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

Background

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

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

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

single prebiotics (31, 33, 36, 37), or tested different prebiotics in separate cohorts (30, 32, 38). More rare crossover studies have taken place, but have either not investigated individual variation in prebiotic response (39), or solely utilized *in vitro* assays of prebiotic response (40, 41). If consistent evidence does emerge that responses to different prebiotics vary within people, then effective prebiotic therapies will face the challenging task of personalizing prebiotic choices for each recipient. Alternatively, if responses to different prebiotics are conserved within individuals, effective prebiotic therapy could primarily focus on selecting individuals for treatment. Evidence supporting this latter scenario include studies showing that individual identity is a stronger predictor of SCFA concentrations than dietary intervention (42), and that individual characteristics like habitual food choice can predict changes to microbiota community composition following prebiotic supplementation (37).

To directly investigate the interaction of prebiotic and individual variation in prebiotic response, we report here the results of a randomized, fully balanced, crossover trial where a single set of individuals was treated with multiple prebiotics. Our primary lines of inquiry were to assess the relative contribution of individual and prebiotic differences to SCFA responses to supplementation, with an emphasis on butyrate production. Secondary analyses included determining what host and microbiological factors shaped variation in prebiotic response.

Methods

Participant recruitment:

This study was conducted at Duke University in Durham, NC, USA, and was registered with ClinicalTrials.gov (Identifier: [NCT03595306](https://clinicaltrials.gov/ct2/show/study/NCT03595306)). All recruitment and study procedures involving human subjects were approved by the Duke University Institutional Review Board

(Pro00087214). Study participants were recruited by flyers, email list-servs, and word-of-mouth at Duke University Campus. Individuals with a self-reported history of irritable bowel syndrome, inflammatory bowel disease, type-2 diabetes, kidney disease, intestinal obstruction, or colorectal cancer were excluded. Participants were also excluded if they had taken oral antibiotics within the past 6 months, had any known food allergies, reported dietary intolerances of any kind, or were pregnant or breastfeeding. If individuals identified as female, their urine was subjected to a pregnancy test (Quidel QuickView One-Step hCG Urine Test) to minimize the risk that prebiotic treatment would impact newborn or infant health. Participants who tested positive were deemed ineligible for participation in the study. Inclusion criteria were that participants were between the ages of 18 and 70, and able to provide stool samples at no risk to themselves. Fiber consumption, prebiotic/probiotic usage, and dietary habits were not used as inclusion/exclusion criteria, but participants were asked to report via surveys as part of the study requirements. During this period, 81 individuals set up a phone interview to determine eligibility and interest to participate in the study. Of these, 41 completed electronic informed consent to the study and were subsequently enrolled. Ultimately, 8 subjects were lost to follow-up, 2 withdrew, and 3 were omitted from some or all analyses due to inability to collect sufficient usable sample, resulting in 28 subjects included in all final data analyses.

Study design:

The three-period crossover longitudinal study was conducted from May of 2018 to March of 2019. The crossover design allowed investigation of three prebiotic interventions while also allowing participants to serve as their own controls (Figure 1). There were six arms of the study, to cover all possible orders of prebiotic consumption and to eliminate the need to account for

uneven carry-over effects. The study consisted of an initial study visit, followed by a 6-week study period with alternating weeks of baseline (no prebiotic consumption) and intervention (prebiotic consumption). Participants were added sequentially to an arm group as they enrolled; meaning the first participant was enrolled in Arm 1, the second to Arm 2 and so on. Participants were blinded to the prebiotics that they were consuming until the end of the study. The prebiotics were taken twice a day, Monday to Friday during the intervention weeks. Monday was a half-dose, to help allow participants to adjust to the increase in fiber. Following consent, participants had an initial study visit, to have their baseline physiology assessed by height, weight, and blood pressure measurement, as well as provide a urine sample and receive the study kit and materials. Participants were asked to self-collect stool at home three times a week each week during the study period. Participants also completed 4 diet surveys throughout the study; one instance of the Diet History Questionnaire Version 3 (DHQ3) (43), covering the one month period prior to the study, and three instances of the Automated Self-Administered 24-hour (ASA24) Dietary Assessment Tool, version 2017, developed by the National Cancer Institute, Bethesda, MD (44). Two ASA24's were completed during the week, and one on a weekend. The DHQ3 was completed prior to the 6-week study period. Additionally, participants completed online-surveys about their experiences with the prebiotics following the intervention weeks.

