1   Short-chain fatty acid production by gut microbiota from children with obesity is linked to

2   bacterial community composition and prebiotic choice

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23   **Abstract**

24   Pediatric obesity remains a public health burden and continues to increase in

25   prevalence. The gut microbiota plays a causal role in obesity and is a promising

26   therapeutic target. Specifically, the microbial production of short-chain fatty acids

27   (SCFA) from the fermentation of otherwise indigestible dietary carbohydrates may

28   protect against pediatric obesity and metabolic syndrome. Still, it has not been

29   demonstrated that therapies involving microbiota-targeting carbohydrates, known as

30   prebiotics, will enhance gut bacterial SCFA production in children and adolescents with

31   obesity (age 10-18). Here, we used an *in vitro* system to examine the SCFA production

32   by fecal microbiota from 17 children with obesity when exposed to five different

33   commercially available over-the-counter (OTC) prebiotic supplements. We found

34   microbiota from all 17 patients actively metabolized most prebiotics. Still, supplements

35   varied in their acidogenic potential. Significant inter-donor variation also existed in SCFA

36   production, which 16S rRNA sequencing supported as being associated with differences

37   in the host microbiota composition. Last, we found that neither fecal SCFA

38   concentration, microbiota SCFA production capacity, nor markers of obesity positively

39   correlated with one another. Together, these *in vitro* findings suggest the hypothesis that

40   OTC prebiotic supplements may be unequal in their ability to stimulate SCFA production

41   in children and adolescents with obesity, and that the most acidogenic prebiotic may

42   differ across individuals.

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45   **IMPORTANCE** Pediatric obesity remains a major public health problem in the US, where

46   17% of children and adolescents are obese, and rates of pediatric 'severe obesity' are

47   increasing. Children and adolescents with obesity face higher health risks, and non-

48   invasive therapies for pediatric obesity often have limited success. The human gut

49   microbiome has been implicated in adult obesity, and microbiota-directed therapies can

50   aid weight loss in adults with obesity. However, less is known about the microbiome in

51   *pediatric* obesity, and microbiota-directed therapies are understudied in children and

52   adolescents. Our research has two important findings: 1) dietary prebiotics (fiber) cause

53   the microbiota from adolescents with obesity to produce more SCFA, and 2) the

54   effectiveness of each prebiotic is donor-dependent. Together, these findings suggest

55   that prebiotic supplements could help children and adolescents with obesity, but that

56   these therapies may not be one-size-fits-all.

Introduction

Approximately 17% of children in the United States have obesity, and the prevalence continues to increase among all ages and populations (1). The prevalence of pediatric obesity is even higher in Hispanic and African American populations in the United States, where rates of severe obesity continue to increase (1). Children with obesity have an increased risk of adverse health events and incur higher healthcare costs (2-4). Despite the severity of the pediatric obesity epidemic, current common treatment strategies centered around lifestyle changes, including behavioral, dietary, and exercise interventions, often fail or have limited success (5). The high prevalence of pediatric obesity, coupled with the low success rate of common interventions, highlights the need for more efficacious, safe strategies to lower BMI in children and adolescents.

The human gut microbiome has emerged as a promising therapeutic target in pediatric obesity. Over the past decade, differences in gut microbial community composition and metabolic activity between obese and lean individuals have been observed (6-8). Causal links have also been established; fecal transplantation can transfer the obesity phenotype from obese donors to lean recipients and recapitulate some key metabolic changes in human obesity (9). Multiple mechanisms for this link have been proposed, including increased energy harvest by obese microbiota (10), activation of enteroendocrine signaling pathways by SCFA (11-13), modulation of glucose and energy homeostasis through bile acid signaling (14), and increased local and systemic inflammation caused by a variety of microbial metabolites (15).

78   Recent attention in obesity research has been specifically drawn to the role of

79   microbially-derived short-chain fatty acids (SCFA). SCFAs, primarily acetate, propionate,

80   and butyrate, are produced by enteric microbes as end products of anaerobic

81   fermentation of undigested, microbially-accessible dietary carbohydrates, and serve a

82   variety of important roles in the gut. Of particular interest is the SCFA butyrate, which

83   serves as the primary nutrient source for colonocytes (16) and functions as a histone

84   deacetylase inhibitor (17, 18). Through its inhibition of NF-κB signaling in colonocytes,

85   butyrate contributes to barrier integrity maintenance and reduces levels of intestinal

86   inflammation markers (19-22). Acetate, propionate, and butyrate also each activate G-

87   protein coupled receptors (GPR) that modulate key metabolic hormones including peptide

88   YY (PYY) and GLP-1 (12, 23). Consistent with these mechanistic findings, mouse studies

89   have shown that supplementation with acetate, propionate, butyrate, or some mixture of

90   these can protect against weight gain, improve insulin sensitivity, and reduce obesity-

91   associated inflammation (24-29). Given the experimental evidence for SCFA

92   supplementation having an anti-obesogenic effect in a murine system, maintaining high

93   levels of SCFA during a weight loss treatment may improve results (27).

94   If increasing SCFA levels is a potential approach to promote weight loss in

95   children, prebiotic supplementation may provide an effective and low-risk adjunctive

96   therapy. Prebiotics are dietary carbohydrates that are indigestible by human-produced

97   enzymes and thus survive transit to the lower GI tract. Once in the colon, prebiotics serve

98   as carbon sources for bacterial fermentation, which in turn yield SCFAs as metabolic end

99   products (30, 31). Multiple types of prebiotics (*e.g.* fructooligosaccharides (FOS), and

100 inulin-type fructans) have been tested in children with obesity ranging from ages 7-18. In

101 select cases, these treatments have been associated with smaller increases in BMI and

102 fat mass (32), and reductions in body weight z-scores, body fat, and trunk fat (33). Still,

103 other prebiotic trials in overweight children have reported no significant beneficial effects

104 (34).

105   Interpreting the mixed outcomes of prior prebiotic clinical trials in pediatric obesity

106 though is complicated by several challenges. First, *in vivo* studies in pediatric obesity to

107 date have each used only one prebiotic supplement due to the logistical constraints of

108 clinical trials (32-34). Trials employing testing only a single type of supplement hinder the

109 ability to generalize conclusions regarding the efficacy of prebiotics and also make it

110 challenging to determine whether some prebiotics are inherently more acidogenic than

111 others. Second, *in vivo* trials in healthy adults have shown substantial inter-individual

112 variation in the single prebiotic effects on stool SCFA concentration (30, 31, 35). Variation

113 in the primary and secondary outcomes could be due to differences in microbial SCFA

114 production; or differences in host physiology, such as SCFA absorption potential. Third,

115 while SCFA concentrations have been shown to be altered in children with overweight or

116 obesity (36), changes in fecal SCFA during dietary intervention have not been measured

117 in past *in vivo* studies in pediatric populations. If prebiotics mediate their effects through

118 SCFA (33, 34, 37), directly tracking SCFAs could help determine treatment success.

119 Fourth, *in vivo* studies in adults, especially those with obesity, may be confounded by the

120 concurrence of chronic disease and the medications a person may be taking to treat

121 chronic disease.

122   In this study, we have taken an *in vitro* approach to address the limitations of prior

123   human studies. An *in vitro* approach facilitates more direct comparisons of different

124   prebiotic supplements: the higher-throughput of *in vitro* experiments allows wider variety

125   of prebiotics to be tested; and, the effects of these supplements can be tested on identical

126   microbiota samples, rather than over time within subjects, which is confounded by

127   microbiota drift over time (38), as well as inconsistencies in dietary composition. Taking

128   an *in vitro* approach to studying the effects of prebiotics on gut microbiota allows a more

129   direct investigation of microbial SCFA production, as we can study the effects of prebiotic

130   supplementation independent of the effects of host absorption (39, 40). Using a preclinical

131   *in vitro* fermentation model, and samples from adolescents with obesity who have not

132   developed long-term complications, we pursued three specific lines of inquiry: 1) whether

133   different types of prebiotics lead to differences in SCFA production by gut microbiota from

134   adolescents with obesity; 2) whether the effects of prebiotics are shaped by inter-

135   individual differences in gut microbiota structure; and, 3) whether fecal SCFA production

136   is associated with protection from obesity.

Results

SCFA production capacity

To measure SCFA production by gut microbiota, we adapted the *in vitro* approach of Edwards et al. 1996 (41). This method was specifically designed to study fermentation of starch in the human lower GI tract, and has since been used to measure metabolite production from human stool samples when exposed to prebiotic fiber (42-44). In brief, we homogenized previously frozen feces in reduced phosphate buffered saline (pH 7.0 $\pm$ 0.1) to create a fecal slurry with a final concentration of 100g/L (Figure 1). These fecal slurries were then supplied with each of five prebiotic carbon sources, and a carbon-free control, and allowed to ferment at 37°C in anaerobic conditions for 24 hours, to approximate colonic transit time (45). Following the incubation period, the concentrations of SCFA in the samples were measured by gas chromatography. To control for differences in overall cell viability or stool slurry nutrient content between donors, we corrected measurements of SCFA concentration by dividing the treatment SCFA concentration by the control SCFA concentration.

