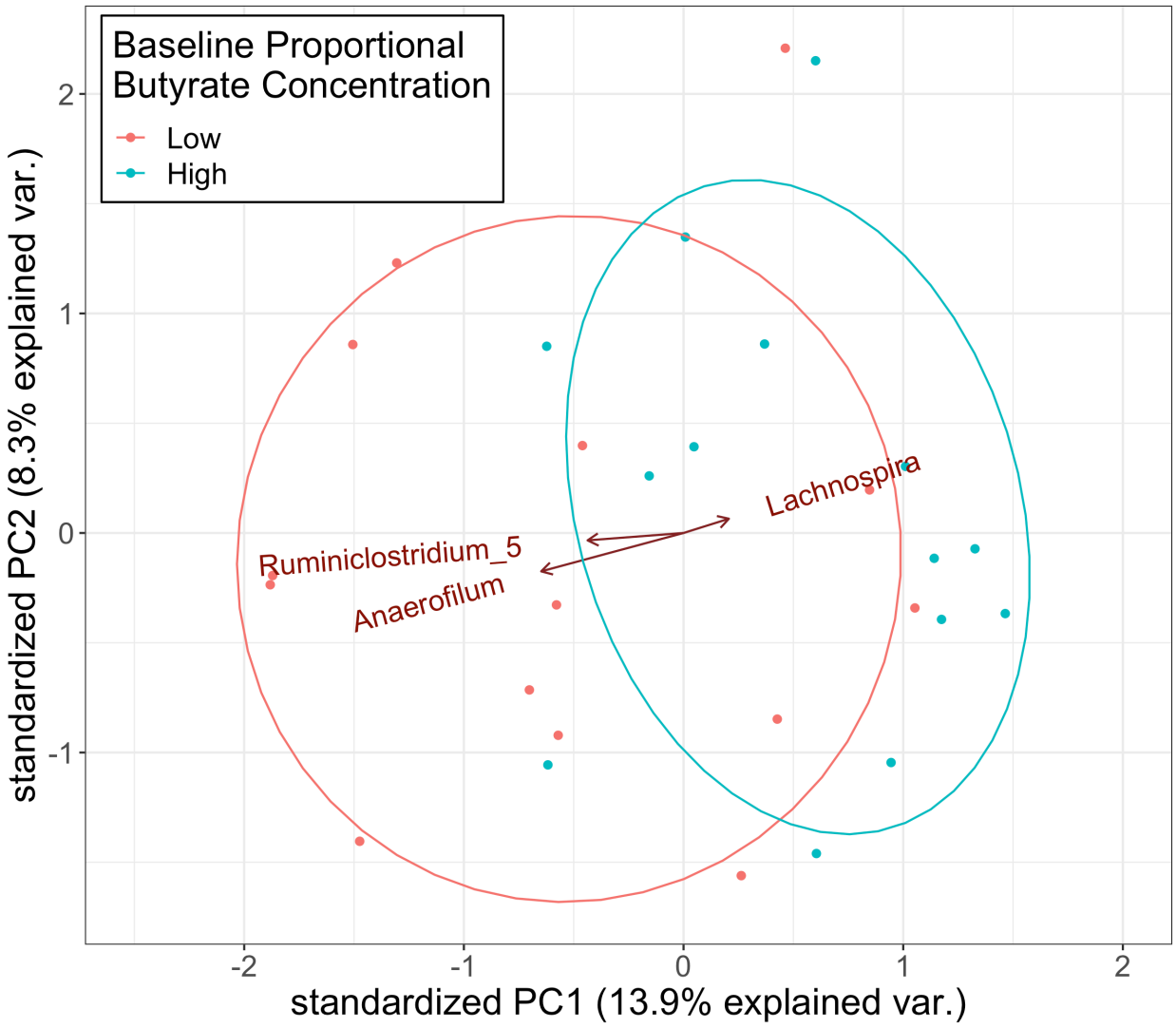


Figure S3: Biplot of stool 16S community composition showing samples (dots) plotted on two principal component axes along with corresponding taxonomic variables (arrows), agglomerated to genus level. Samples are colored by group (high and low; > median baseline %butyrate and <= median baseline %butyrate, respectively) and ellipses represent 95% confidence intervals of the centroid for each group. Centroids are significantly different ($p = 0.0022$; PERMANOVA using unweighted UniFrac distance). Only significantly differentially abundant genera (Benjamini-Hochberg corrected $q < 0.05$, ALDEx2) are represented by arrows and labels.

