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 are
73 a promising microbiota-targeting approach for promoting health (34).

74 A key challenge of using prebiotics to promote SCFA production is heterogeneity in
75 treatment response. Many distinct prebiotic formulations exist (35, 36), 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, 37, 38), or tested different prebiotics in separate cohorts (30, 32, 39).
81 More rare crossover studies have taken place, but have either not investigated individual
82 variation in prebiotic response (40), or solely utilized *in vitro* assays of prebiotic response (41,
83 42). 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 (43), and that
89 individual characteristics like habitual food choice can predict changes to microbiota community
90 composition following prebiotic supplementation (38).
91 To directly investigate the interaction of prebiotic and individual variation in prebiotic
92 response, we report here the results of a randomized, three-way, crossover trial where a single set
93 of individuals was treated with multiple prebiotics. The design was uniform in period (each
94 intervention occurring the same number of times in an intervention period) and in series (each
95 intervention occurring the same number of times in a intervention series), and fully balanced
96 with respect to carryover effects (each intervention is preceded by each other intervention an
97 equal number of times across the study). Our primary lines of inquiry were to assess the relative
98 contribution of individual and prebiotic differences to SCFA responses to supplementation, with
99 an emphasis on butyrate production. Secondary analyses included determining what host and
100 microbiological factors shaped variation in prebiotic response.
101
102 **Methods**

103 Participant recruitment:

104 This study was conducted at Duke University in Durham, NC, USA, and was registered
105 with ClinicalTrials.gov (Identifier: NCT03595306). All recruitment and study procedures
106 involving human subjects were approved by the Duke University Institutional Review Board
107 (Pro00087214). Study participants were recruited by flyers, email list-servs, and word-of-mouth
108 at Duke University Campus. Individuals with a self-reported history of irritable bowel syndrome,
109 inflammatory bowel disease, type-2 diabetes, kidney disease, intestinal obstruction, or colorectal
110 cancer were excluded. Participants were also excluded if they had taken oral antibiotics within
111 the past six months, had any known food allergies, reported dietary intolerances of any kind, or
112 were pregnant or breastfeeding. If individuals identified as female, their urine was subjected to a
113 pregnancy test (Quidel QuickView One-Step hCG Urine Test) to minimize the risk that prebiotic
114 treatment would impact newborn or infant health. Participants who tested positive were deemed
115 ineligible for participation in the study. Inclusion criteria were that participants were between
116 the ages of 18 and 70, and able to provide stool samples at no risk to themselves. Fiber
117 consumption, prebiotic/probiotic usage, and dietary habits were not used as inclusion/exclusion
118 criteria, but participants were asked to report via surveys as part of the study requirements.
119 During this period, 81 individuals set up a phone interview to determine eligibility and interest to
120 participate in the study. Of these, 41 completed electronic informed consent to the study and
121 were subsequently enrolled. Ultimately, eight subjects were lost to follow-up, two withdrew, and
122 three were omitted from some or all analyses due to inability to collect sufficient usable sample,
123 resulting in 28 subjects included in all final data analyses.

124

125 Study design:

126 The three-period crossover longitudinal study was conducted from May of 2018 to March
127 of 2019. The crossover design allowed investigation of three prebiotic interventions while also
128 allowing participants to serve as their own controls (Figure 1). There were six arms of the study,
129 to cover all possible orders of prebiotic consumption and to eliminate the need to account for
130 uneven carry-over effects. The study consisted of an initial study visit, followed by a 6-week
131 study period with alternating weeks of baseline (no prebiotic consumption) and intervention
132 (prebiotic consumption). Participants were added sequentially to an arm group as they enrolled;
133 meaning the first participant was enrolled in Arm one, the second to Arm two and so on.
134 Participants were blinded to the prebiotics that they were consuming until the end of the study.
135 The prebiotics were taken twice a day, Monday to Friday during the intervention weeks. Monday
136 was a half-dose, to help allow participants to adjust to the increase in fiber. Following consent,
137 participants had an initial study visit, to have their baseline physiology assessed by height,
138 weight, and blood pressure measurement, as well as provide a urine sample and receive the study
139 kit and materials. Participants were asked to self-collect stool at home three times a week each
140 week during the study period. Participants also completed four diet surveys throughout the study;
141 one instance of the Diet History Questionnaire Version 3 (DHQ3) (44), covering the one month
142 period prior to the study, and three instances of the Automated Self-Administered 24-hour
143 (ASA24) Dietary Assessment Tool, version 2017, developed by the National Cancer Institute,
144 Bethesda, MD (45). Two ASA24's were completed during the week, and one on a weekend. The
145 DHQ3 was completed prior to the 6-week study period. We found that correlations of total
146 calories, protein, fat, carbohydrate, and dietary fiber between the two tools were robust (Calories;
147 $p = 0.032$, $r = 0.42$: Protein; $p = 0.021$, $r = 0.45$: Fat; $p = 0.036$, $r = 0.41$: Carbohydrates; **p =**
148 **0.069**, $r = 0.38$: Dietary fiber; $p = 0.001$, $r = 0.59$: Pearson correlations). Additionally,

149 participants completed online-surveys about their experiences with the prebiotics following the
150 intervention weeks.
151
152 Prebiotic supplementation:
153 Three prebiotics were tested in this study: inulin (manufactured by Now Foods,
154 Bloomingdale, IL), wheat dextrin (Benefiber, manufactured by GSK Consumer Healthcare,
155 Warren, NJ), and galactooligosaccharides (GOS) (Bimuno, manufactured by Clasado Limited,
156 Shinfield, UK). In a clean non-laboratory space, prebiotics were weighed into individual doses
157 and packaged into pre-labeled food-safe sealable plastic bags. Each participant received all three
158 prebiotics throughout the study; inulin at 9g / day, wheat dextrin at 9g / day, and
159 galactooligosaccharides (GOS) at 3.6g / day. These doses correspond to the recommended daily
160 intake on the product label. In order to further minimize the possibility of discomfort associated
161 with consuming prebiotics, the first day of dosing for each prebiotic was a half dose to allow
162 acclimation (4.5g for inulin and dextrin, 1.8g for GOS). Participants consumed one of the three
163 prebiotics at a time, twice a day, for a duration of five days, followed by a week of washout. The
164 order was determined by their assigned study arm. Participants were instructed to consume the
165 prebiotics by mixing the powders in any kind of beverage (such as water) or food (such as
166 applesauce), so long as it was cold or room temperature.
167
168 Compliance and side effects:
169 Surveys suggested participants complied with the design of the prebiotic intervention.
170 After each prebiotic intervention week (weeks 2, 4, and 6), participants were sent a survey that
171 asked about missed doses. 26 of 28 participants responded to the surveys, with an average of

172 2.77 out of 3 surveys being returned. Of the 72 person-weeks surveyed, 18 included missed
173 doses. Of 720 doses surveyed, 26 were missed, indicating a study average missed dose rate of
174 3.6%. No respondent missed more than 14% of doses over the course of the study, and none
175 missed more than 30% of doses during any intervention week.

176 Side-effects reporting also provided evidence that participants consumed the prebiotics.
177 After each prebiotic intervention week, participants were sent a survey that asked about side
178 effects. When asked to identify what extent they agree with the statement "The prebiotic caused
179 my gastrointestinal discomfort, 7 out of 23 respondents indicated that they 'agree' with this
180 statement (as opposed to 'neither agree nor disagree', 'disagree', and 'strongly disagree') during
181 the inulin supplementation week. For GOS and dextrin, 3 of 25 and 4 of 24 indicated agreement.
182 When asked whether the prebiotic affected the specific symptoms of abdominal pain, bloating,
183 flatulence, nausea, reflux/heartburn, and borborygmi, fewer than 20% of participants reported
184 'increased' or 'greatly increased' symptoms, except in the case of flatulence (all prebiotics) and
185 abdominal pain (inulin). 12 of 23 respondents indicated increased or greatly increased flatulence
186 on inulin, 6 of 25 on GOS, and 8 of 24 on dextrin. Six of 23 respondents indicated increased
187 abdominal pain on inulin, compared of none on GOS and 2 on dextrin. There were no differences
188 in self-reported weekly average Bristol stool hardness between baseline and any of the three
189 prebiotic interventions ($p = 0.61$, 0.20, and 0.38 for inulin, GOS, and dextrin, respectively; paired
190 t tests).

191 Consistent with the notion that prebiotic impacts are stronger in individuals who tend to
192 consume less fiber, we found evidence of a higher likelihood for side effects in those individuals
193 who habitually consumed less fiber. Participants who indicated that GOS or Dextrin increased
194 ('greatly increased' or 'increased') flatulence had significantly lower habitual fiber consumption

195 than did those participants who indicated that GOS or Dextrin decreased or did not affect
196 flatulence ('did not affect' or 'reduced') ($p = 0.006$ and $p = 0.045$ for GOS and Dextrin,
197 respectively; t-test). This affect was not present for inulin ($p = 0.45$). Similarly, Participants who
198 indicated that GOS or Dextrin increased ('greatly increased' or 'increased') borborygmi had
199 significantly or nearly significantly lower habitual fiber consumption than did those participants
200 who indicated that GOS or Dextrin decreased or did not affect borborygmi ('did not affect' or
201 'reduced') ($p = 0.065$ and $p = 0.019$ for GOS and Dextrin, respectively; t-test). This affect was
202 not present for inulin ($p = 0.64$). Further, fiber consumption other than the provided supplement
203 did not change over the course of the study ($p = 0.15$, ANOVA).
204
205 Stool collection:
206 At the pre-study visit, participants were provided with a standard urine specimen collection cup
207 and shown to a bathroom to collect the sample. Urine samples were also sent to LabCorp in a BD
208 Vacutainer UA tube to measure metabolic variables, including routine urinalysis, sodium,
209 potassium, urea nitrogen, and creatinine. Consented participants were asked to self-collect stool
210 three times a week at home using sampling kits provided to them. Participants were asked to
211 immediately store samples in their personal freezers (-20C). Participants were asked to bring
212 stored samples to the David Lab freezers on a weekly basis. Stool samples were transferred and
213 stored at -80C until thawed for DNA extraction and other sample processing.
214
215 *In vitro* fermentation:
216 *In vitro* fermentation of stool microbiota was conducted following protocols previously
217 published (41). This method has been shown to be robust to the effects to the effects of freezing