To validate our assay, we ran a series of experiments using feces from validation sample sets. We verified that our control-corrected SCFA production data was not influenced by bacterial abundance ($p = 0.38$, $\rho = 0.14$, Spearman correlation; Figure S1). Absolute (not relativized to control) SCFA concentrations are supplied in the supplement (Figures S2 and S3). As our fermentation experiments used previously frozen fecal samples, we verified that total SCFA production was strongly correlated between fresh samples and twice freeze-thawed samples ($p < 0.0001$, $\rho = 0.75$, Spearman correlation;

159 Figure S4A). Since we elected to not provide our fermentation reactions with nutrients in

160 excess of what was contained in the fecal slurries, we verified that there existed strong

161 correlation in total SCFA production between PBS-grown and colonic medium-grown

162 cultures (46), both when supplied with dextrin and inulin (Dextrin: $p = 0.001$, $\rho = 0.68$;

163 inulin: $p = 0.02$, $\rho = 0.51$; Spearman correlations; Figure S5). We found that total SCFA

164 production over control was positively correlated with the pH of starting fecal slurries ($p =$

165 0.003, $\rho = 0.46$; Spearman correlation; Figure S6A). A weaker correlation may exist

166 between SCFA production and the final pH of the fermentation vessels ($p = 0.067$, $\rho =$

167 0.29, Spearman correlation; Figure S6B).

168   We subsequently applied our assay to fecal microbiota from a cohort of 17 children

169 ranging in age from 10 – 18, Tanner stages 2 – 5, and body-mass index (BMI) from 25.9

170 – 75.3 (Table 1). We found all 17 individuals demonstrated a net gain of SCFA relative to

171 the control in at least one prebiotic treatment, which led us to conclude that all tested

172 cultures were viable and metabolically active (Figure 2).

173

Donor and prebiotic both impact SCFA production *in vitro*

175   We next tested the hypothesis that different prebiotics equally promote the

176 production of SCFA by performing statistical analysis of SCFA production as a function

177 of the prebiotic type and individual identity. Our analysis revealed heterogeneity in the

178 efficacy of prebiotic supplements (two-way ANOVA, $p < 0.001$; Table S1; Figure 2a),

179 ranging from inulin, which resulted in a 2.35 mean fold change in total SCFA, to GOS,

180 which resulted in 3.55 mean fold change in total SCFA. Frequently, only two or three of

181   the five tested prebiotics resulted in increased total SCFA production within an individual.

182   Our statistical testing also revealed consistent patterns between individuals' gut

183   microbiota in terms of SCFA production (two-way ANOVA, p < 0.001; Table S1; Figure

184   2b), with mean fold change in SCFA over control ranging from 2.37 to 6.12. Within

185   individuals, the average fold change in SCFA concentration in the prebiotic treatments

186   often appeared to be driven by a few strongly acidogenic prebiotics. Last, our analysis

187   indicated a significant interaction between prebiotic type and individual identity (two-way

188   ANOVA, p< 0.001; Table S1; Figure 2c). Because our statistical analysis considered

189   technical replicates as separate experimental conditions, this result suggests the

190   presence of consistent prebiotic/individual responses across *in vitro* assay replicate runs

191   – not whether such interactions are consistent within an individual over time.

192

193   **SCFA production *in vitro* predicts the abundance of bacteria in the starting culture**

194   If inter-individual differences in gut microbiota mediated responses to prebiotic

195   treatment, we would expect that specific bacterial taxa, which varied between individuals,

196   could also be associated with SCFA production. To evaluate this hypothesis, we used the

197   R package *stray* (47) to create a Bayesian multinomial logistic normal linear regression

198   (*pibble*) model that tested for correlations between *in vitro* SCFA production in response

199   to each prebiotic and 16S rRNA community composition of patient stool used in the

200   fermentations, at the genus level. This analysis revealed that SCFA production from

201   prebiotics was correlated with the relative abundances of 18 different bacterial genera

202   (95% credible interval not covering 0, Figure 3). Of the 13 genera positively associated

203   with SCFA production, 9 are known or likely fiber degraders (48-52), *Akkermansia*, is

204   often observed to increase in abundance after prebiotic treatment (53), and one,

205   *Methanobrevibacter*, an archaeon hydrogenotrophic methanogen, is known to increase

206   the efficiency of carbohydrate metabolism by the microbiota (54) (Table 3). Most genera

207   identified by *stray* were associated with SCFA production in a limited set of prebiotic

208   treatments. One genus, *Lactobacillus*, is positively associated with SCFA production on

209   XOS, but were negatively associated with SCFA production on GOS. Overall, the

210   presence of specific associations between bacterial taxa and different prebiotics supports

211   a model where different individuals vary in their levels of prebiotic degrading gut bacteria.

212

213   **Metrics of obesity do not appear to correlate with SCFA production capacity of**

214   **stool**

215   Finally, we tested the hypothesis that *in vitro* SCFA production would be

216   associated with obesity-related phenotypes. We compared clinical metadata from

217   individuals, which included BMI, insulin, and HbA1c, with average total SCFA production

218   across prebiotics and found no significant correlations in our population (Spearman

219   correlation; Table 2). Fecal microbial SCFA production capacity may not be directly

220   associated with obesity though because rates of host SCFA uptake likely vary, and this

221   variance may influence host intestinal physiology (55-57). Indeed, in support of the idea

222   that SCFA absorption rate (which was not measured in this study) shape metabolic

223   homeostasis and host health, we observed a negative association between fecal SCFA

224   concentrations and *in vitro* SCFA production across the range of tested prebiotics (Figure

225   4). Furthermore, if SCFA absorption efficiencies varied by individual, residual fecal SCFA

226   concentrations may not directly reflect the complete effect of bacterial metabolism on

227   obesity. Consistent with this notion, no significant relationships were apparent between

228   concentrations of SCFA in patient stool and clinical markers of obesity measured at

229   enrollment, including BMI, insulin levels, and HbA1c (Table 2), although this may also be

230   explained by uncontrolled patient parameters.

Discussion

In this study we found that the microbiota of all tested adolescents with obesity increased total SCFA production when exposed *in vitro* to at least one prebiotic. Both donor and prebiotic were significant factors in determining SCFA production *in vitro*, as was their interaction. Our modeling revealed distinct associations between specific microbial taxa and SCFA production on different prebiotics. We interpret this result as suggesting that the associated bacteria play a role in the fiber fermenting capacity of the community. We observed no correlations between either stool SCFA concentrations or *in vitro* acidogenic capacity of communities and any metrics of obesity (Table 2).

We have recapitulated previous findings that both donor and prebiotic are important in determining the SCFA production from *in vitro* prebiotic supplementation (31, 50, 58), and we found that not all prebiotics appear equally acidogenic (50). Since our *in vitro* system removes the host as a potential source of variation, our data support a gut microbial role for inter-donor variation in fecal SCFA production. In addition, the strength of the interaction between donor and prebiotic strongly suggests that prebiotics are not one-size-fits-all; rather, inconsistent results from prior studies of prebiotics in pediatric obesity (32, 34, 59) may be due to variation in the SCFA production capacity of individuals' gut microbiota across the tested prebiotics. Future therapeutic efforts involving prebiotics in patients with obesity may benefit from stratified or personalized treatments. Nutritional therapies that are personalized to individuals' microbiota are already in development (60).

252   Murine and *in vitro* studies show that increased signaling through GPRs, mediated

253   by acetate, propionate, and butyrate, increases satiety and insulin sensitivity, while

254   decreasing adipogenesis (12, 23, 61), yet, we did not observe associations between fecal

255   SCFA levels and metrics of obesity. The effects of SCFA on obesity may be masked by

256   uncontrolled patient factors, such as differences in caloric intake and variation in

257   individual nutrient harvest and utilization. In order to observe the effects of SCFA on

258   obesity, it would be necessary to control for these variable physiological and lifestyle

259   parameters, which we did not attempt. These patient factors may also have influenced

260   our inability to observe an association between acidogenic capacity of microbiota and

261   fecal SCFA concentrations. However, this may also be explained by the potential

262   uncoupling of fecal SCFA production and fecal SCFA concentration. *In vitro,* increased

263   luminal concentrations of butyrate have been shown to upregulate the sodium-coupled

264   monocarboxylae transporter SLC5A8 (55), and addition of physiological mixtures of

265   SCFA has been shown to upregulate the monocarboxylate transporter SLC16A1 (62),

266   both of which uptake acetate, propionate, and butyrate from the lumen. Since gut epithelia

267   have the capacity to absorb up to 95% of SCFA before excretion (63), increased host

268   SCFA uptake (triggered by increased gut bacterial production) could, therefore, lead to

269   constant or even decreased fecal SCFA concentrations. This complex relationship could

270   explain the absence of positive correlations we observed between stool SCFA levels and

271   the acidogenic capacity of gut microbiota. It may be necessary to delve further upstream

272   of fecal SCFA concentration by measuring proxies for host SCFA uptakes, such as the

273   expression of SCFA transporters (SLC5A8 and SLC16A1) and SCFA receptors (GPR43,

274   GPR41, and GPR109A) (55).

275   The primary limitations of this study involve constraints common to *in vitro* culture

276   studies. First, many factors affecting bacterial SCFA production *in vivo* are difficult to

277   replicate *in vitro*, including the availability of nutrients such as nitrogen, the starting

278   concentration of SCFA, the redox state of the environment, and the efficiency of cross-

279   feeding interactions (64, 65). Different metabolic results between prebiotics may have

280   occurred if we provided alternative co-metabolites or nutrients, in addition to the tested

281   prebiotics. We chose our culture conditions, namely a media-free approach that does not

282   add any nutrients beyond what is present in the stool, in an effort to avoid inducing artificial

283   selective conditions within our cultures. Prior experimental digestion studies have shown

284   that prebiotic response patterns can be recapitulated across varying culture conditions

285   (42, 44). Indeed, we found strong correlation in SCFA production between cultures grown

286   with our media-free approach and those grown in a more conventional medium containing

287   added nitrogen, vitamins, minerals, and acetate. Further, this approach allowed us to

288   minimize the influence of the host on measurements of microbiota production of SCFA.