Prebiotic supplementation:

Three prebiotics were tested in this study: inulin (manufactured by Now Foods, Bloomingdale, IL), wheat dextrin (Benefiber, manufactured by GSK Consumer Healthcare, Warren, NJ), and galactooligosaccharides (GOS) (Bimuno, manufactured by Clasado Limited, Shinfield, UK). In a clean non-laboratory space, prebiotics were weighed into individual doses

and packaged into pre-labeled food-safe sealable plastic bags. Each participant received all three prebiotics throughout the study; inulin at 9g / day, wheat dextrin at 9g / day, and galactooligosaccharides (GOS) at 3.6g / day. These doses correspond to the recommended daily intake on the product label. In order to further minimize the possibility of discomfort associated with consuming prebiotics, the first day of dosing for each prebiotic was a half dose to allow acclimation (4.5g for inulin and dextrin, 1.8g for GOS). Participants consumed one of the three prebiotics at a time, twice a day, for a duration of 5 days, followed by a week of washout. The order was determined by their assigned study arm. Participants were instructed to consume the prebiotics by mixing the powders in any kind of beverage (such as water) or food (such as applesauce), so long as it was cold or room temperature.

Stool collection:

At the pre-study visit, participants were provided with a standard urine specimen collection cup and shown to a bathroom to collect the sample. Urine samples were also sent to LabCorp in a BD Vacutainer UA tube to measure metabolic variables, including routine urinalysis, sodium, potassium, urea nitrogen, and creatinine. Consented participants were asked to self-collect stool three times a week at home using sampling kits provided to them. Participants were asked to immediately store samples in their personal freezers (-20C). Participants were asked to bring stored samples to the David Lab freezers on a weekly basis. Stool samples were transferred and stored at -80C until thawed for DNA extraction and other sample processing.

In vitro fermentation:

In vitro fermentation of stool microbiota was conducted following protocols previously published (40). In brief, a 10% w/v fecal slurry was made using thawed week-1 stool and anaerobic 1X PBS (pH 7.0 ± 0.1). This slurry was filtered through 0.33mm pore size mesh, then mixed 1:1 with a 1% w/v solution of prebiotic and incubated in duplicate in a 24-well cell culture plate for 24hr. Three prebiotic treatments were used, corresponding to the three prebiotics used in the *in vivo* portion of this study: inulin (Now Foods Inulin Powder, part #2944), galactooligosaccharides (GOS; Bimuno Powder), and wheat dextrin (Benefiber Original). The resulting fermentation conditions were therefore 5% fecal slurry with 0.5% prebiotic (w/v) and occurred at 37°C for 24 hours. Following fermentation, a 1mL aliquot was taken from each well for SCFA quantification.

Quantification of SCFA:

Quantification of SCFA by gas chromatograph flame ionization detection (GCFID) was performed following protocols previously published (40). In brief, 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 (25m x .2mm id x .3µm film). Acetate, propionate, isobutyrate, butyrate, isovalerate, and valerate were identified and quantified in each sample by comparing to an 8-point standard curve that encompassed the

sample concentration range. Standards contained 0.1mM, 0.2mM, 0.5mM, 1mM, 2mM, 4mM, 8mM, and 16mM concentrations of each SCFA.