218 stool, with total SCFA production remaining correlated between fresh stool and stool frozen and
219 thawed up to two times (41), and butyrate production remaining correlated between fresh stool
220 and stool frozen once ($p = 0.0004$, $\rho = 0.49$, Spearman correlation), as were samples in this
221 study. In brief, a 10% w/v fecal slurry was made using thawed week-1 stool and anaerobic 1X
222 PBS (pH 7.0 ± 0.1). This slurry was filtered through 0.33mm pore size mesh, then mixed 1:1
223 with a 1% w/v solution of prebiotic and incubated in duplicate in a 24-well cell culture plate for
224 24hr. We expect these *in vitro* fermentations to represent the non-adherent microbial fraction of
225 stool, as well as bacteria adhered to smaller particles that pass through a 0.33mm filter. Three
226 prebiotic treatments were used, corresponding to the three prebiotics used in the *in vivo* portion
227 of this study: inulin (Now Foods Inulin Powder, part #2944), galactooligosaccharides (GOS;
228 Bimuno Powder), and wheat dextrin (Benefiber Original). The resulting fermentation conditions
229 where therefore 5% fecal slurry with 0.5% prebiotic (w/v) and occurred at 37°C for 24 hours.
230 Following fermentation, a 1mL aliquot was taken from each well for SCFA quantification.
231
232 Quantification of SCFA:
233 Quantification of SCFA by gas chromatograph flame ionization detection (GCFID) was
234 performed following protocols previously published (41). In brief, a 1mL aliquot of either 10%
235 fecal slurry in PBS or a 10% solution of the fermentation vessel contents was obtained. To this,
236 50 μL of 6N HCl was added to acidify the solution to a pH below 3. The mixture was vortexed,
237 centrifuged at 14,000rcf for five minutes at 4°C to remove particles. Avoiding the pellet, 750 μL
238 of this supernatant was passed through a 0.22μm spin column filter. The resulting filtrate was then
239 transferred to a glass autosampler vial (VWR part #66009-882).

240 Filtrates were analyzed on an Agilent 7890b gas chromatograph (GC) equipped with a
241 flame-ionization detector (FID) and an Agilent HP-FFAP free fatty-acid column (25m x .2mm id
242 x .3µm film). Acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate were identified
243 and quantified in each sample by comparing to an 8-point standard curve that encompassed the
244 sample concentration range. Standards contained 0.1mM, 0.2mM, 0.5mM, 1mM, 2mM, 4mM,
245 8mM, and 16mM concentrations of each SCFA. Once measured, the SCFA concentrations were
246 multiplied by a scaling factor of 10 to return them to their undiluted value. We report SCFA
247 concentrations as mM, however, based on our methodology using a standardized stool mass /
248 diluent ratio, these mM measurements are quantitatively equivalent to mmol/kg (*e.g.* 5mM is equal
249 to 5mmol/kg).
250
251 DNA extraction, PCR amplification, and sequencing:
252 We performed 16S rRNA gene amplicon sequencing on human stool samples to determine
253 microbiota community composition following protocols previously published (41). DNA was
254 extracted from frozen fecal samples with the Qiagen DNeasy PowerSoil DNA extraction kit (ID
255 12888-100). Amplicon sequencing was performed using custom barcoded primers targeting the
256 V4 region of the 16S rRNA gene (46), using published protocols (46-48). The sequencing library
257 was diluted to a 5pM concentration and sequenced using an Illumina MiniSeq and a MiniSeq Mid
258 Output Kit (FC420-1004) with paired-end 150bp reads.
259
260 Identifying sequence variant and taxonomy assignment:
261 We used an analysis pipeline with DADA2 (49) to identify and quantify sequence
262 variants, as previously published (41, 50). To prepare data for denoising with DADA2, 16S

263 rRNA gene primer sequences were trimmed from paired sequencing reads using Trimmomatic
264 v0.36 without quality filtering (51). Barcodes corresponding to reads that were dropped during
265 trimming were removed using a custom python script. Reads were demultiplexed without quality
266 filtering using python scripts provided with Qiime v1.9 (52). Bases between positions 10 and 150
267 were retained for the forward reads and between positions 0 and 140 were retained for the
268 reverse reads. This trimming, as well as minimal quality filtering of the demultiplexed reads was
269 performed using the function fastqPairedFilter provided with the DADA2 R package (v1.8.0).
270 Sequence variants were inferred by DADA2 independently for the forward and reverse reads of
271 each of the two sequencing runs using error profiles learned from all 20 samples. Forward and
272 reverse reads were merged. Bimeras were removed using the function removeBimeraDenovo
273 with default settings. Taxonomy was assigned using the function assignTaxonomy from
274 DADA2, trained using version 123 of the Silva database.
275
276 Statistics and modelling:
277 All statistical analyses and modelling for this study were conducted in R (R version 3.4).
278 T-tests, Wilcoxon Rank-Sum tests, Spearman correlations, Pearson correlations, and ANOVA
279 tests were conducted with base R. PERMANOVAs were carried out with the 'adonis2' function
280 from the R package 'vegan'. Differential abundance tests were carried out using the R package
281 'ALDEx2'. Linear models, and generalized linear models were implemented with the 'lm' and
282 'glm' functions from base R. Generalized linear mixed models (GLMM) were implemented with
283 the 'glmer' function from R package 'lme4'. P values for effects in linear models and
284 generalized linear models were calculated using the 'summary' function in base R on fitted 'lm'
285 and 'glm' objects and are based on F-tests. P values for effects in GLMMs were calculated using

286 the 'summary' function (base R) on fitted 'glmer' objects, and are based on Wald Z-tests. All R
287 code used to analyze the data presented in this manuscript is freely available on a publicly
288 accessible repository (https://doi.org/10.6084/m9.figshare.c.5405505).
289
290 **Results**
291 **Study Participants.** We enrolled participants into a six-week, three-period prebiotic intervention
292 study (Figure 1). We used a crossover design to compare multiple prebiotics across a subject
293 pool. We selected inulin, wheat dextrin, and galactooligosaccharides (GOS) as our prebiotics, as
294 these are commonly consumed (53), commercially available dietary supplements, and are
295 monomerically distinct. Our crossover was standardized in series and period, with each prebiotic
296 occurring the same number of times within each series (once) and study period (twice). The
297 design was also balanced with respect to first-order carry-over effects, meaning we shuffled the
298 order in which prebiotics were administered so as to minimize the interactive effects between
299 any two pairs of consecutive prebiotics. Each study period consisted of an intervention-free
300 baseline week and a prebiotic intervention week. Stool samples were collected on days three,
301 four, and five of each of the six study weeks (baselines and intervention). Of the 41 participants
302 who initially enrolled, two withdrew, three were unable to collect at least one usable sample (i.e.,
303 insufficient volume or inadequate labeling) per week, and eight were lost to follow-up. We
304 focused our subsequent analyses on the remaining 28 participants who completed the study.
305 These participants in total collected 413 stool samples (mean=2.46 stool samples per person per
306 week).
307

308 **Prebiotic supplementation affected SCFA production at the cohort-level.** We first sought to
309 examine differences in microbiome response during each of the different prebiotic treatments.
310 Analyses of gut microbial community composition did not reveal associations with inulin, GOS,
311 or wheat dextrin treatment ($p > 0.05$, PERMANOVA using unweighted UniFrac distance at
312 species level). In addition, no species were statistically differentially abundant during any
313 prebiotic intervention and its corresponding prebiotic-free baseline ($p > 0.05$, Benjamini-
314 Hochberg corrected Wilcoxon Rank Sum tests using the R package ALDEx2). While previous
315 studies have observed changes in community composition following prebiotic administration,
316 such effects have been observed in a subset of tested prebiotics (30, 32), and with different
317 dosing schedules (30-32, 38). We also did not observe lasting shifts in community composition
318 over the course of the study at the cohort level, as baseline weeks three and five did not differ
319 from baseline week one by PERMANOVA ($p = 0.995$ and 0.999, respectively).
320 Still, in contrast to the lack of a coherent 16S rRNA gene shifts among participants, we
321 found the three prebiotics varied according to resulting fecal SCFA profiles relative to baseline.
322 GOS was associated with decreased total stool SCFA concentrations (Table 1, Figure S1C, effect
323 size = -0.071, $p = 0.038$, GLMM), an outcome that has been previously observed (54).
324 Differences within individual SCFAs were also apparent when analyzing absolute fecal SCFA
325 concentrations (Table 1). Dextrin increased propionate concentration (effect size = 0.074, $p =$
326 0.038, GLMM), and GOS decreased both propionate and butyrate concentrations (effect size = -
327 0.087, $p = 0.015$; effect size = -0.14, $p = 0.002$, GLMMs, respectively). Still, changes in total and
328 individual SCFA concentrations may be caused by effects of stool hydration, which can vary up
329 to three-fold and directly impacts measures of SCFA concentration in feces (55, 56). To assess
330 the relative production of each SCFA, we therefore normalized levels of individual SCFAs to

account for differences in stool hydration. Our resulting analysis found that inulin supplementation increased proportional butyrate concentrations (butyrate concentration / total SCFA concentration) in participants' stool (effect size = 0.082, $p = 0.007$, GLMM; Figure 2, Table 1). These findings are consistent with prior reports that inulin supplementation may increase butyrogenesis (57-59). Our analyses also suggested GOS and wheat dextrin decreased proportional butyrate concentration in participants' stool (GOS; effect size = -0.074, $p = 0.012$. Dextrin; effect size = -0.071, $p = 0.015$, GLMM; Figure 2, Table 1). These findings align with prior trials that found both GOS (54, 60, 61) and dextrin (62, 63) increases acetate production over more reduced SCFAs, including butyrate and propionate, potentially by shunting nutrients away from butyrate production. No prebiotic altered proportional acetate or proportional propionate concentration at the cohort level ($p > 0.05$, GLMM; Figures S1A and S1B, Table 1).