289   We did observe shifts in community composition during the 24 hour fermentations (Figure

290   S7); however, we remained able to find statistical associations between SCFA production

291   capacity and pre-fermentation community composition. A second set of limitations in this

292   study involves our reliance on patient collection of stool. Inter-donor variation in prebiotic

293   response could have originated in technical variation between how patients exposed stool

294   to aerobic conditions (66) or how they froze their samples (67), which in turn could have

295   affected the fraction of viable microbial cells in stool samples. Still, we found a significant

296   correlation between *in vitro* total SCFA production from fresh stool and stool that had

297   been frozen and thawed twice. Variation in donor prebiotic response could also have

298   biological origins due to physiological differences between people (*e.g.* efficiency of food

299   digestion, consistency of stool (68)) or differences in diet, which can lead to variation in

300   stool microbial load and nutrient content (69). Rather than control for a myriad of different

301   sources of variation whose origins we did not measure, we chose the straightforward

302   approach of standardizing donor samples by employing a consistent concentration of

303   stool slurry (5% w/v stool in PBS) in our experiments.

304   Future work to address these limitations could test multiple stool samples per

305   subject to confirm whether the observed variation in prebiotic response is durable

306   between individuals over time. Future studies could also examine the correlation between

307   the metabolic effects of prebiotic supplementation *in vitro* and *in vivo* using randomized

308   human trials that couple human prebiotic supplementation, *in vivo* measurement of SCFA

309   production, and *in vitro* tests of microbiota metabolic activity. It would also be useful for

310   such studies to explore the impact of prebiotic supplementation on host physiology, both

311   *in vitro* and *in vivo*. Specifically, the effects of prebiotic supplementation on colonic

312   epithelial barrier integrity, SCFA receptor (GPR41, 43, 109A) expression, and SCFA

313   transporter (MCT1, SMCT1) expression could provide greater insight into the health

314   impacts of prebiotic supplementation, as well as explain why fecal SCFA concentrations

315   may not mirror the metabolic capacity of gut microbiota.

Methods

Cohort

Stool was collected from human donors under a protocol approved by the Duke

Health Institutional Review Board (Duke Health IRB Pro00074547) for a prospective

longitudinal cohort study and biorepository. Participants whose samples were used in

this study were treatment-seeking adolescents with obesity who were newly enrolled in

a multi-disciplinary weight management program. All subjects received family-based

intensive lifestyle modification. Based on clinical necessity, some participants also were

placed on a low-carbohydrate diet, medications to facilitate weight loss, or underwent

weight loss surgery (Table S2). Due to the low number of patients assigned to each

treatment arm, we did not attempt to base any analyses on patient treatment plan.

Patients were aged 10-18, with BMI ≥ 95th percentile. None had prior antibiotic use in

the 1 month prior to enrolment, used medications known to interfere with the intestinal

microbiome, and did not have other significant medical problems. Stool samples used in

this study were from enrollment, 3-month, 4.5-month, and 6-month follow-up visits

(Table S2). The clinical metadata used for correlations was collected at enrollment, 3

months, and 6 months. The metadata collected nearest to the stool sample collection

date was used in our analyses.

Stool Collection

Patients collected intact stool samples in the clinic or at home using a plastic stool

collection container (Fisher Scientific: 02-544-208) and were asked to immediately store

338   this container in their home freezer. Patients then returned the sample by either bringing

339   it to the study team or scheduling a home pickup within 18 hours of stooling. Stool was

340   transported frozen in an insulated container with an ice pack. Upon receipt in the lab,

341   samples were placed on dry ice until transferred to a -80°C freezer for long term storage.

342   All patient samples were frozen at -80°C within 19 hours of stooling (range, 0.08hr –

343   18.83hr; median, 11.42hr) except one which was stored 44.03hr after stooling. The time

344   between stooling and freezing at -80°C did not have a significant effect on average SCFA

345   production (p = 0.58, ρ = -0.15, Pearson correlation). Stool samples for analysis were

346   processed by removing containers from -80°C storage and thawing on ice in a biological

347   safety cabinet until soft enough to aliquot. Thawed containers of stool were opened to

348   atmosphere for a maximum of 10 minutes while samples were aliquoted. After primary

349   aliquoting, the remaining stool was transferred to an anaerobic chamber (COY Laboratory

350   Products, 5% hydrogen, 5% CO2, 90% Nitrogen) and further portioned into approximately

351   2g aliquots for this study. These aliquots were then stored as solid stool pellets at -80°C

352   until used for this study.

353

354   ***In vitro* fermentation**

355   See figure 1 for an overview of *in vitro* fermentation methods. Aliquoted stool was

356   thawed at room temperature in an anaerobic chamber. Once thawed, stool was weighed

357   and placed into a polyethylene filter bag with 0.33mm pore size (Whirl-Pak B01385) and

358   10mL of anaerobic 1X PBS was added for each gram of stool, resulting in a 10% w/v fecal

359   slurry, similar to previous studies (41, 42, 70, 71). During our validation experiments, a

360   medium designed to simulate colonic contents was used in place of 1X PBS to create

361   stool slurries (46). The filter bag was then closed and placed into a stomacher (Seward

362   Stomacher 80) where the contents were homogenized on the medium speed setting for

363   60 seconds. The liquid fraction was removed from the downstream side of the filter

364   membrane, and the solid fraction was discarded. A 1mL aliquot of this liquid fraction was

365   removed for analysis of SCFA concentration, to determine the SCFA concentration of the

366   starting stool sample. During our validation experiments, two separate 1mL aliquots of

367   this liquid fraction were removed: one was used to estimate relative bacteria abundance

368   of starting fecal slurries using total DNA, as has been previously published (72); the

369   remaining aliquot was used to determine the pH of the starting fecal slurry using a

370   handheld pH meter (Elite pH Spear, Thermo-Fischer Scientific). The remaining liquid

371   fraction was incubated in duplicate across six different treatments, either supplemented

372   with inulin (Now Foods Inulin Powder, part #2944), fructooligosaccharides (FOS; Cargill,

373   part   #100047199),   galactooligosaccharides   (GOS;   Bimuno   Powder),

374   xylooligosaccharides (XOS; BioNutrition prebiotic with Llife-Oligo, part #359), wheat

375   dextrin (Benefiber Original), or unsupplemented. For each reaction, 1mL of 10% fecal

376   slurry was placed in one well of a 24-well cell culture plate. Each well was then delivered

377   1mL of 1% (w/v) prebiotic solution in 1X PBS; or 1mL of 1X PBS without prebiotic. During

378   our validation experiments, prebiotics were dissolved in colonic medium instead of 1X

379   PBS. The resulting fermentation conditions where therefore 5% fecal slurry with 0.5%

380   prebiotic (w/v). A 5% fecal slurry was selected because its fermentative capacity has been

381   previously demonstrated to be insensitive to small variations in concentration and is

382   feasible to work with using this method (42). A 0.5% final concentration of prebiotic in the

383   context of a 5% fecal slurry is analogous to an average adult consuming 20g of dietary

384   fiber per day, assuming an average daily stool mass of 200g (73). Fermentation reactions

385   were carried out in an anaerobic chamber at 37°C for 24 hours. Following fermentation,

386   1mL media was taken from each reaction vessel for SCFA quantification. During our

387   validation experiments, a separate 1mL aliquot was taken for pH measurement.

388

Simulation of Freeze/Thaws Experienced by Study Samples

390   To test the effects of freeze/thaw cycles on *in vitro* SCFA production, we collected

391   fresh, whole fecal samples from four healthy adults who were not patients in the study

392   cohort. Informed consent was obtained from volunteers and the protocol was approved

393   by the Duke Health Institutional Review Board. Samples were brought into an anaerobic

394   chamber after voiding. Once in anaerobic conditions, these samples were divided into

395   three aliquots. One aliquot was processed immediately following the same *in vitro*

396   fermentation protocol used in our study. transferred to -80C storage. After a minimum of

397   24 hours, one of these two aliquots was removed from the freezer and thawed at room

398   temperature for 2 hours, before being returned to -80C for an additional minimum of 24

399   hours. Each of these frozen aliquots was thawed and processed following the same *in*

400   *vitro* fermentation protocol. This allowed direct comparison of samples that had been used

401   in fermentations immediately after voiding to those that had been frozen and thawed one

402   and two times.