DNA extraction, PCR amplification, and sequencing:

We performed 16S rRNA gene amplicon sequencing on human stool samples to determine microbiota community composition following protocols previously published (40). DNA was extracted from frozen fecal samples with the Qiagen DNeasy PowerSoil DNA extraction kit (ID 12888-100). Amplicon sequencing was performed using custom barcoded primers targeting the V4 region of the 16S gene (45), using published protocols (45-47). The sequencing library was diluted to a 5pM concentration and sequenced using an Illumina MiniSeq and a MiniSeq Mid Output Kit (FC420-1004) with paired-end 150bp reads.

Identifying sequence variant and taxonomy assignment:

We used an analysis pipeline with DADA2 (48) to identify and quantify sequence variants, as previously published (40, 49). To prepare data for denoising with DADA2, 16S rRNA primer sequences were trimmed from paired sequencing reads using Trimmomatic v0.36 without quality filtering (50). Barcodes corresponding to reads that were dropped during trimming were removed using a custom python script. Reads were demultiplexed without quality filtering using python scripts provided with Qiime v1.9 (51). Bases between positions 10 and 150 were retained for the forward reads and between positions 0 and 140 were retained for the reverse reads. This trimming, as well as minimal quality filtering of the demultiplexed reads was performed using the function fastqPairedFilter provided with the DADA2 R package (v1.8.0). Sequence variants were inferred by DADA2 independently for the forward and reverse reads of

each of the two sequencing runs using error profiles learned from all 20 samples. Forward and reverse reads were merged. Bimeras were removed using the function `removeBimeraDenovo` with default settings. Taxonomy was assigned using the function `assignTaxonomy` from DADA2, trained using version 123 of the Silva database.

Results

Prebiotic supplementation affected SCFA production at the cohort-level. We enrolled participants into a six-week, three-period prebiotic intervention study (Figure 1). We used a crossover design to compare multiple prebiotics across a subject pool. We selected inulin, wheat dextrin, and galactooligosaccharides (GOS) as our prebiotics, as these are commonly consumed (52), commercially available dietary supplements, and are monomerically distinct. Our crossover was standardized in series and period, with each prebiotic occurring the same number of times within each series (once) and study period (twice). The design was also balanced with respect to first-order carry-over effects, meaning we shuffled the order in which prebiotics were administered so as to minimize the interactive effects between any two pairs of consecutive prebiotics. Each study period consisted of an intervention-free baseline week and a prebiotic intervention week. Stool samples were collected on Wednesday, Thursday, and Friday of each of the six study weeks (baselines and intervention). Of the 41 participants who initially enrolled, 2 withdrew, 3 were unable to collect at least one usable sample (i.e., insufficient volume or inadequate labeling) per week, and 8 were lost to follow-up. We focused our subsequent analyses on the remaining 28 participants who completed the study. These participants in total collected 413 stool samples (mean=2.46 stool samples per person per week).

We first sought to examine differences in microbiome response during each of the different prebiotic treatments. Analyses of gut microbial community composition did not reveal associations with inulin, GOS, or wheat dextrin treatment ($p > 0.05$, PERMANOVA using unweighted UniFrac distance at species level). In addition, no species were statistically differentially abundant during any prebiotic intervention and its corresponding prebiotic-free baseline ($p > 0.05$, Benjamini-Hochberg corrected Wilcoxon Rank Sum tests using the R package ALDEx2). While previous studies have observed changes in community composition following prebiotic administration, such effects have been observed in a subset of tested prebiotics (30, 32), and with different dosing schedules (30-32, 37).

Still, in contrast to the lack of a coherent 16S rRNA shifts among participants, we found the three prebiotics varied according to resulting fecal SCFA profiles relative to baseline. GOS was associated with decreased total stool SCFA concentrations (Table 1, Figure S1C, effect size = -0.071, $p = 0.038$, GLMM), an outcome that has been previously observed (53). Differences within individual SCFAs were also apparent when analyzing absolute fecal SCFA concentrations (Table 1). Dextrin increased propionate concentration (effect size = 0.074, $p = 0.038$, GLMM), and GOS decreased both propionate and butyrate concentrations (effect size = -0.087, $p = 0.015$; effect size = -0.14, $p = 0.002$, GLMMs, respectively). Still, changes in total and individual SCFA concentrations may be caused by effects of stool hydration, which can vary up to three-fold and directly impacts measures of SCFA concentration in feces (54, 55). To assess 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 (56-58). 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 (53, 59, 60) and dextrin (61, 62) 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, 63-66). 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, 42). In line with prior studies of prebiotic response, (40) 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 (pseudo- $R^2 = 0.39$, $p < 0.0001$; GLM F-test) were much stronger than prebiotic-specific effects (pseudo- $R^2 = 0.05$, $p = 0.001$; GLM F-test). Additionally, individuals who exhibited the largest response in proportional stool butyrate concentration on a given prebiotic were more likely to