Butyrogenic responses are more strongly predicted by individual identity than by prebiotic. We next investigated how prebiotic responses varied among individuals. We focused our analysis on prebiotic impacts on butyrate production because it was the sole SCFA that was altered at the cohort-level, and it is also the microbially derived SCFA most commonly implicated in enteric health (2, 6-8, 64-67). We also focused on proportional butyrate concentrations to avoid the effects of stool hydration on our analysis and to be consistent with previous individual-level analyses of fecal SCFAs (32, 43). In line with prior studies of prebiotic response, (41) statistical modeling yielded evidence for individual-specific butyrogenic responses to each of the prebiotics (that is, an interaction between individual and prebiotic terms in a GLMM). Nonetheless, further analyses suggested that the strongest determinant of proportional butyrate responses was shaped by individual identity: individual-specific effects

354 (pseudo-R^2 = 0.39, $p < 0.0001$; GLM F-test) were much stronger than prebiotic-specific effects
355 (pseudo-R^2 = 0.05, p = 0.001; GLM F-test). Additionally, individuals who exhibited the largest
356 response in proportional stool butyrate concentration on inulin were more likely to exhibit larger
357 responses on dextrin (ρ = 0.45, p = 0.015, Spearman correlation; Figure 3). We observed a
358 similar, positive, non-significant correlation between dextrin and GOS (ρ = 0.35, p = 0.065).
359 This relationship was not observed for inulin and GOS (ρ = 0.12, p = 0.53). This pattern was
360 apparent in spite of correcting for baseline differences in SCFA production between individuals,
361 which were also consistent over time and between baseline and treatment weeks.
362
363 **Individuals consistently varied according to SCFA metabolic state, microbiota community**
364 **composition, and SCFA production potential**. Why might individual-specific effects be
365 stronger than prebiotic-specific effects in our cohort? We observed that individuals were
366 idiosyncratic in their SCFA profiles over time. Both proportional butyrate concentration and
367 butyrate concentration were correlated within individuals between the first and last baseline
368 weeks (ρ = 0.49, $p < 0.007$; and ρ = 0.63, p = 0.0003, respectively, Spearman correlations;
369 Figure S2). Variation between individuals' SCFA profiles could also be linked to inter-individual
370 microbiota variation, a well-known factor in prebiotic response (43). When we divided
371 participants into 'high' (> median) and 'low' (<= median) fecal butyrate groups, we observed a
372 significant effect of this grouping on microbiota community composition (R^2 = 0.075, p =
373 0.0022, PERMANOVA using unweighted UniFrac distance; Figure S3). We identified three
374 specific genera that were differentially abundant between the groups: *Anaerofilum* and
375 *Ruminiclostridium_5* were decreased in the high butyrate group, and *Lachnospira* was increased
376 in the high butyrate group (p = 0.009, .043, and 0.030, respectively; Benjamini-Hochberg

377 corrected Wilcoxon Rank Sum tests; Figure S3). We additionally observed a relationship
378 between baseline microbiota community structure and average response to all three prebiotics
379 (Δ%butyrate). After dividing participants into 'responders' and 'non-responders', based on
380 whether average Δ%butyrate was positive or negative, we found a significant effect of this
381 grouping on microbiota community composition ($R^2 = 0.059$, $p = 0.028$, PERMANOVA using
382 unweighted UniFrac distance; Figure S4). Thus, individuals' starting microbiome composition
383 was associated with prebiotic treatment outcome.
384
385 **Baseline intake patterns of dietary fiber negatively correlated with response to prebiotic**
386 **intervention.**
387 Next, we sought to understand what host factors might influence individuals' microbiomes and
388 ultimately shape their butyrogenic responses across the tested prebiotics. We focused on fiber
389 intake, which has been shown to shape microbiota community composition and metabolic output
390 (68, 69). We measured fiber consumption using a dietary questionnaire spanning the month
391 preceding the study (44), and we compared fiber intakes to each individual's average response to
392 the three prebiotic interventions (average Δ%butyrate). Despite prior studies suggesting that
393 plant or fiber intake would augment gut microbial metabolic capacity (38, 69-71), our
394 comparison revealed that dietary fiber consumption was negatively correlated with average
395 Δ%butyrate ($\rho = -0.40$, $p = 0.046$; Spearman correlation; Figure 4A). Thus, higher habitual fiber
396 intake was associated with a lower average Δ%butyrate during the prebiotic intervention. To
397 corroborate these findings using an objective biomarker, we also compared average Δ%butyrate
398 to baseline spot urinary potassium, a known marker for fruit and vegetable intake (72), as well as
399 for general healthy eating (73, 74). We validated that baseline spot urinary potassium was

400 correlated with dietary fiber intake in our study ($\rho = 0.47$, $p = 0.031$; Spearman correlation;
401 Figure S5A). While we found no significant correlation between baseline spot urinary potassium
402 and average Δ%butyrate ($\rho = -0.38$, $p = 0.071$; Spearman correlation; Figure S5B), the
403 magnitude and direction of effect suggests a statistical relationship may have been apparent with
404 a larger sample size.
405
406 **Observational evidence that baseline SCFA metabolic state and dietary history limits**
407 **prebiotic response.** To understand why habitual fiber intake would be negatively associated
408 with prebiotic responses, we considered recent ecological studies suggesting that nutrient
409 limitation is a key factor shaping the metabolism of mammalian gut microbiota (75, 76). Even if
410 excess fiber is available to polysaccharide utilizing microbes, fixed caps in the availability of
411 other nutrients involved in carbohydrate fermentation could ultimately affect the relative
412 production of different SCFA species. Such relative changes would be caused by differences in
413 the oxidation state of SCFAs. Acetate is the most oxidized SCFA and tends to be produced first
414 and in higher quantities during fermentation (76-78). By contrast, butyrate is the most reduced
415 (76, 78) and the least energetically favorable (76) primary SCFA for the microbe to produce.
416 Thus, changes to overall SCFA production should be associated with changes in proportional
417 butyrate (76-78); and, by extension, individuals who naturally consume high levels of fiber and
418 already activate SCFA production pathways would exhibit reduced capacity to increase
419 proportional butyrate levels following prebiotic treatment.
420 We identified three findings that were consistent with a threshold model of metabolic
421 capacity limitation. First, we observed that individuals with higher starting proportional
422 concentrations of butyrate exhibited the weakest butyrogenic prebiotic responses. ($p = 0.008$, ρ

423 = -0.49, Spearman correlation; Figure 4B). In our study, no participant ultimately exhibited
424 butyrate concentrations in excess of 29mM following prebiotic treatment (though we note that
425 higher upper limits (e.g. greater than 40mM) have been previously observed (30)).
426 A second finding linking background nutrient intake and metabolic limitation involved
427 baseline proportional butyrate concentrations and habitual diet. Dietary fiber consumption was
428 positively correlated with proportional butyrate concentration at baseline week 1, prior to any
429 prebiotic intervention (1g fiber/2000 kilocalorie diet led to an increase in proportional butyrate
430 concentration of 0.010, p = 0.012, beta regression; Figure S6) as has been previously suggested
431 (33, 37, 79). Furthermore, using an ecological technique for identifying relationships between
432 multivariate datasets known as co-inertia analysis (80), we found a significant interaction
433 between baseline proportional SCFA concentrations and food choices(p = 0.031, coinertia
434 analysis). This analysis involved comparing patterns in the abundance of short chain acids and
435 food intake across matched samples (Figure 5A). Coinertia analysis associated the intake of
436 several fiber-rich food groups with baseline proportional butyrate concentrations including
437 legumes, berry fruits, and dark-green vegetables (vectors pointing towards the top-left in Figures
438 5B, C).
439
440 **Culture-based experimental evidence links fiber intake to limits on microbiome metabolic**
441 **response**. A third line of experimental evidence arose linking habitual nutrient intake and
442 microbial SCFA production capacity. We hypothesized that if higher baseline fiber consumption
443 reduced the capacity of gut microbiota to increase proportional butyrate production from
444 prebiotics, the metabolic activity of fecal microbiota would exhibit a negative relationship with
445 fiber intake. To test this hypothesis, we applied a recently developed an *ex vivo* fermentation

446 model (41) to baseline stool samples collected from each study participant. This model
447 experimentally tests the metabolic effects of supplementing a microbial community using a
448 given prebiotic as the sole additional carbon source, independent of potentially confounding host
449 factors like SCFA absorption, and has been shown to be robust to the effects of freezing stool
450 (41). We note that while our *in vivo* measurements rely on proportional SCFA concentrations
451 due to the potentially confounding effects of factors like stool hydration, these *in vitro* measures
452 allow us to directly measure absolute SCFA levels as an indicator of metabolic activity and
453 prebiotic utilization. In support of the model's capability to produce SCFAs following prebiotic
454 administration, we observed an increase in overall SCFA production relative to control ($p <$
455 0.0001, t-test; average of 4.41-fold increase across participants, calculated using the average
456 total SCFA concentration of three separate prebiotic treatments) when the *in vitro* assays were
457 supplemented with prebiotics as the sole carbon source. When we compared SCFA production
458 to fiber intake, we observed as expected a negative correlation between fiber consumption and
459 SCFA production ($p = 0.021$, $\rho = -0.46$, Spearman correlation using average of three prebiotic
460 treatments; Figure 4C). We also observed a negative correlation between fiber consumption and
461 SCFA production in prebiotic-free control treatments ($p = 0.019$, $\rho = -0.47$, Spearman
462 correlation; Figure S7). Thus, individuals who tended to eat more fiber in our cohort also
463 harbored gut microbiota that produced less SCFA when exposed to prebiotics. Still, we note that
464 a relationship was not observed when *in vitro* %butyrate was compared to baseline fiber
465 consumption ($p = 0.37$, Spearman correlation). This negative result may reflect the inability of
466 our *in vitro* assays to capture metabolic tradeoffs affecting the relative production of different
467 SCFAs following prebiotic challenge *in vivo*.
468