403

Media Preparation

To validate our methods, namely our use of a 5% fecal slurry in PBS, without supplementation of other nutrient components, we compared SCFA production with our methods to SCFA production when stool was instead resuspended in a medium designed to simulate the large intestine. We used a slightly modified medium derived from Gamage et al. 2017 (46). The media contained, per liter: peptone 0.5g, yeast extract 0.5g, $NaHCO_3$ 6g, hemin solution (0.5% (w/v) hemin and 0.2% (w/v) NaOH) 100uL, L-cysteine HCl monohydrate 0.53g, bile salts 0.5g, Vitamin Supplement (ATCC MD-VS) 1mL, K_2HPO_4 0.228g, KH_2PO_4 0.228g, $(NH_4)_2SO_4$ 0.228g, NaCl 0.456g, $MgSO_4$ 0.0456g, $CaCl_2$ 0.0460g, Trace Mineral Supplement (ATCC MD-TMS) 1mL, and 287uL glacial acetic acid. The pH of the medium was adjusted to 7.0±0.1.

Quantification of SCFA

The SCFA concentration of fecal slurries and fermentation vessels was determined following a protocol adapted from Zhao, Nyman, and Jönsson (74). First, a 1mL aliquot of either 10% fecal slurry in PBS or the fermentation vessel contents was obtained. To this, 50 μL of 6N HCl was added to acidify the solution to a pH below 3. The mixture was vortexed, centrifuged at 14,000rcf for 5 minutes at 4°C to remove particles. Avoiding the pellet, 750 μL of this supernatant was passed through a 0.22μm spin column filter. The resulting filtrate was then transferred to a glass autosampler vial (VWR part #66009-882).

Filtrates were analyzed on an Agilent 7890b gas chromatograph (GC) equipped with a flame-ionization detector (FID) and an Agilent HP-FFAP free fatty-acid column

426   (25m x .2mm id x .3μm film). A volume of 0.5μL of the filtrate was injected into a sampling

427   port heated to 220°C and equipped with a split injection liner. The column temperature

428   was maintained at 120°C for 1 minute, then ramped to 170°C at a rate of 10°C/min, then

429   maintained at 170°C for 1 minute. The helium carrier gas was run at a constant flow rate

430   of 1mL/min, giving an average velocity of 35 cm/sec. After each sample, we ran a one

431   minute post-run at 220°C and a carrier gas flow rate of 1mL/min to clear any residual

432   sample. All C2:C5 short-chain fatty acids were identified and quantified in each sample

433   by comparing to an 8-point standard curve that encompassed the sample concentration

434   range. Standards contained 0.1mM, 0.2mM, 0.5mM, 1mM, 2mM, 4mM, 8mM, and 16mM

435   concentrations of each SCFA.

436

437   **DNA Extraction, PCR Amplification, and Sequencing**

438   We performed 16S rRNA gene amplicon sequencing on human stool samples to

439   determine microbiota community composition. DNA was extracted from frozen fecal

440   samples with the Qiagen DNeasy PowerSoil DNA extraction kit (ID 12888-100). Amplicon

441   sequencing was performed using custom barcoded primers targeting the V4 region of the

442   16S gene (75), using published protocols (75-77). The sequencing library was diluted to

443   a 10nM concentration and sequenced using an Illumina MiniSeq and a MiniSeq Mid

444   Output Kit (FC420-1004) with paired-end 150bp reads.

445

446   **Identifying Sequence Variants and Taxonomy Assignment**

447 We used an analysis pipeline with DADA2 (78) to identify and quantify sequence

448 variants, as previously published by Silverman et al. (79). To prepare data for denoising

449 with DADA2, 16S rRNA primer sequences were trimmed from paired sequencing reads

450 using Trimmomatic v0.36 without quality filtering (80). Barcodes corresponding to reads

451 that were dropped during trimming were removed using a custom python script. Reads

452 were demultiplexed without quality filtering using python scripts provided with Qiime v1.9

453 (81). Bases between positions 10 and 150 were retained for the forward reads and

454 between positions 0 and 140 were retained for the reverse reads. This trimming, as well

455 as minimal quality filtering of the demultiplexed reads was performed using the function

456 fastqPairedFilter provided with the DADA2 R package (v1.8.0). Sequence variants were

457 inferred by DADA2 independently for the forward and reverse reads of each of the two

458 sequencing runs using error profiles learned from all 20 samples. Forward and reverse

459 reads were merged. Bimeras were removed using the function removeBimeraDenovo

460 with default settings. Taxonomy was assigned using the function assignTaxonomy from

461 DADA2, trained using version 123 of the Silva database.

462

463 **Modeling Microbial Composition Data**

464 To associate microbial genera to SCFA production on different prebiotics, the

465 sequence variant table was amalgamated to the genus level using the R package

466 *phyloseq* (81). Genera that were observed with at least 3 counts in at least 3 samples

467 were retained. This filtering step retained 99.3% of sequence variant counts and a total

468 of 97 genera.

469   To associate microbial composition to SCFA production on different prebiotics we

470   made use of Bayesian Multinomial Logistic-Normal linear regression implemented in the

471   R package *stray* as the function *pibble* (82). We chose this method to account for

472   uncertainty due to counting, and compositional constraints as motivated in Silverman,

473   Durand (79) and Grantham, Reich (83). Our regression model was defined for the j-th

474   sample by the covariate vector $x_j = \left[1, x_{j(Inulin)}, x_{j(GOS)}, x_{j(XOS)}, x_{j(Dextrin)}\right]^T$ where

475    $x_{j(Inulin)}$ is the amount of total SCFA produced by the community in sample j as assessed

476   by our *in vitro* assay and the preceding 1 represents a constant intercept. The regression

477   model priors required that 4 hyperparameters *Gamma*, *Theta, Xi,* and *upsilon* be

478   specified. We set the hyperparameters *Theta* to an $D \times Q$ matrix of zeros (where $D = 97$,

479   the number of sequence variants; and $Q = 5$, the number of covariates) representing our

480   prior assumption that, on average, the association between each prebiotic and each taxon

481   is zero.

482   We set the hyperparameters *Gamma* to be the matrix

483
$$
\begin{bmatrix}
5 & 0 & 0 & 0 & 0 \\
0 & 2 & .6 & .6 & .6 \\
0 & .6 & 2 & .6 & .6 \\
0 & .6 & .6 & 2 & .6 \\
0 & .6 & .6 & .6 & 2
\end{bmatrix}
$$

484   which was chosen to reflect the following prior information: (1) the relative scale of

485    $Gamma_{11}$ to $Gamma_{kk}$ (for $k \in {\sim}2, \dots, 5\}$) implies that we have little knowledge regarding

486   the mean composition between individuals but that we conservatively expect that the

487   association between butyrate production and microbial composition is small

488   comparatively. (2) the value of 0.6 state that, on average across genera, we assume that

489　the effects each prebiotic are correlated with an average correlation of 0.3, (3) in concert

490　with our prior choices for *Xi* and *upsilon* (below), the scale of *Gamma* represents our

491　assumption that the technical noise in our community measurements is smaller (by a

492　factor of $\approx e^2$) than the magnitude of the biological variation between samples. This later

493　prior regarding technical versus biological variation was informed by Silverman, Durand

494　(79). All prior choices were further investigated using prior predictive checks (84). To

495　reflect a weak prior assumption that the absolute abundance of each taxon is uncorrelated

496　we choose $upsilon = D + 3$ and *Xi* to be the $(D-1) \times (D-1)$ matrix with elements $Xi_{ii} =$

497　$(upsilon - D)$ and $Xi_{ij} = (upsilon - D)/2$, for $j \neq i$ (85). While the model fit by *stray* and

498　our corresponding priors were specified with respect to additive log-ratio coordinates, we

499　utilized theory from compositional data analysis to transform these results into centered

500　log-ratio coordinates for interpretation (79). Credible intervals and figures reflect 2000

501　samples from the posterior distribution of the corresponding multivariate regression

502　model.

503

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513

514   **Conflicts of Interest**

515   L.A.D. was a member of the Kaleido Biosciences Strategic Advisory Board and

516   retains equity in the company.

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810

811   **FIGURES AND TABLES**

812

Variable	Male (n)	Female (n)	BMI Range	Average BMI	Age Range	Average Age	Total
Value	6	10	25.9 – 75.3	34.9	10-18	15.7	17

813   **Table 1: Demographic characteristics of participants in this study.** One patient

814   provided samples used in all analyses but was lost to follow-up before providing clinical

815   metadata; that patient is only counted in the total column.