exhibit larger responses on the other two prebiotics ($\rho = 0.49, 0.37$, and 0.28 , $p = 0.008, 0.05$, and 0.15 , respectively for inulin vs dextrin, dextrin vs GOS, and inulin vs GOS, Spearman correlations; Figure 3). This pattern was apparent in spite of correcting for baseline differences in SCFA production between individuals, which were also consistent over time and between baseline and treatment weeks.

Individuals consistently varied according to gut metabolic state, microbiota community composition, and metabolic potential. Why might individual-specific effects be stronger than prebiotic-specific effects in our cohort? We observed that individuals were idiosyncratic in their SCFA profiles over time. Both proportional butyrate concentration and butyrate concentration were correlated within individuals between the first and last baseline weeks ($\rho = 0.49$, $p < 0.007$; and $\rho = 0.63$, $p = 0.0003$, respectively, Spearman correlations; Figure S2). Variation between individuals' SCFA profiles could also be linked to inter-individual microbiota variation, a well-known factor in prebiotic response (42). When we divided participants into 'high' ($>$ median) and 'low' ($\leq$ median) fecal butyrate groups, we observed a significant effect of this grouping on microbiota community composition ($R^2 = 0.075$, $p = 0.0022$, PERMANOVA using unweighted UniFrac distance; Figure S3). We identified three specific genera that were differentially abundant between the groups: *Anaerofilum* and *Ruminiclostridium_5* were decreased in the high butyrate group, and *Lachnospira* was increased in the high butyrate group ($p = 0.009, .043$, and 0.030 , respectively; Benjamini-Hochberg corrected Wilcoxon Rank Sum tests; Figure S3). We additionally observed a relationship between baseline microbiota community structure and average response to all three prebiotics ($\Delta\%$ butyrate). After dividing participants into 'responders' and 'non-responders', based on whether average $\Delta\%$ butyrate was positive or

negative, we found a significant effect of this grouping on microbiota community composition ($R^2 = 0.059$, $p = 0.028$, PERMANOVA using unweighted UniFrac distance; Figure S4). Thus, individuals' starting microbiome composition was associated with prebiotic treatment outcome.

Intake patterns of dietary fiber negatively correlated with response to prebiotic intervention.

Next, we sought to understand what host factors might influence individuals' microbiomes and ultimately shape their butyrogenic responses across the tested prebiotics. We focused on fiber intake, which has been shown to shape microbiota community composition and metabolic output (67, 68). We measured fiber consumption using a dietary questionnaire spanning the month preceding the study (43), and we compared fiber intakes to each individual's average response to the three prebiotic interventions (average $\Delta\%$ butyrate). Despite prior studies suggesting that plant or fiber intake would augment gut microbial metabolic capacity (37, 68-70), our comparison revealed that dietary fiber consumption was negatively correlated with average $\Delta\%$ butyrate ($\rho = -0.40$, $p = 0.046$; Spearman correlation; Figure 4A). Thus, higher habitual fiber intake was associated with a lower average $\Delta\%$ butyrate during the prebiotic intervention. To corroborate these findings using an objective biomarker, we also compared average $\Delta\%$ butyrate to spot urinary potassium, a known marker for fruit and vegetable intake (71), as well as for general healthy eating (72, 73). We validated that spot urinary potassium was correlated with dietary fiber intake in our study ($\rho = 0.47$, $p = 0.031$; Spearman correlation; Figure S5A) and found evidence for a negative correlation between spot urinary potassium and average $\Delta\%$ butyrate ($\rho = -0.38$, $p = 0.071$; Spearman correlation; Figure S5B).