469 **Consistent growth of specific microbial genera supports individual-specific prebiotic**
470 **response.** Next, we used our *ex vivo* fermentations to associate specific gut bacterial taxa with
471 prebiotic response. Following 24hr fermentation, microbial community composition was
472 assayed from each prebiotic-supplemented culture and compared to no-carbon-added control
473 cultures, for each of the 28 participants, using ALDEx2. This analysis revealed multiple genera
474 that were over or under-represented in each prebiotic treatment (Figure 6).
475 Additionally, most genera that were differentially abundant in one prebiotic treatment
476 exhibited similar differential abundance in the other prebiotic treatments. This result is
477 consistent with prior studies finding that fiber-degrading bacteria capable of degrading a breadth
478 of dietary carbohydrates ("generalists") are abundant in the gut (81). Indeed, all genera
479 exhibiting conserved higher abundance in two or three prebiotic treatments compared to control
480 (*Collinsella*, *Faecalibacterium*, *Lachnospiraceae_UCG-005*, *Marvinbryantia*, and
481 *Ruminiclostridium*) are known degraders of common dietary fibers with broad specificity (82-
482 84). By contrast, of the genera exhibiting conserved lower abundance in two or three prebiotic
483 treatments compared to control (*Alistipes*, *Anaerofilum*, *Flavonifractor*, *Holdemania*, and
484 *Parabacteroides*), we are not aware of reports linking them to degradation of inulin, dextrin, or
485 GOS, and only *Parabacteroides* has been shown to degrade any dietary fiber (82, 84). We do
486 note though that we did not observe the aforementioned genera to be associated with prebiotic
487 responses in our *in vivo* data. Such findings suggest that other behavioral or lifestyle factors not
488 captured by *in vitro* assay contribute to variation in microbiome response to prebiotics in
489 humans.

490 Overall, the consistent response of select bacterial taxa to prebiotic addition *in vitro*
491 further supports the conclusion that responses across a range of prebiotics will be linked to
492 individual-specific factors like gut microbial community composition.
493
494 **Discussion**
495 In this study, we administered the prebiotics inulin, GOS, and dextrin to a set of
496 individuals using a fully balanced crossover design. We found that these prebiotics varied in
497 their impact on fecal SCFA concentrations when data was agglomerated at the population scale,
498 with inulin increasing proportional butyrate and both GOS and wheat dextrin decreasing
499 proportional butyrate. Yet, our analysis also suggested that individual identity exerted a much
500 stronger effect on prebiotic response, and we observed that responses to each prebiotic were
501 correlated within individuals. Individual response variation was associated with differences in
502 baseline microbiological and dietary factors. In particular, we found that proportional
503 butyrogenic response to prebiotics was anti-correlated with habitual fiber intake. We confirmed
504 the involvement of the microbiota (as opposed to the host) in this relationship using *in vitro*
505 fermentation experiments. This negative relationship may be explained by limitations in the
506 butyrate production capacity of human gut microbiota.
507 From a microbial ecological perspective, our findings support a model where microbial
508 metabolic repertoires within individuals' microbiota have the capacity to utilize a diverse array
509 of chemically distinct fiber types. Evidence in favor of this model include *in vitro* prebiotic
510 screens suggesting that individuals uniformly harbor bacteria capable of degrading a wide variety
511 of carbohydrate species (81). Such metabolic plasticity may benefit gut microbiota by enhancing
512 community stability and invasion resistance (85-87), and could be enabled by substrate-

513 switching at the level of strains (87, 88) or cross-feeding interactions (89). Still, differences in
514 the levels of strains underlying these metabolic interactions may vary between individuals (81,
515 90), which could in turn lead to inter-individual variation in the capacity of individuals'
516 microbiota to ferment prebiotics.

517 The nutritional implications of our work were, in our view, more surprising. A positive
518 relationship between dietary fiber consumption and microbiota community structure (91), and
519 metabolic function (69, 92-94) is well established, with higher fiber intake linked to greater
520 SCFA and, specifically, butyrate production. Thus, we expected that individuals habituated to a
521 high-fiber diet would have a greater capacity to utilize incoming novel fiber towards increasing
522 proportional butyrate production, not a lower capacity as we observed. Still, prior studies
523 reporting links between diet patterns and microbiota response have specifically associated
524 changes to microbial community composition with baseline fiber intake (38). Associations
525 between fiber consumption and SCFA production, by contrast, have either not been tested for in
526 prebiotic trials (95, 96) or not revealed a positive relationship (38). Additionally, while our
527 analyses confirmed an association between baseline proportional butyrate levels and intake of
528 plant foods groups linked to dietary fiber (e.g. legumes and dark green vegetables, Figure 5), it
529 also linked food groups typically associated with a Mediterranean diets (e.g. seafood, dairy, fruit,
530 and poultry (97)) with higher baseline levels of proportional acetate (Figure 5).

531 In light of the surprising nature of our findings, it is necessary to fully explore other
532 potential drivers of this negative relationship between baseline fiber consumption and prebiotic
533 response. One major concern is the potential for biased study compliance, where those
534 individuals consuming the most fiber habitually were the least compliant to consuming prebiotic
535 supplements. We found evidence to the contrary and observed an overall missed-dose rate of

536 only 3.6% based on self-report during post-intervention surveys. Importantly, participants were
537 not compensated for their participation in this study, so falsifying compliance surveys was
538 minimally incentivized. We also found that side-effects were not more likely in high baseline
539 fiber consumers, providing further evidence that high fiber consumers were not more likely to
540 skip prebiotic doses. Another concern is that the prebiotic supplements caused behavioral or
541 physiological alterations disproportionately. However, we found that habitual fiber consumption
542 did not decline throughout the study, and no differences in self-assessed Bristol stool hardness
543 were observed. Together, this evidence bolsters our confidence in concluding that the observed
544 negative relationship between baseline fiber consumption and butyrogenic response to prebiotics
545 is real and driven by microbiological factors. We also acknowledge our modest sample size of 28
546 individuals, and note that some additional relationships may become apparent with greater
547 statistical power. Future and larger prebiotic trials are needed to determine the generality of our
548 observed association between habitual diet and SCFA production.
549

550 **Conclusions**

551 Our study provides evidence that the responsiveness of an individual to prebiotic
552 treatment may be predictable from diet and baseline concentrations of SCFA in stool. We
553 emphasize that we do not believe these predictions should be used to modify the diets of
554 individuals who normally consume high levels of fiber or have higher concentrations of SCFA in
555 stool. Ample evidence supports the benefits of both a high fiber diet and adequate microbial
556 SCFA production (71, 98, 99). Rather, we argue our findings support strategies for personalizing
557 microbiota therapy that focus on individual selection. Prior work on prebiotic responses have
558 primarily used human studies with parallel design (30, 32, 39), which do not allow analysis of

559 individualized response across prebiotics. Here we show, we believe for the first time, that the
560 responses within individuals are correlated across at least some pairs of prebiotics, and that
561 average response correlates with host factors. Ongoing prioritization efforts are being used to
562 identify patients most likely to respond to microbiota-directed and microbiota-dependent disease
563 therapies (100-103), and are also being explored to enhance prebiotic therapy (30, 70, 93, 94).
564 Our study here suggests that such efforts to personalize prebiotic supplementation can rely on
565 dietary history and fecal metabolite levels as informative and non-invasive biomarkers of
566 treatment outcome.
567

568 **List of Abbreviations**

569 DHQ3: diet history questionnaire, version 3
570 GCFID: gas chromatograph – flame ionization detector
571 GLM: generalized linear model
572 GLMM: generalized linear mixed-effects model
573 GOS: galactooligosaccharides
574 SCFA: short-chain fatty acids
575 USDA: United States Department of Agriculture
576

577 **Declarations**

578 Ethics approval and consent to participate

579 All recruitment and study procedures involving human subjects were approved by the
580 Duke University Institutional Review Board (Pro00087214). All subjects provided electronic
581 informed consent prior to enrollment.

582

583 Consent for publication

584 Not applicable.

585

586 Availability of data and materials

587 The datasets generated and analyzed during the current study, along with analytical code,
588 are either included in the supplement or available in the figshare repository,
589 https://doi.org/10.6084/m9.figshare.c.5405505. The raw sequencing data is available on the
590 National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the
591 accession PRJNA834995.

592

593 Competing interests

594 The authors declare that they have no competing interests.

595

596 Funding

597 This study was supported by funding from the Beckman Young Investigator program, the
598 Damon Runyon Cancer Research Foundation, the NASA Translational Research
599 Institute through Cooperative Agreement NNX16AO69A, and NIH 1R01DK116187-01. This
600 work used a high-performance computing facility partially supported by grant 2016-IDG-1013
601 (HARDAC+: Reproducible HPC for Next-generation Genomics"). ZH was funded by the
602 National Defense Science and Engineering Graduate fellowship. MV was funded by a
603 Postdoctoral Enrichment Program Award from the Burroughs Wellcome Fund. JS and BP were
604 funded by the Duke School of Medicine Medical Scientist Training Program. BP was

additionally funded by the Integrative Bioinformatics for Investigating and Engineering Microbiomes (IBIEM) program. No funding body had any role in the design of the study nor the collection, analysis, or interpretation of the data nor in writing the manuscript.

Authors' contributions

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

Acknowledgements

The authors acknowledge the Stedman Nutrition Center at Duke University for lending kitchen facilities for food-safe repackaging of prebiotics, and Jeffrey Letourneau, Rachael Bloom, and Savita Gupta for assistance with study coordination and logistics.