816
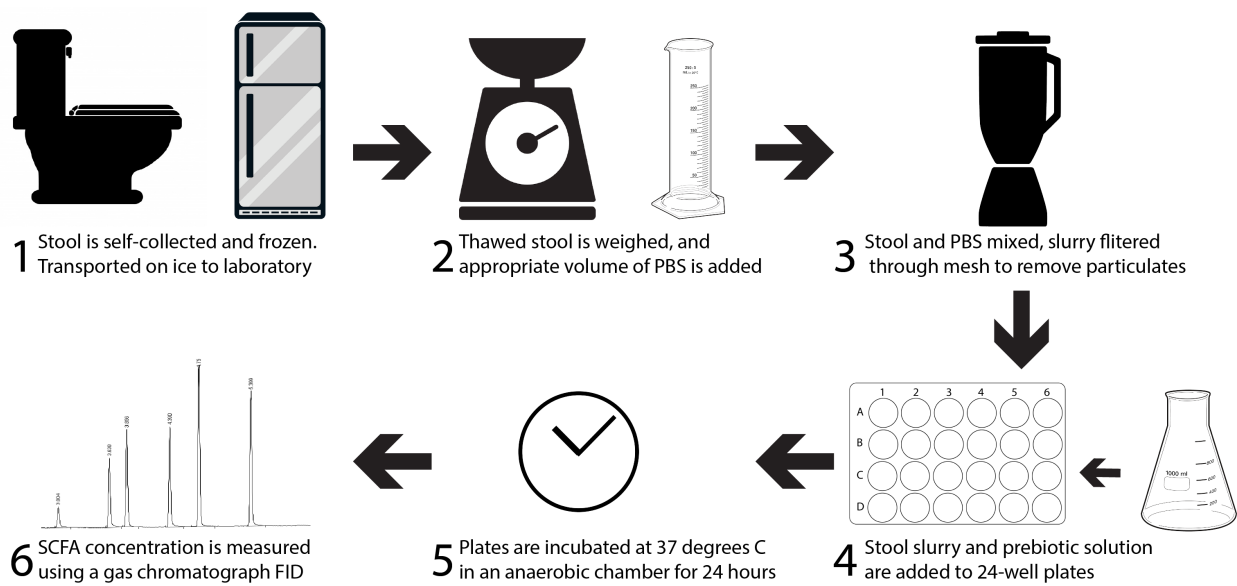
Figure 1: Overview of *in vitro* fermentation methods.

818
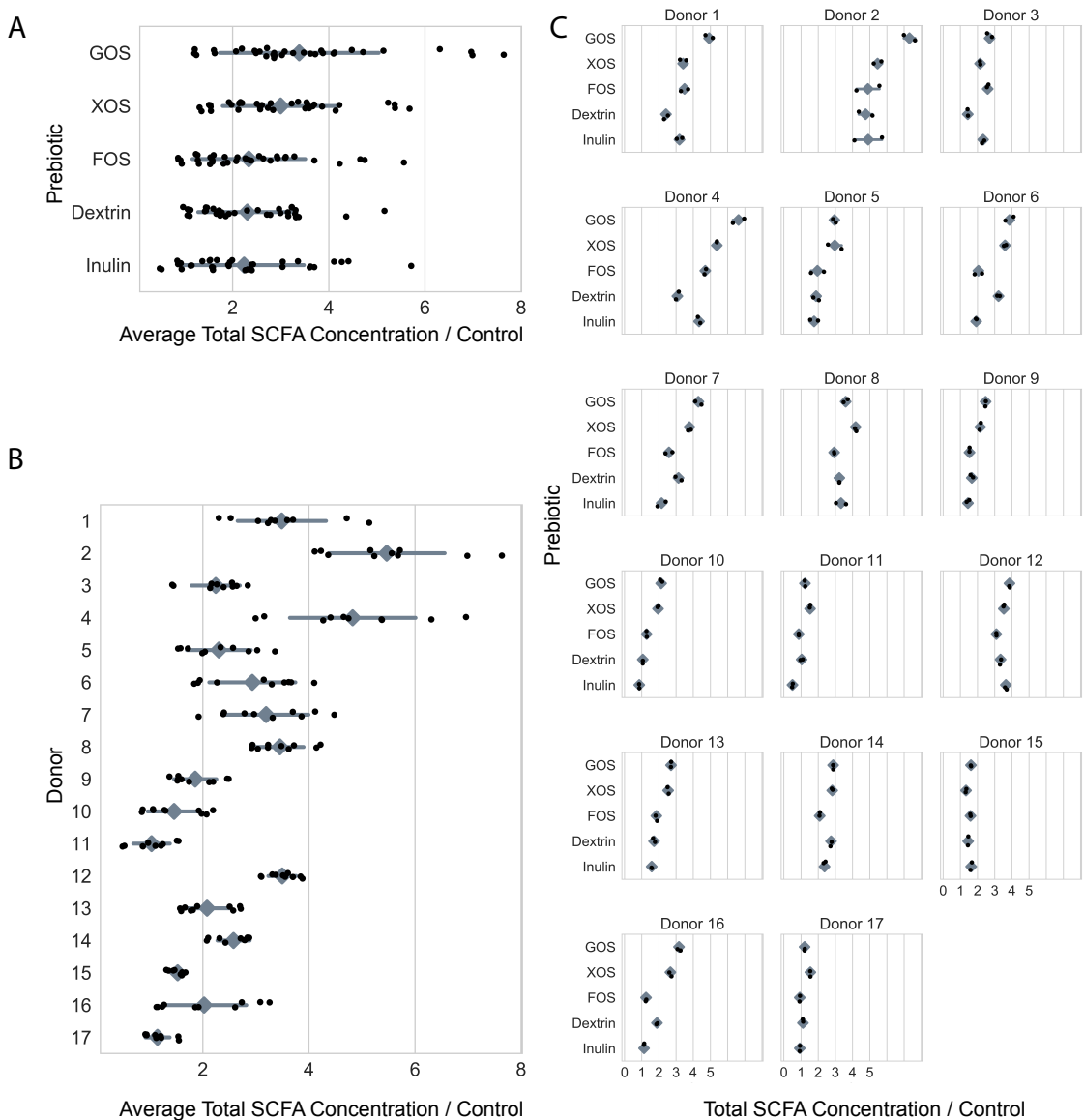
Figure 2: In vitro SCFA production by prebiotic (a), donor (b), and individually (c).

In a two-way ANOVA of the effects of 'Donor' and 'Prebiotic' on 'SCFA Concentration /

Control', 'Donor', 'Prebiotic', and their interaction were all statistically significant

($p < 0.0001$, $p < 0.0001$, $p < 0.0001$, respectively). Shown is the total SCFA concentration of

an in vitro culture after 24hrs of anaerobic incubation, divided by the SCFA

concentration of the corresponding prebiotic-free control culture, for each of five

825   prebiotic growth conditions across 17 donors (black dots). Grey diamonds are means

826   and grey bars are standard deviations. (Absolute SCFA concentrations are depicted in

827   Figure S3).
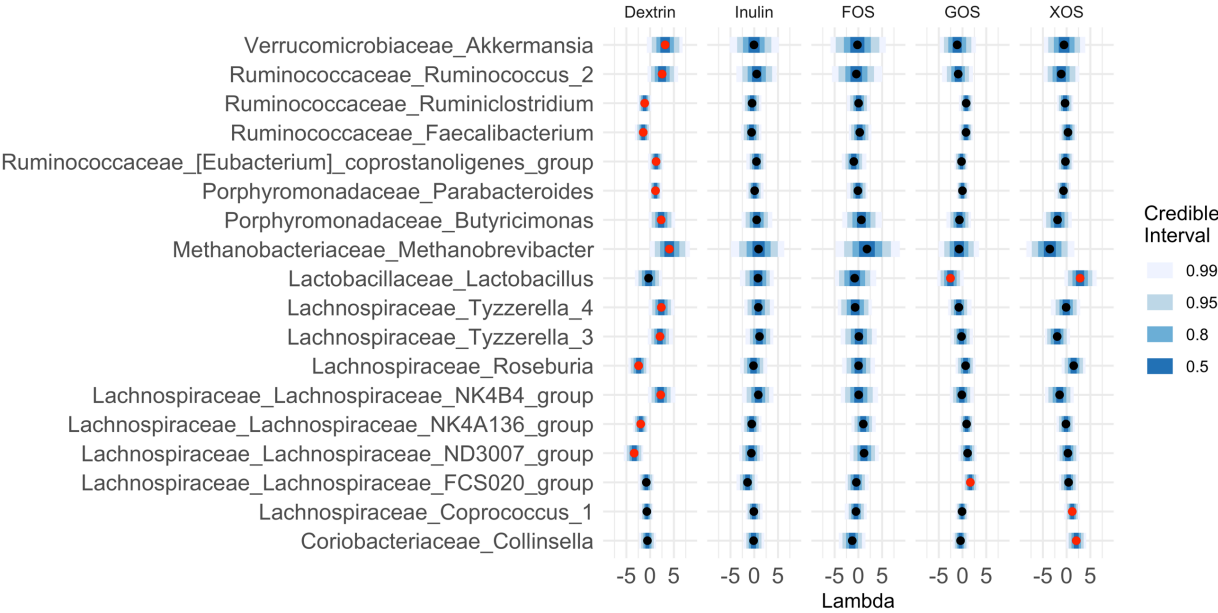
Figure 3: 18 genera were found to be credibly associated with SCFA production in at least one of our five prebiotic growth conditions. Shown are the mean Lambda and 99%, 95%, 80%, and 50% credible intervals for all 18 genera credibly associated with at least one prebiotic growth condition, plotted on centered log-ratio (CLR) coordinates. Red centers denote associations with 95% credible intervals that do not cover 0. Lambda represents the strength of the effect of each covariate on each taxa. A lambda of one reflects a unit fold-change in SCFA concentration over control as being associated with a unit fold-change in the CLR-transformed relative abundance of the genus.