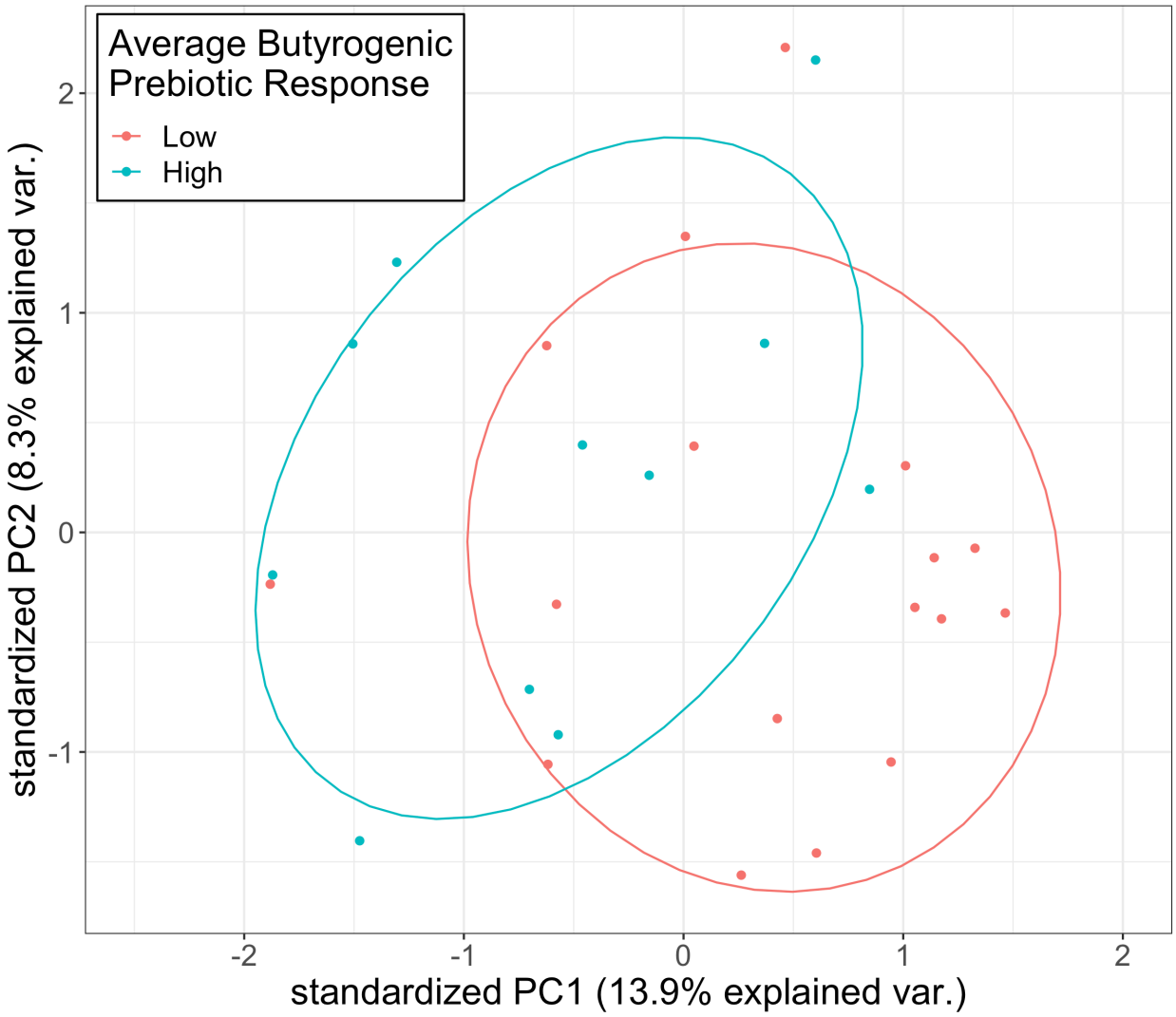


Figure S4: PCA of stool 16S community composition showing samples plotted on two principal component axes. No genera were significantly differentially abundant (Benjamini-Hochberg corrected q < 0.05, ALDEx2), so no variables are plotted**.** Samples are colored by group (high and low; > 0 average butyrogenic response to prebiotics and < 0 average butyrogenic response to prebiotics, respectively) and ellipses represent 95% confidence intervals of the centroid for each group. Centroids are significantly different (p = 0.028; PERMANOVA using unweighted UniFrac distance).