Figures

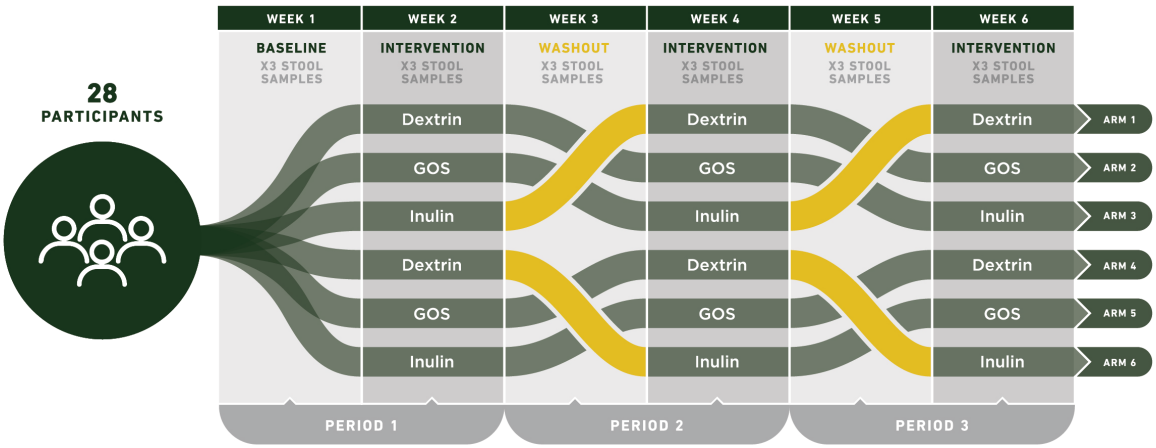


Figure 1: Prebiotic trial design. A three-period crossover design was standardized in series and period, with each prebiotic occurring the same number of times within each arm (once) and study period (twice). The design was also balanced with respect to first-order carry-over effects, meaning prebiotics 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.

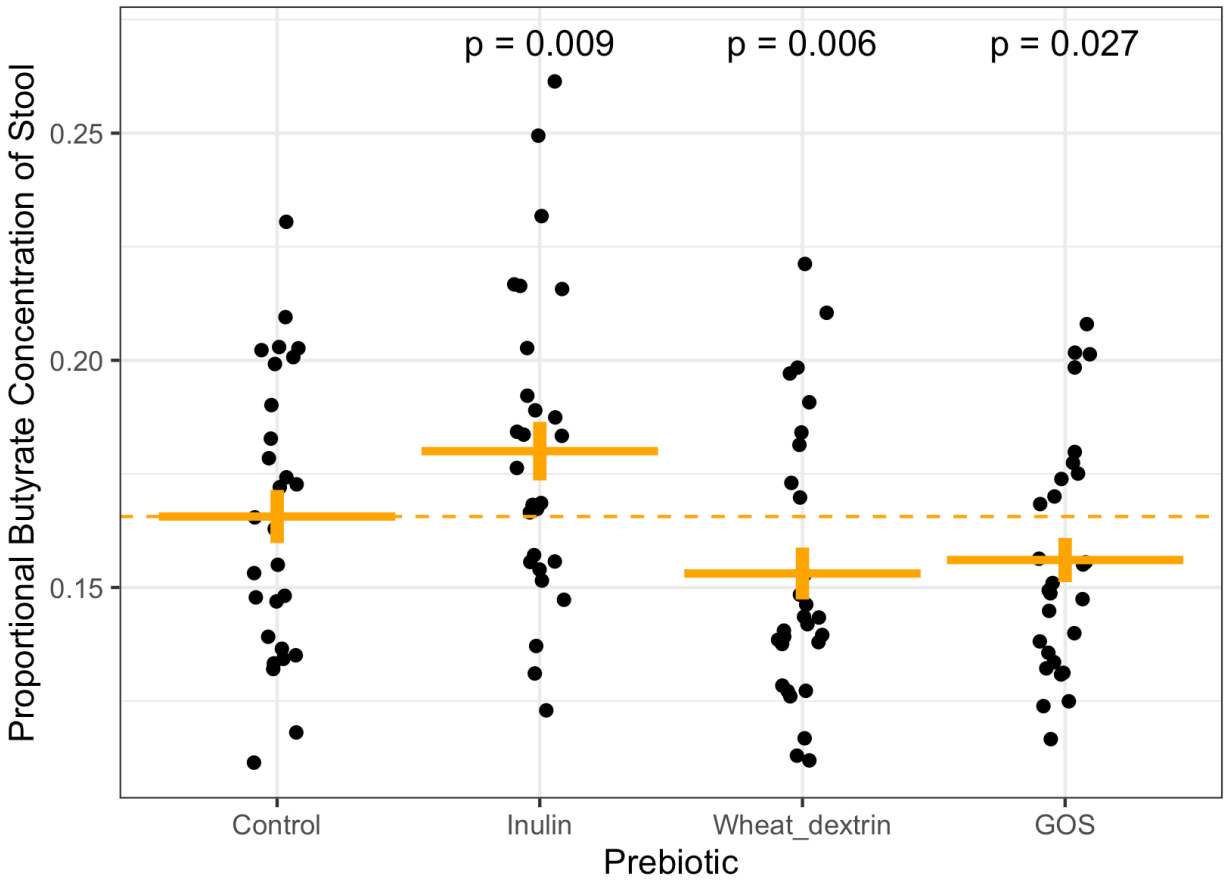


Figure 2: Effects of prebiotic supplementation on proportional butyrate concentration of stool. Plotted are the average proportional butyrate concentrations for each study participant during each prebiotic intervention period (up to three samples per person per week), and the average of all three baseline weeks (up to nine 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). A proportional butyrate concentration value of 1 = 100%.

657

	Control	Inulin		GOS		Dextrin	
	Median [min – max]	Median [min – max]	p	Median [min – max]	p	Median [min – max]	p
CONCENTRATION							
Acetate (mM)	47.4 [5.6 – 102.1]	46.9 [11.6 – 94.1]	0.52	47.2 [14.8 – 80.0]	0.18	50.6 [10.9 – 94.7]	0.39
Propionate (mM)	13.1 [3.2 – 37.8]	13.1 [5.7 – 21.8]	0.41	12.3 [2.6 – 26.0]	**0.015**	14.4 [5.7 - 29.6]	**0.038**
Butyrate (mM)	12.1 [2.8 – 40.3]	12.4 [4.1 – 34.5]	0.26	10.2 [4.1 – 29.5]	**0.002**	10.9 [4.6 – 31.3]	0.29
Total SCFA (mM)	75.1 [15.6 – 153.7]	72.7 [27.9 – 131.1]	0.67	68.6 [25.6 – 114.4]	**0.038**	76.4 [21.1 – 145.4]	0.51
PROPORTION							
Acetate (%)	64.6 [41.9 - 77.9]	63.7 [22.1 - 74.6]	0.21	65.9 [45.9 – 86.8]	0.19	65.7 [44.5 – 74.9]	0.43
Propionate (%)	18.4 [9.6 - 36.2]	19.6 [8.1 – 34.7]	0.63	18.8 [4.5 – 35.4]	0.70	20.1 [10.3 – 36.4]	0.31
Butyrate (%)	16.1 [8.0 – 32.9]	18.0 [11.2 – 55.1]	**0.009**	15.3 [8.6 - 28.0]	**0.027**	14.6 [10.4 – 30.4]	**0.006**

658

659 **Table 1: Concentrations and proportions of SCFA in stool during prebiotic and control**
660 **weeks, averaged across individuals**. Significance was determined by generalized linear mixed
661 models (see methods). Concentrations of stool expressed as mM are equivalent to mmol/kg (see
662 methods).

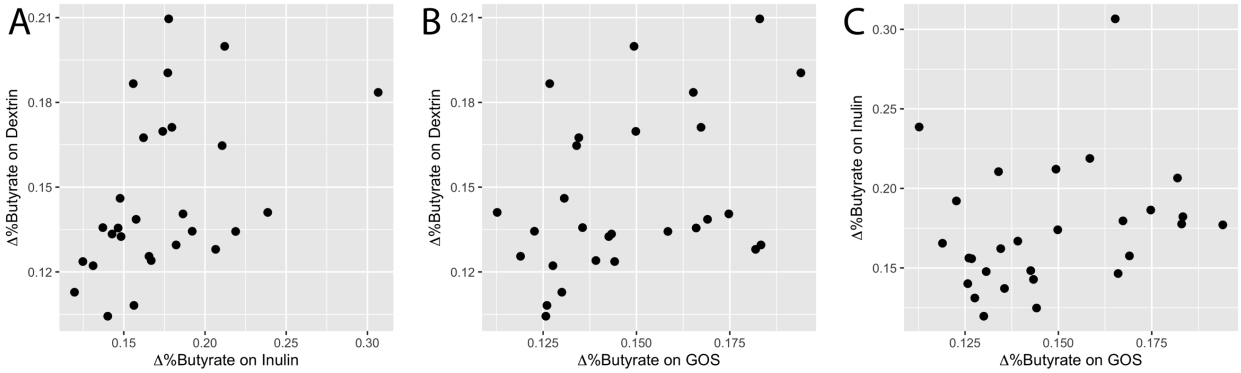


Figure 3: Individual changes in proportional butyrate concentration (Δ%butyrate) compared across multiple prebiotics. Changes in Δ%butyrate were positively correlated when comparing responses to dextrin and inulin ($\rho = 0.45$, $p = 0.015$; Spearman correlation), as well as responses to dextrin and GOS ($\rho = 0.35$, $p = 0.065$). The relationship between inulin and GOS response trended towards a positive association ($\rho = 0.12$, $p = 0.53$). A proportional butyrate concentration value of 1 = 100%.