	BMI	Insulin	HbA1c
Average Net SCFA Production	p = 0.98 ρ = -0.007	p = 0.63 ρ = 0.13	p = 0.75 ρ = 0.083
Fecal SCFA	p = 0.65 ρ = -0.12	p = 0.61 ρ = 0.13	p = 0.72 ρ = -0.09

838 **Table 2: Neither average SCFA production *in vitro* nor fecal SCFA concentration**

839 **correlated with metrics of obesity measured in individuals at time of enrollment.** P

840 values and ρ from Spearman correlations.
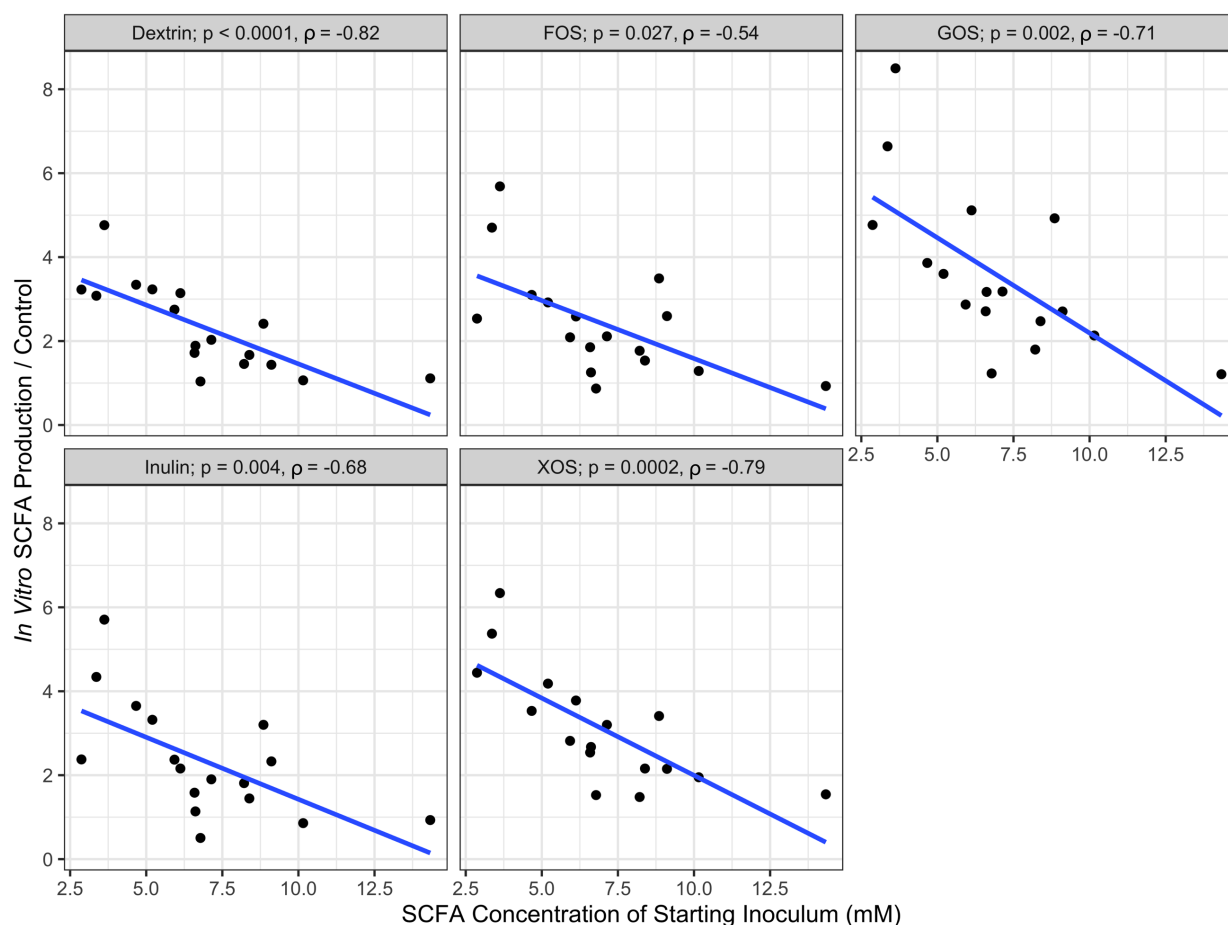
Figure 4: Spearman correlations between *in vitro* SCFA production and SCFA concentration of the starting fecal inoculum. SCFA production is the average of technical replicates, with the linear regression line plotted.

Genus	Association with SCFA production					Fiber Degrader?	Reference
	Dextrin	XOS	GOS	FOS	Inulin		
Akkermansia	+					Supporter	(86)
Ruminococcus_2	+					Degrader	(48)
Coprostanoligenes_group	+					No Evidence	(87)
Parabacteroides	+					Degrader	(49)
Butyricimonas	+					Associated	(50)
Methanobrevibacter	+					Supporter	(88)
Tyzzerella_4	+					Degrader	(51)
Tyzzerella_3	+					Degrader	(51)
Lachnospiraceae_NK4B4	+					Degrader	(51)
Lactobacillus		+	-			Degrader	(49)
Coprococcus_1		+				Degrader	(52)
Collinsella		+				No Evidence	(89)
Lachnospiraceae_FCS020			+			Degrader	(51)

845 **Table 3: Associations between microbial genera and SCFA production on five**

846 **different prebiotic substrates.**

847 **SUPPLEMENTARY FIGURES AND TABLES**
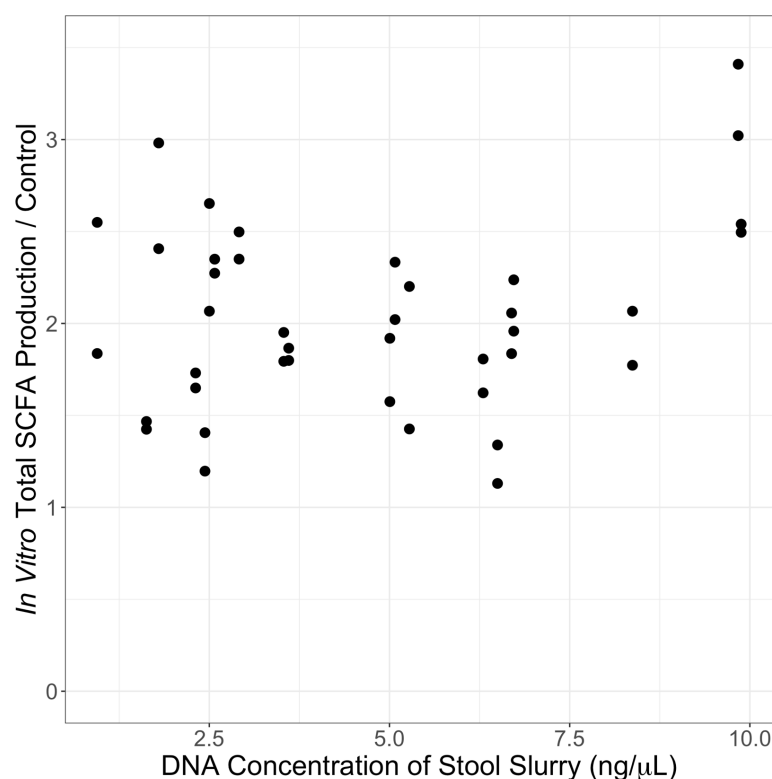
848

849 **Figure S1: Bacteria abundance in stool, as measured by DNA concentration, does**

850 **not correlate with control-corrected total SCFA production in *in vitro* cultures (p =**

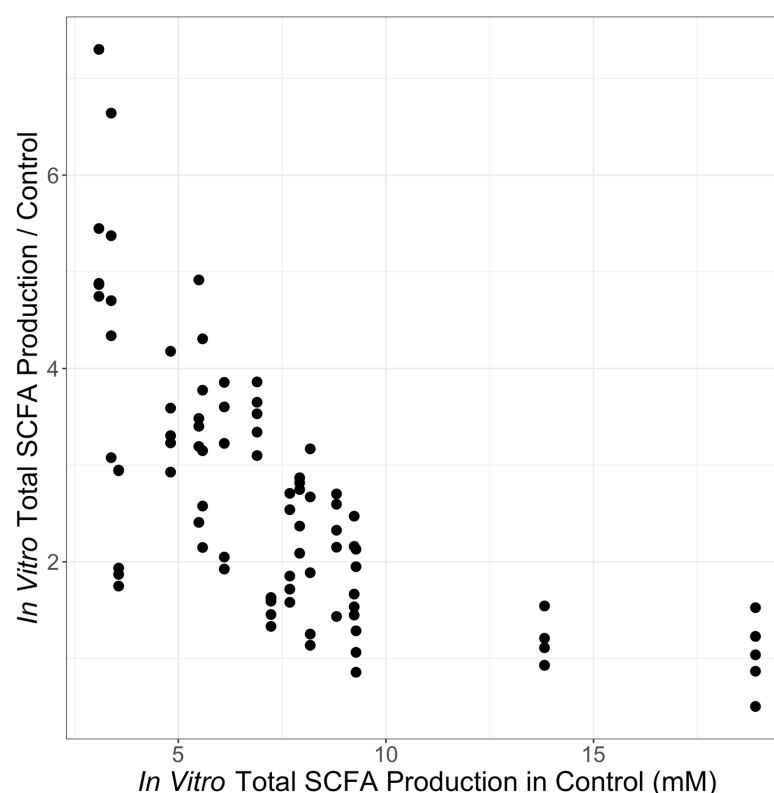851 **0.38, ρ = 0.14; Spearman correlation).**