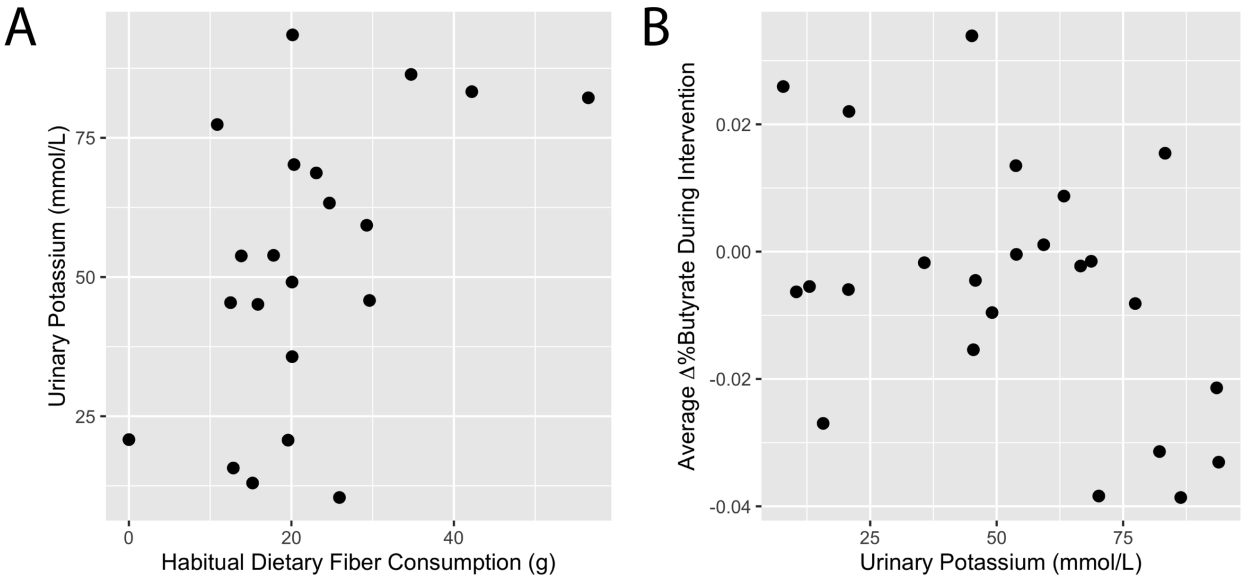


Figure S5: Relationships between urinary potassium and (A) habitual dietary fiber consumption, and (B) average change in proportional butyrate concentration during prebiotic interventions. Urinary potassium was positively correlated with habitual fiber consumption ($\rho = 0.47$, $p = 0.031$; Spearman correlation; A) and showed evidence for a negative correlation with average change in proportional butyrate concertation during prebiotic interventions ($\rho = -0.38$, $p = 0.071$; Spearman correlation; B).