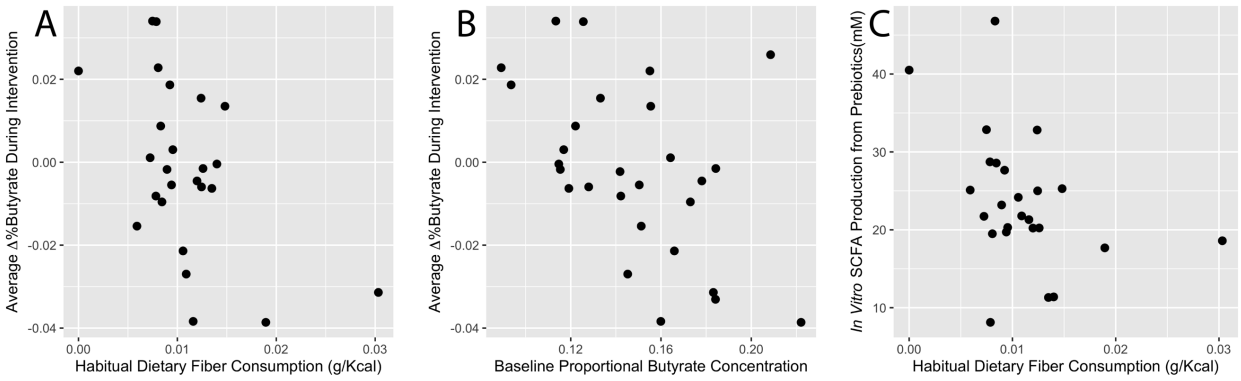


Figure 4: Relationships between habitual diet, baseline proportional butyrate concentration, and metrics of SCFA production. (A) Average change in proportional butyrate concentration of stool during intervention (Δ%butyrate) was negatively correlated with habitual dietary fiber consumption ($\rho = -0.40$, $p = 0.046$; 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).

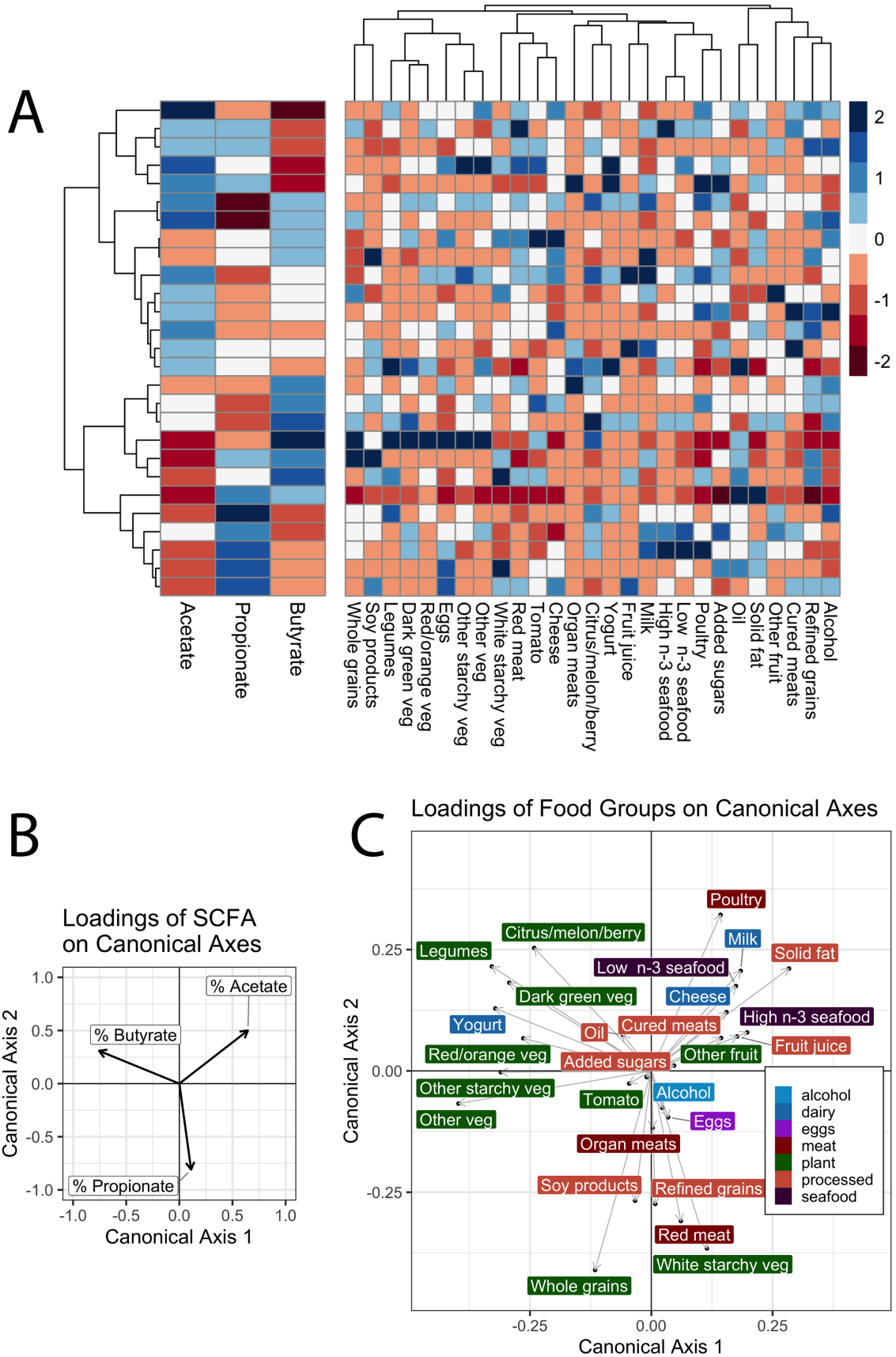
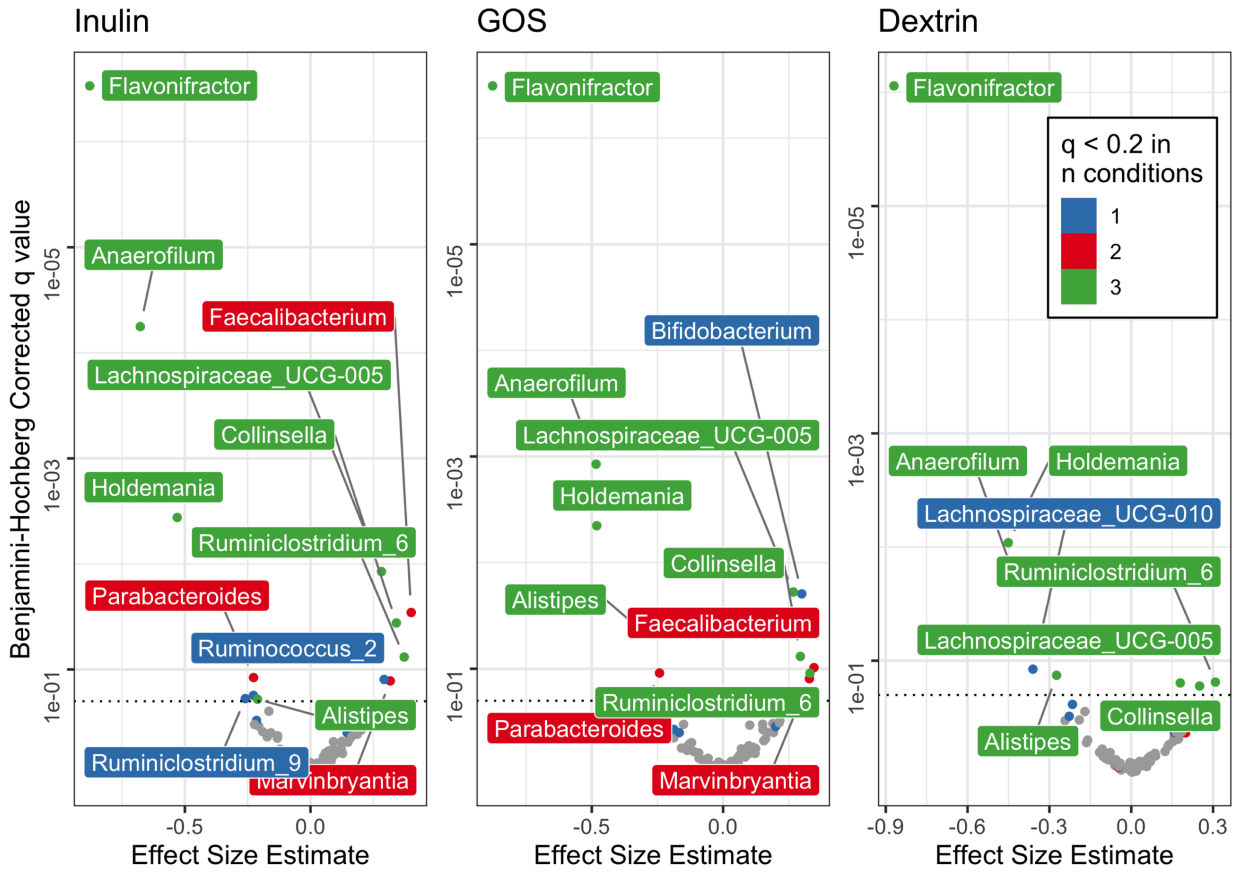

A
Acetate
Propionate
Butyrate
Whole grains
Soy products
Legumes
Dark green veg
Red/orange veg
Eggs
Other starchy veg
Other veg
White starchy veg
Red meat
Tomato
Cheese
Organ meats
Citrus/melon/berry
Yogurt
Fruit juice
Milk
High n-3 seafood
Low n-3 seafood
Poultry
Added sugars
Oil
Solid fat
Other fruit
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