Figure S2: To control for differences in overall cell viability or stool slurry nutrient content between donors, we corrected measurements of SCFA concentration by dividing the treatment SCFA concentration by the control SCFA concentration. The resulting fold-change data do not contain information about absolute SCFA production. We examined the potential for this artifact to influence our interpretation, and found that fold changes of SCFA concentrations after prebiotic treatment relative to the control were correlated with absolute control treatment levels (p<0.0001, ρ = -0.77, Spearman correlation), Absolute (not corrected to control) SCFA concentrations are presented in figure S3.
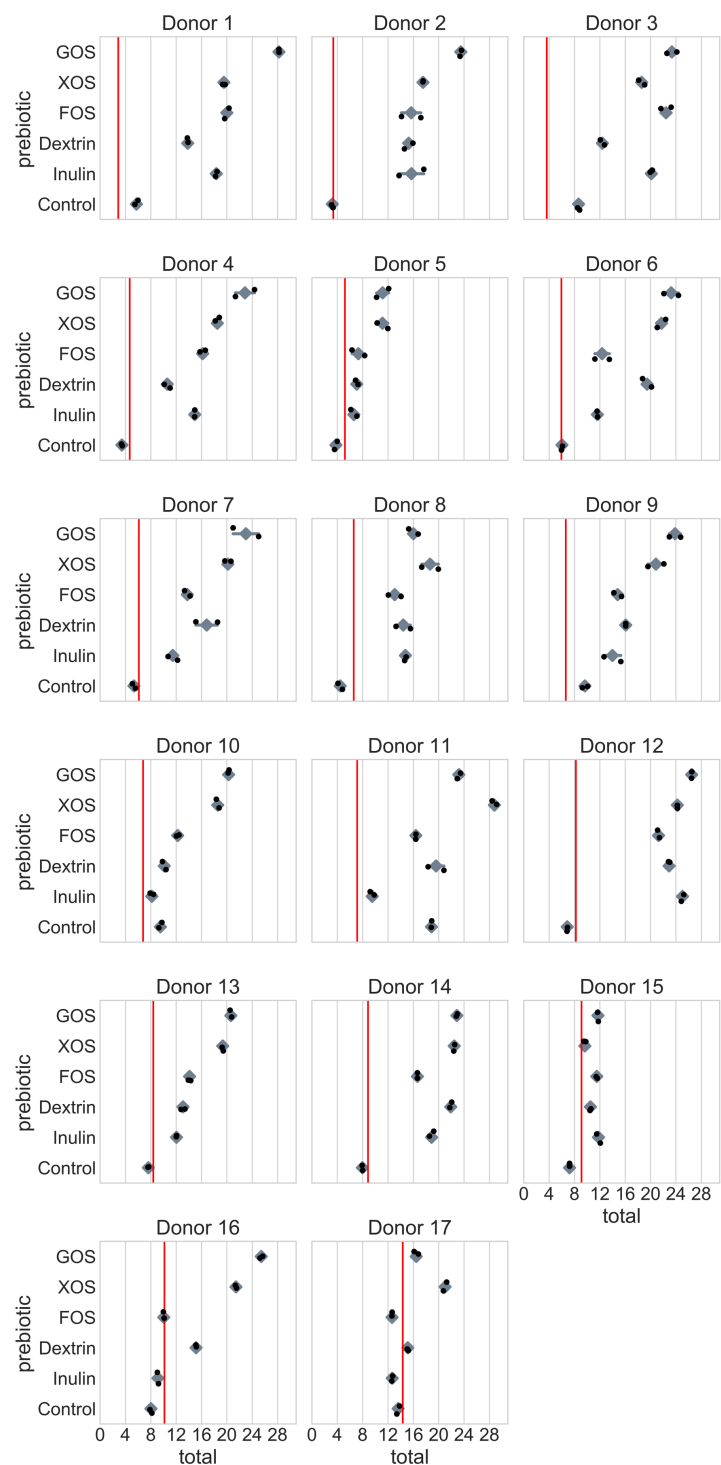
Figure S3: Total SCFA concentration of *in vitro* fermentation vessels after 24hr fermentation, plotted for each donor across five prebiotic treatments and the unsupplemented control vessel. The vertical red line indicates the total SCFA

866   concentration of the starting fecal slurry prior to fermentation. Instances where SCFA

867   concentration decreases during fermentation may be explained by net SCFA

868   consumption by the community when no fermentable carbon is supplied, or by a lack of

869   change in concentration coupled with technical variation in our measurements.

870   Instances where SCFA concentration is increased in the control treatment suggest that

871   some unmetabolized carbohydrate may have remained in the stool to be metabolized
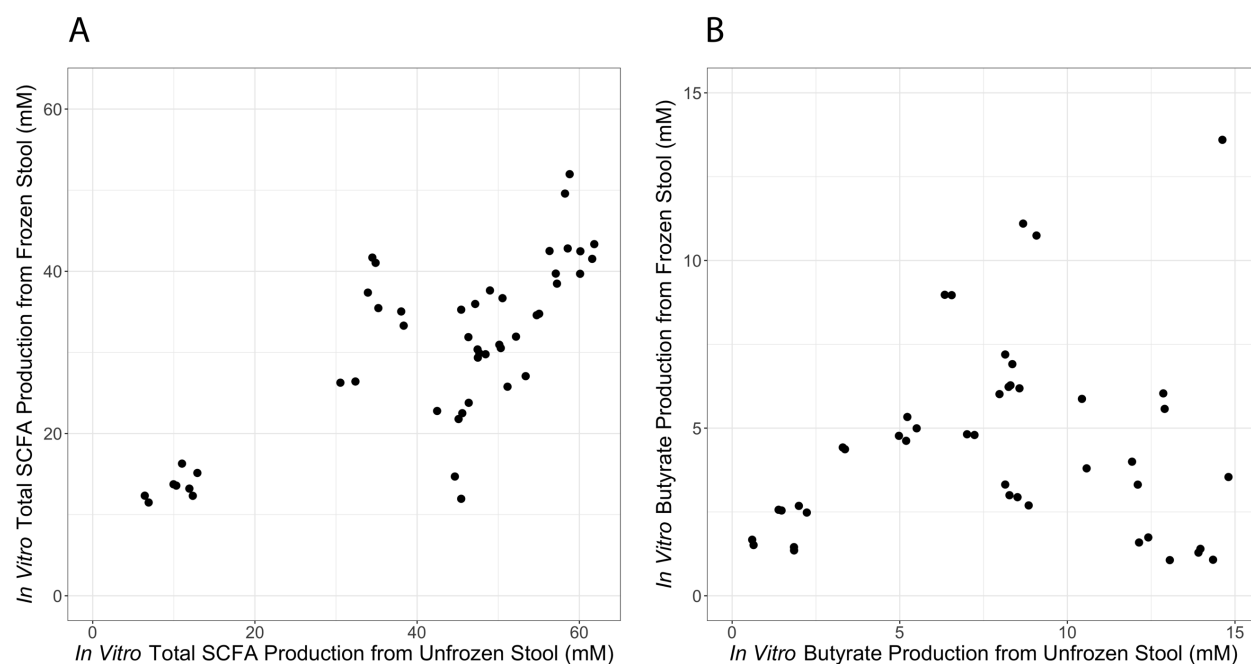
872   during *in vitro* fermentation.

Figure S4: *In vitro* total SCFA production from unfrozen stool samples and from twice frozen stool samples is highly correlated (A, p < 0.0001, ρ = 0.75, Spearman correlation). In contrast, *in vitro* butyrate production is not correlated between unfrozen and twice frozen stool samples (B, p = 0.18, ρ = 0.19 Spearman correlation).
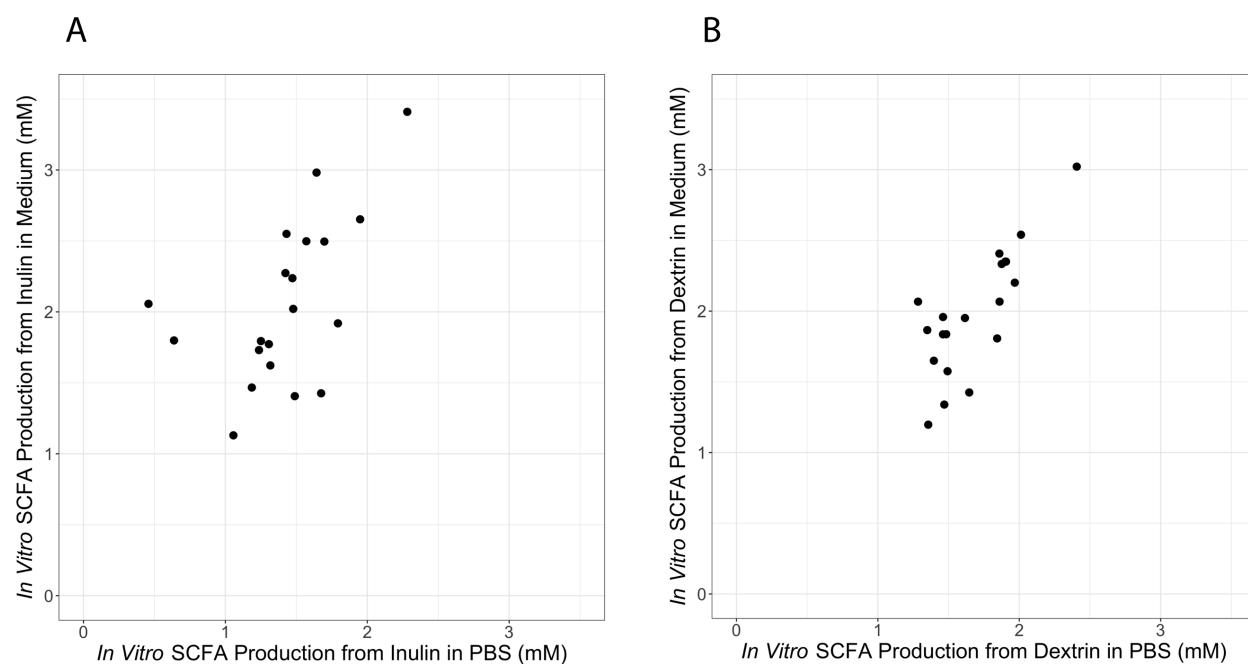
878
879 **Figure S5: *In vitro* total SCFA production from inulin (A) and dextrin (B) is well**