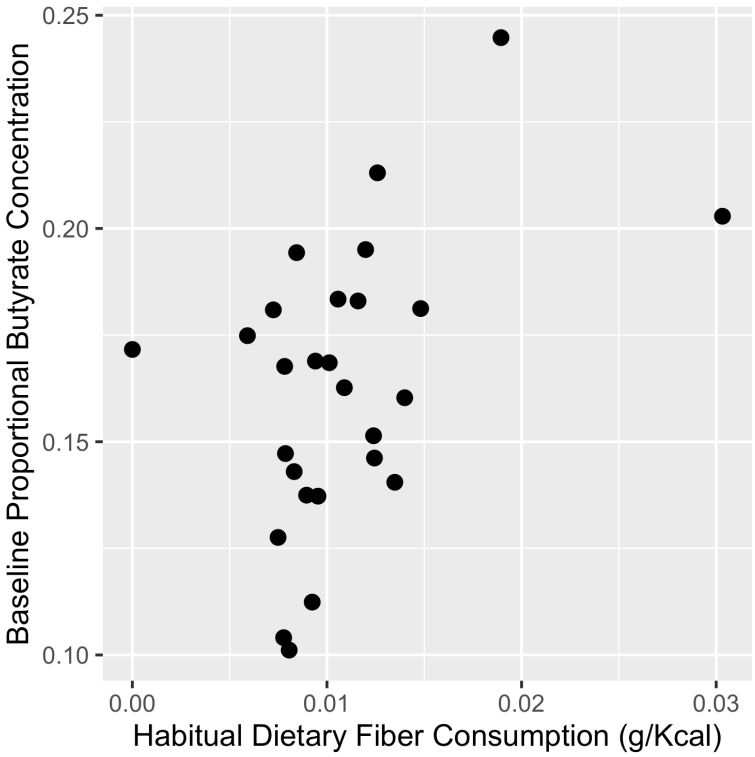


Figure S6: Relationship between habitual dietary fiber consumption and baseline proportional butyrate concentration at week 1 (prior to study intervention). Fiber consumption was positively associated with baseline proportional butyrate concentration, with 1g fiber/2000 kilocalorie diet associated with an increase in proportional butyrate concentration of 0.010 ($p = 0.012$, beta regression).

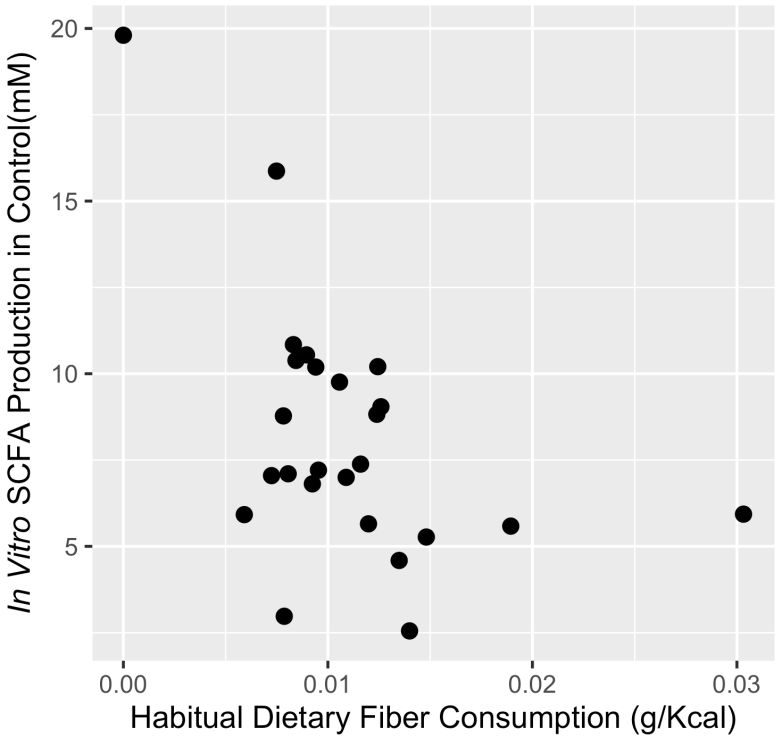


Figure S7: Relationship between *in vitro* SCFA production in prebiotic-free control treatments and habitual dietary fiber consumption. *In vitro* SCFA production was negatively correlated with habitual fiber consumption ($\rho = -0.43$, $p = 0.031$; Spearman correlation).

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