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



693

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

700

Supplementary figures and tables

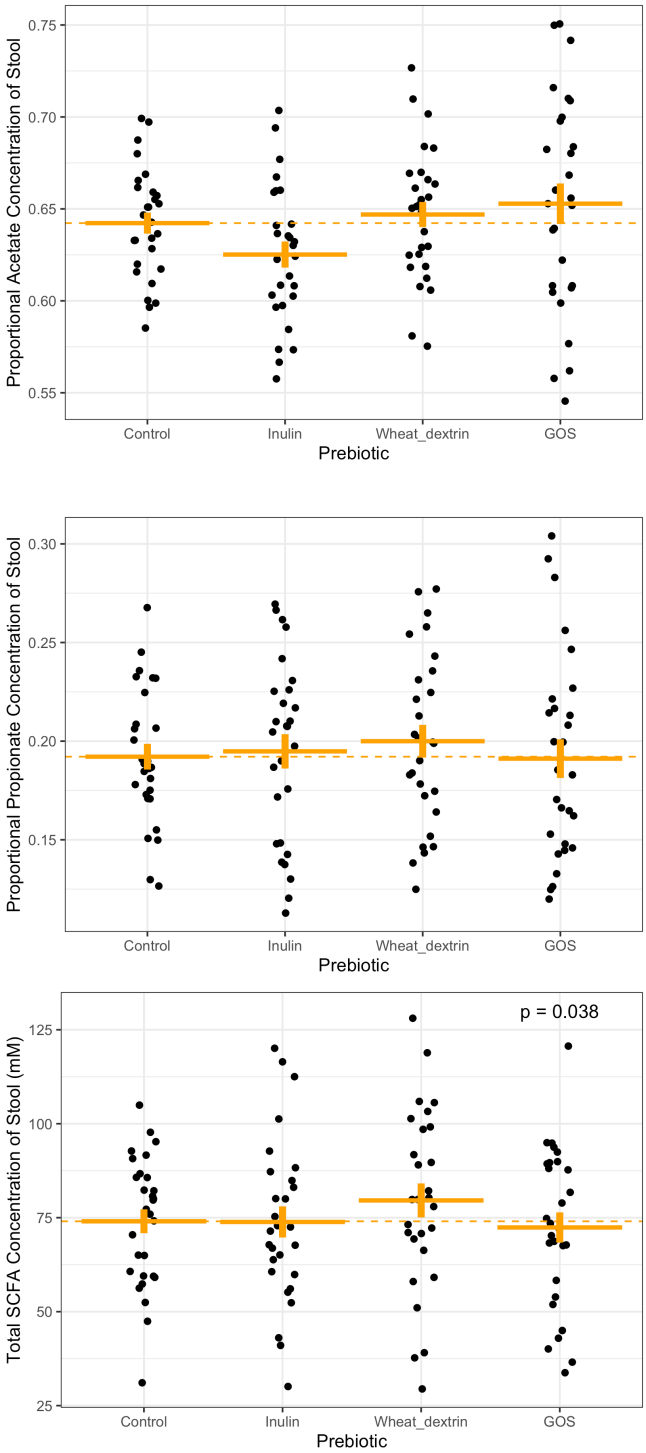


Figure S1: Proportional Acetate (A), proportional propionate (B), and total SCFA (C) concentrations during baseline and prebiotic interventions. All significant differences between prebiotic treatments and control are designated by displayed p values, calculated using a series of generalized linear mixed models. *A proportional SCFA concentration value of 1 = 100%.*

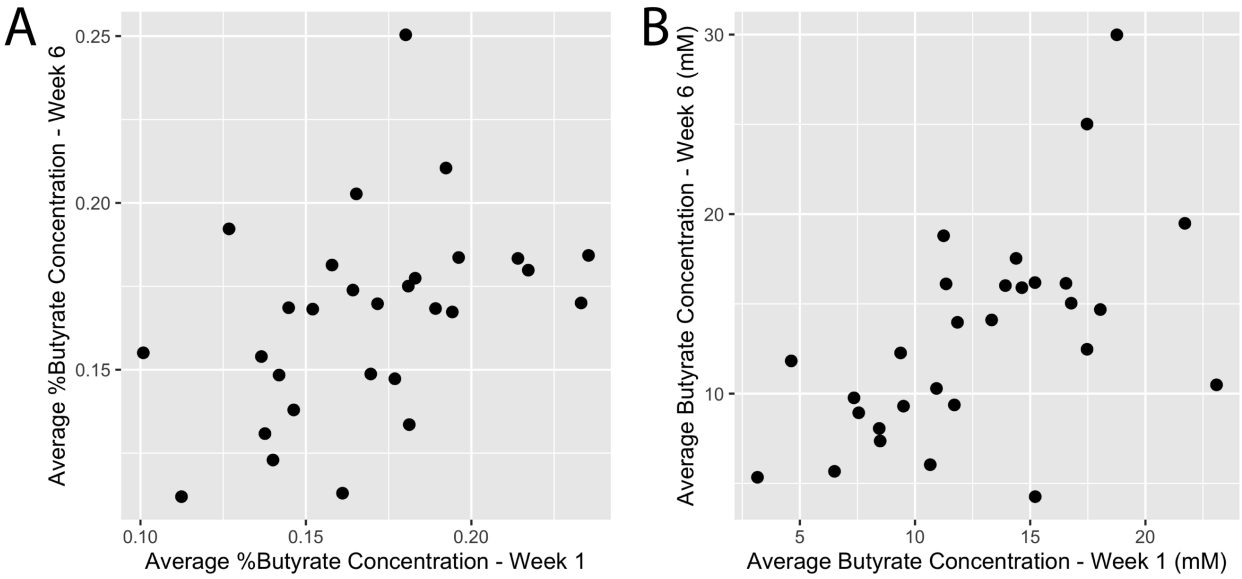


Figure S2: Average proportional butyrate concentration (A) and average butyrate concentration (B) in participant stool during the first and last week of the study. Both proportional concentration and raw concentration were correlated between the first and last weeks ($\rho = 0.49$, $p < 0.007$; and $\rho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

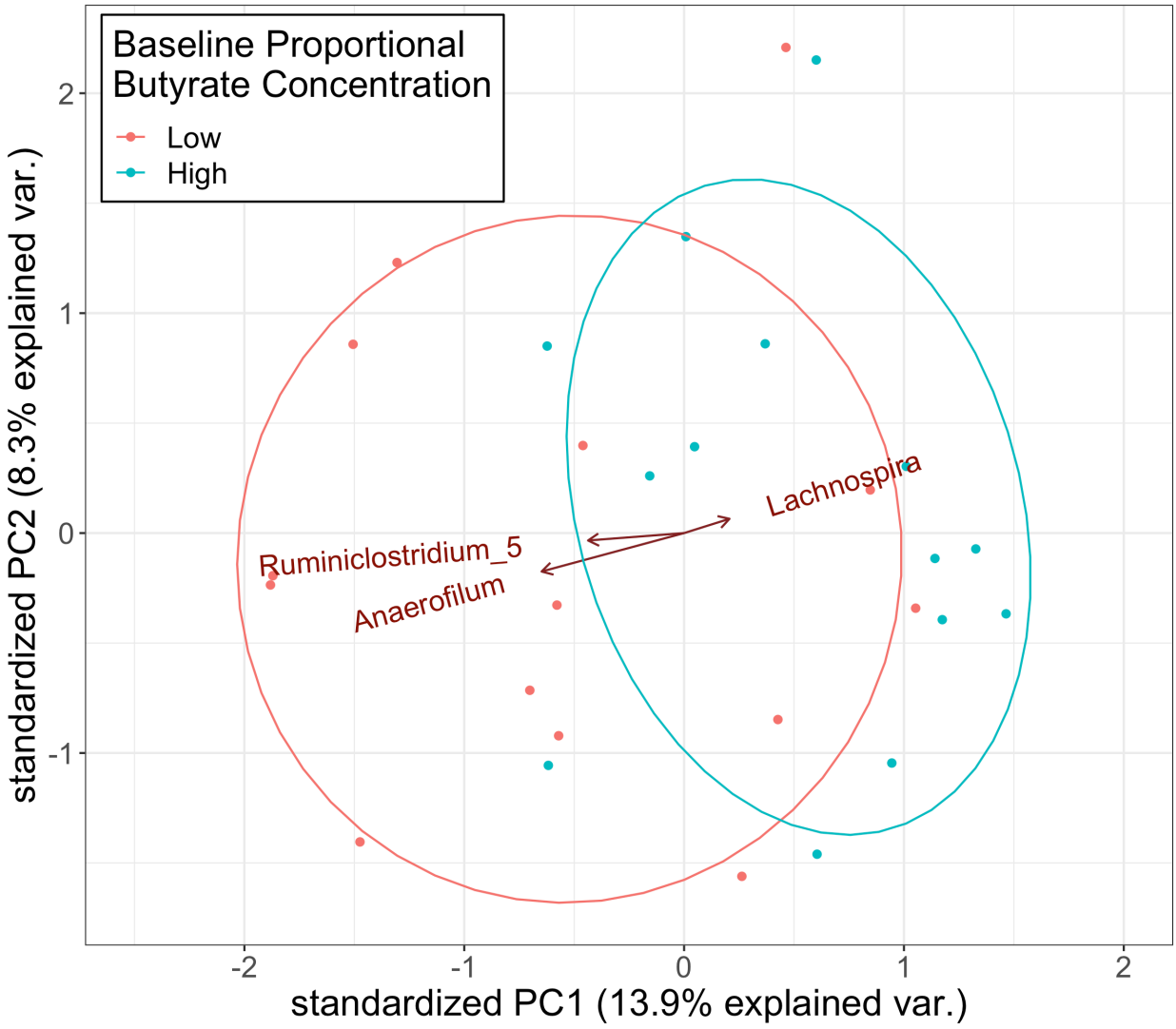


Figure S3: Biplot of stool 16S rRNA gene community composition showing samples (dots) plotted on two principal component axes along with corresponding taxonomic variables (arrows), agglomerated to genus level. Samples are colored by group (high and low; > median baseline %butyrate and <= median baseline %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.