880 **correlated between cultures grown in PBS and cultures grown in a medium**

881 **designed to mimic the colonic environment (p = 0.001, ρ = 0.68 (A); p = 0.02, ρ =**
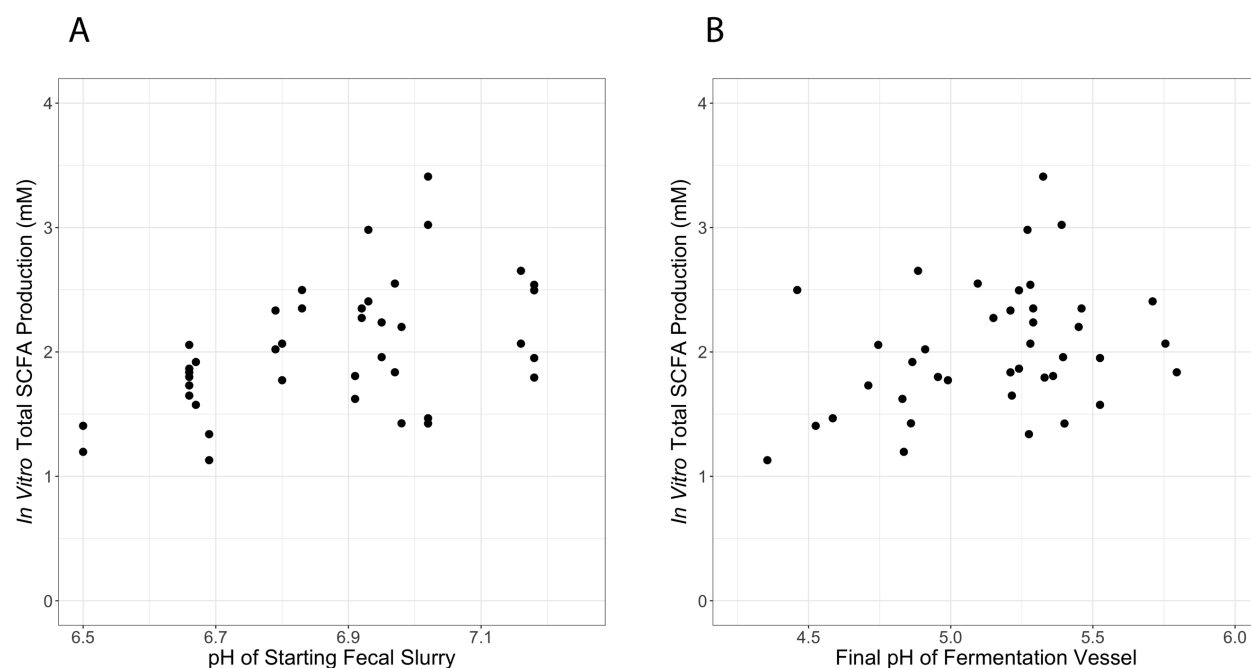
882 **0.51 (B); Spearman correlations).**

Figure S6: *In vitro* total SCFA production over control is positively correlated with

the pH of starting fecal slurries (p = 0.003, ρ = 0.46; Spearman correlation). A

weaker correlation might exist between SCFA production and the final pH of

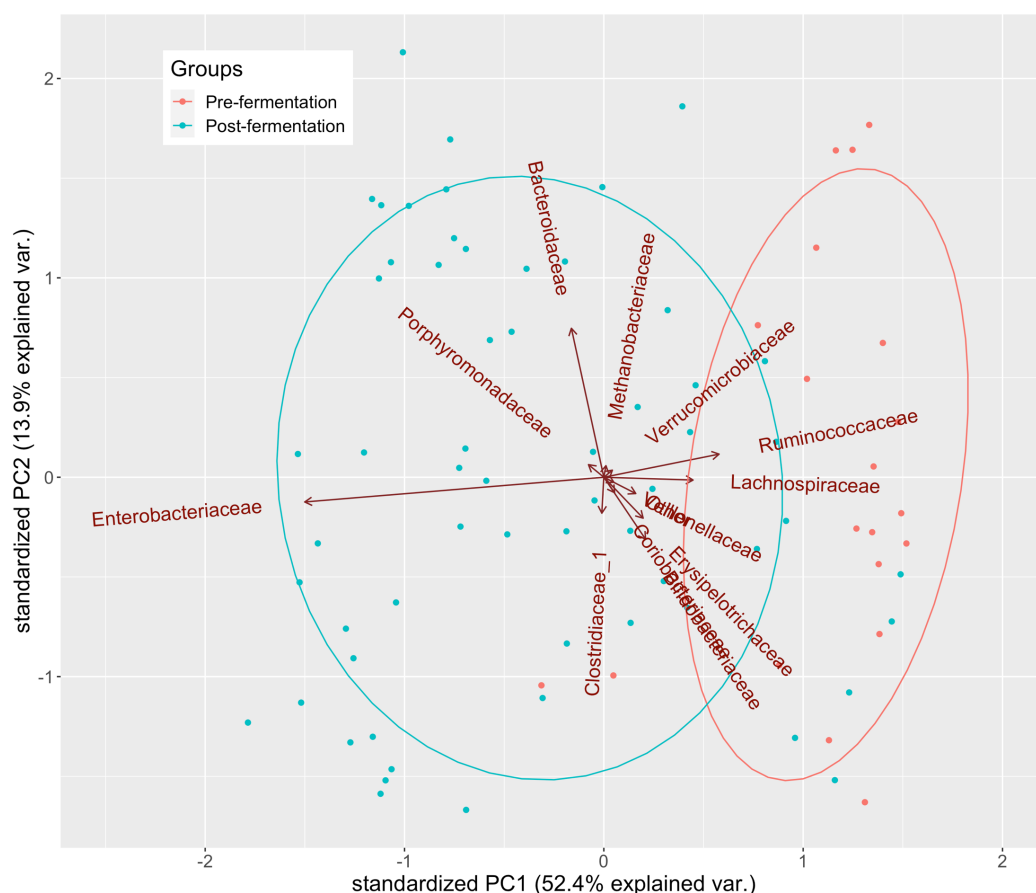fermentation vessels (p = 0.067, ρ = 0.29; Spearman correlation).

Figure S7: Microbial community composition changes during the course of a 24hr fermentation. We performed 16S rRNA sequencing on pre-fermentation and post-fermentation samples from 20 donors across 3 treatments (inulin, dextrin, and control). We then measured the Shannon diversity of the fecal slurries both before and after fermentation (all treatments averaged) and found a significant decrease in Shannon diversity over the course of fermentation ($p < 0.0001$; paired t-test). To characterize the changes in community composition associated with this decrease in diversity, we tested the pre-fermentation and post-fermentation samples for differential abundance of taxa at the species level. We found 10 taxa with significantly different abundances between the two sample sets ($p < 0.05$; Benjamini-Hochberg corrected Wilcoxon rank-sum tests). Of these 10 taxa, only Escherichia/Shigella spp. increased in relative abundance after

900   fermentation, while nine other taxa each decreased in relative abundance (*Anaerostipes*

901   *hadrus, Bacteroides acidifaciens, Blautia faecis, Blautia wexlerae* and two other *Blautia*

902   of undetermined species, two undetermined species in the *Eubacterium hallii* group,

903   and *Ruminococcaceae_UCG-004 spp.*). We attribute these changes to differences in

904   growth rates among bacteria in our in vitro system, and the inability of our fermentation

905   medium to support the growth of some community members.

Source	*df*	*SS*	*F*	*p*
Donor	16	243.1	255.4	<0.0001
Prebiotic	4	35.7	149.9	<0.0001
Donor*Prebiotic	64	28.7	7.5	<0.0001

906 **Table S1: Two-way ANOVA of *in vitro* SCFA production across donors and**

907 **prebiotics.**

Patient	Treatment	Timepoint (month)
1	Lifestyle only	6
2	Lifestyle only	0
3	Lifestyle only	3
4	Lifestyle only	0
5	Lifestyle only	6
6	Lifestyle only	4.5
7	Lifestyle only	0
8	Lifestyle only	3
9	Lost to follow-up	0
10	Lifestyle only	4.5
11	Lifestyle only	3
12	Lifestyle only	4.5
13	Low carb	0
14	Lifestyle only	4.5
15	Metformin	4.5
16	Lifestyle only	4.5
17	Lifestyle only	4.5

908 **Table S2: Treatment arms and sample timepoints of patients in this study.**