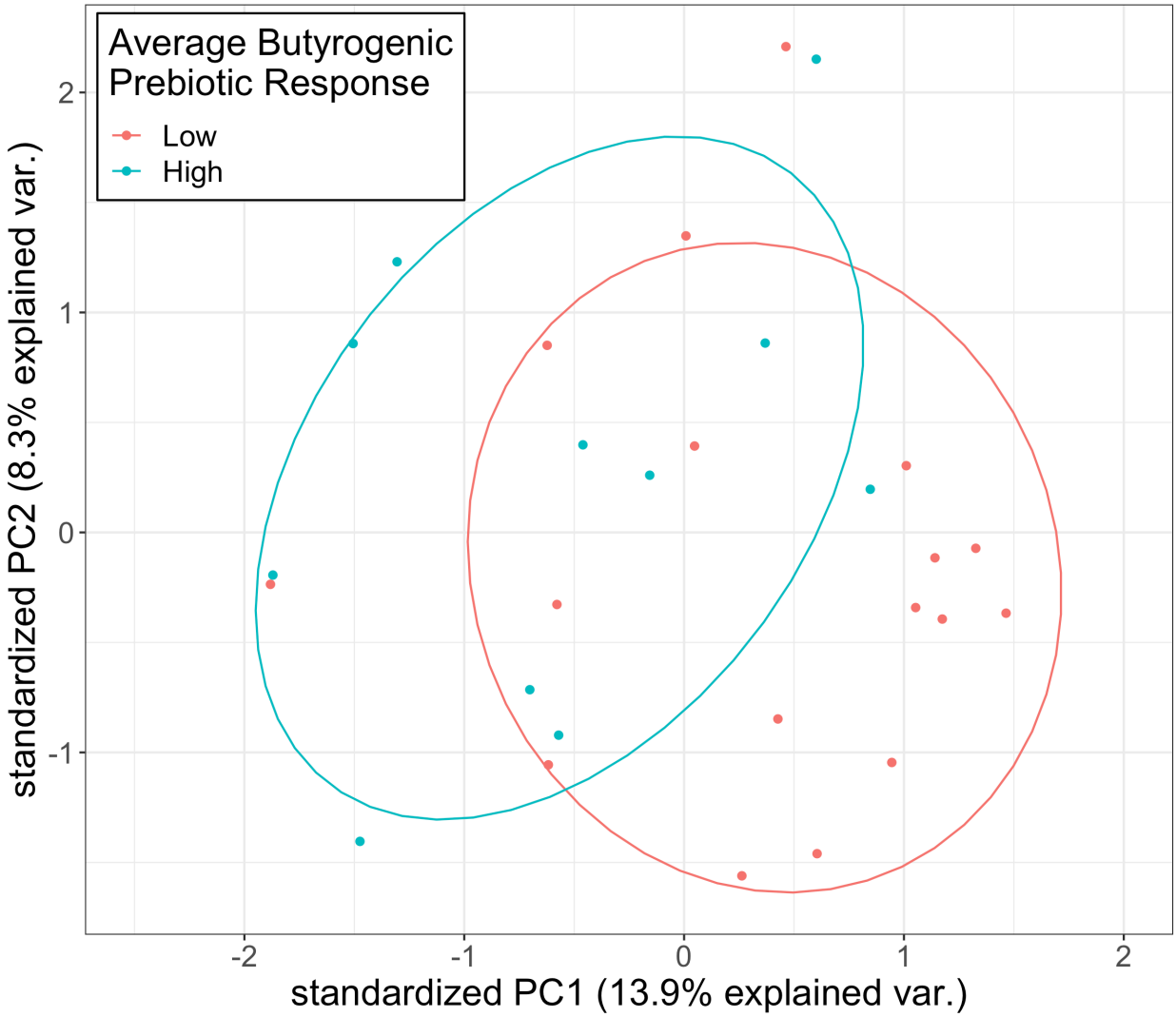


Figure S4: PCA of stool 16S rRNA gene community composition showing samples plotted on two principal component axes. No genera were significantly differentially abundant (Benjamini-Hochberg corrected $q < 0.05$, ALDEx2), so no variables are plotted**.** Samples are colored by group (high and low; > 0 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).

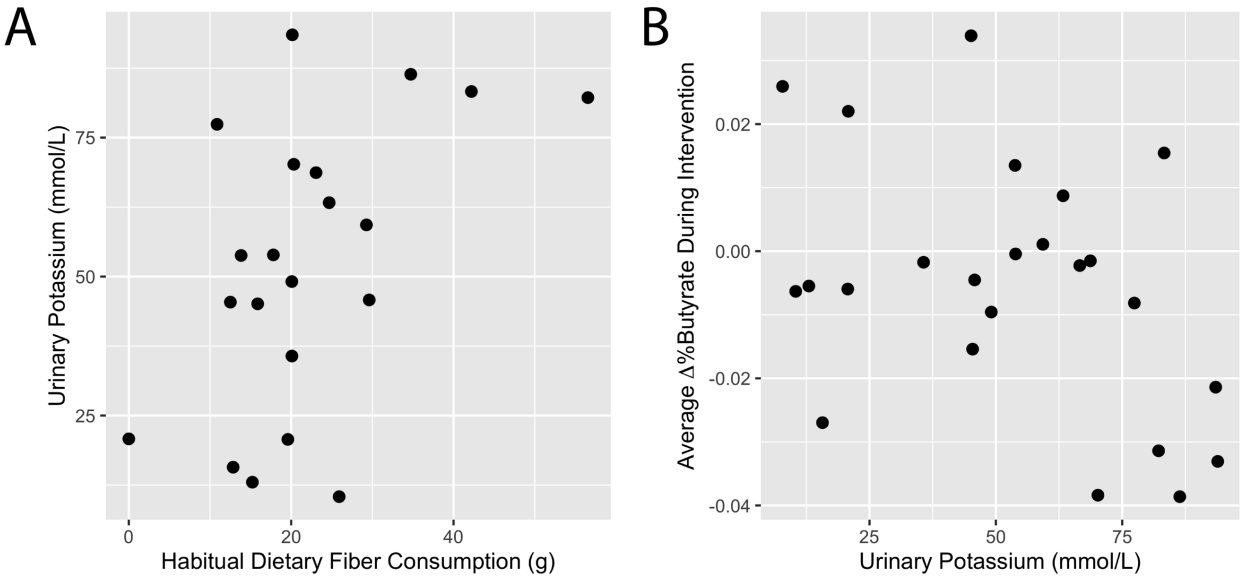


Figure S5: Relationships between baseline urinary potassium and (A) habitual dietary fiber consumption, and (B) average change in proportional butyrate concentration during prebiotic interventions. Baseline urinary potassium was positively correlated with habitual fiber consumption ($\rho = 0.47$, $p = 0.031$; 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).

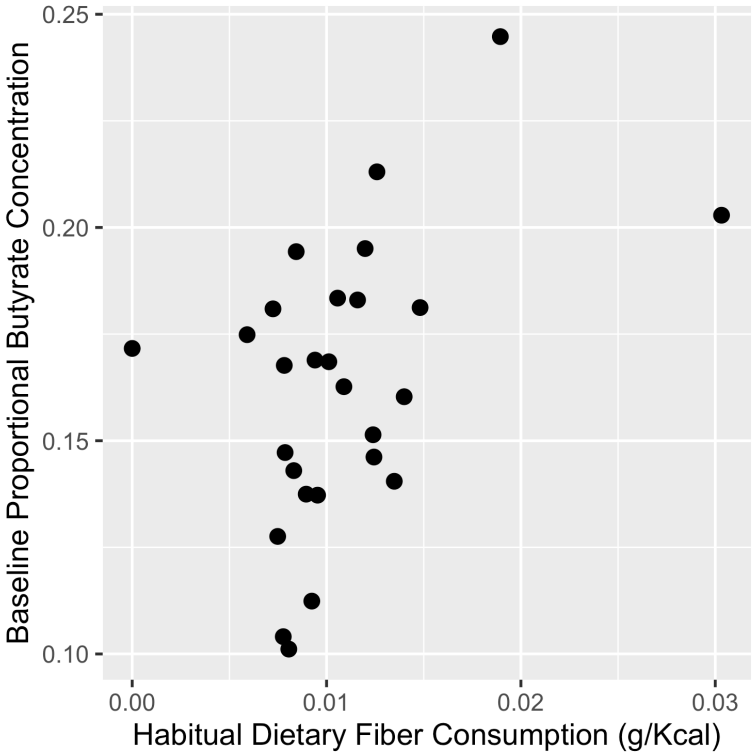


Figure S6: Relationship between habitual dietary fiber consumption and baseline proportional butyrate concentration at week one (prior to study intervention). Fiber consumption was positively associated with baseline proportional butyrate concentration, with 1g fiber/2000 kilocalorie diet associated with an increase in proportional butyrate concentration of 0.010 ($p = 0.012$, beta regression). A proportional butyrate concentration value of 1 = 100%.

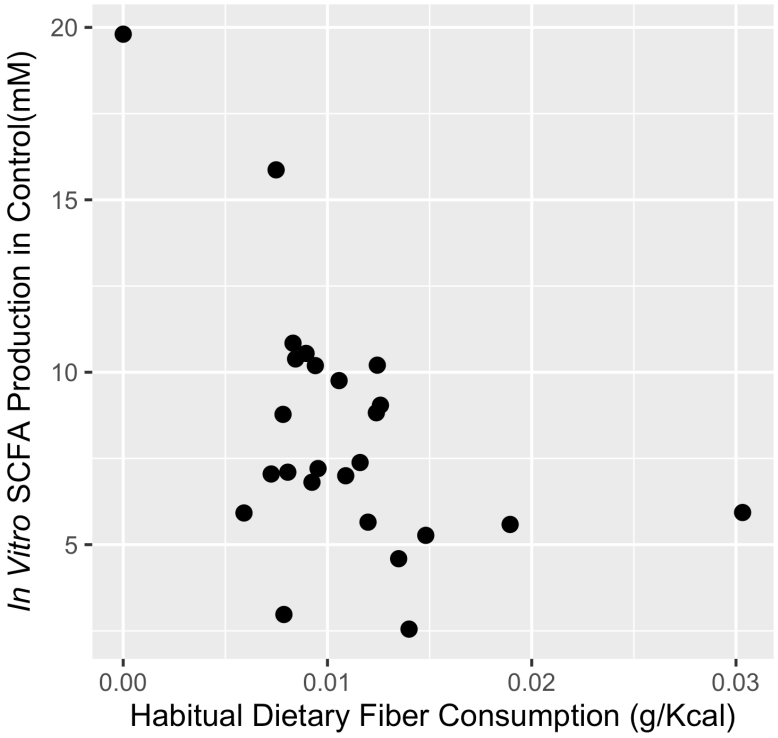


Figure S7: Relationship between *in vitro* SCFA production in prebiotic-free control treatments and habitual dietary fiber consumption. *In vitro* SCFA production was negatively correlated with habitual fiber consumption ($\rho = -0.43$, $p = 0.031$; Spearman correlation).

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