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

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

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

proportional butyrate concentrations including legumes, berry fruits, and dark-green vegetables (Figure 5).

Culture-based experimental evidence links fiber intake to limits on microbiome metabolic

response. A third line of experimental evidence arose linking habitual nutrient intake and microbial SCFA production capacity. We hypothesized that if baseline fiber consumption reduced the capacity of gut microbiota to produce SCFAs, the metabolic activity of fecal microbiota would exhibit a negative relationship with fiber intake. To test this hypothesis, we applied a recently developed an *ex vivo* fermentation model (40) to baseline stool samples collected from each study participant. This model experimentally tests the metabolic effects of supplementing a microbial community using a given prebiotic as the sole additional carbon source, independent of potentially confounding host factors like SCFA absorption. In support of the model's capability to produce SCFAs following prebiotic administration, we observed an increase in overall SCFA production relative to control ($p < 0.0001$, t-test; average of 4.41-fold increase across participants, calculated using the average total SCFA concentration of 3 separate prebiotic treatments) when the *in vitro* assays were supplemented with prebiotics as the sole carbon source. When we compared SCFA production to fiber intake, we observed as expected a negative correlation between fiber consumption and SCFA production ($p = 0.021$, $\rho = -0.46$, Spearman correlation using average of three prebiotic treatments; Figure 4C). We also observed a negative correlation between fiber consumption and SCFA production in prebiotic-free control treatments ($p = 0.019$, $\rho = -0.47$, Spearman correlation; Figure S7. Thus, individuals who tended to eat more fiber in our cohort also harbored gut microbiota that produced less SCFA when exposed to prebiotics.

Consistent growth of specific microbial genera supports individual-specific prebiotic

response. Notably, our *ex vivo* fermentations also associated specific gut bacterial taxa with prebiotic response. Following 24hr fermentation, microbial community composition was assayed from each prebiotic-supplemented culture and compared to prebiotic-free control cultures, for each of the 28 participants, using ALDEx2. This analysis revealed multiple genera that were over or under-represented in each prebiotic treatment (Figure 6).

Additionally, most genera that were differentially abundant in one prebiotic treatment exhibited similar differential abundance in the other prebiotic treatments. All genera exhibiting conserved higher abundance in two or three prebiotic treatments compared to control (*Collinsella*, *Faecalibacterium*, *Lachnospiraceae*, *Marvinbryantia*, and *Ruminiclostridium*) are known degraders of common dietary fibers with broad specificity (78-80). By contrast, of the genera exhibiting conserved lower abundance in two or three prebiotic treatments compared to control (*Alistipes*, *Anaerofilum*, *Flavonifractor*, *Holdemania*, and *Parabacteroides*), we are not aware of reports linking them to degradation of inulin, dextrin, or GOS, and only *Parabacteroides* has been shown to degrade any dietary fiber (78, 80). Overall, the consistent response of select bacterial taxa to prebiotic addition *in vitro* further supports the conclusion that responses across a range of prebiotics will be linked to individual-specific factors like gut microbial community composition.

Discussion

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

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

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

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

high-fiber diet would have a greater capacity to utilize incoming novel fiber, not a lower capacity as we observed. Still, prior studies reporting links between diet patterns and microbiota response have specifically associated changes to microbial community composition with baseline fiber intake (37). Associations between fiber consumption and SCFA production, by contrast, have either not been tested for in prebiotic trials (92, 93) or not revealed a positive relationship (37). Additionally, while our analyses confirmed an association between baseline proportional butyrate levels and intake of plant foods groups linked to dietary fiber (e.g. legumes and dark green vegetables), it also linked food groups typically associated with a Mediterranean diets (e.g. seafood, dairy, fruit, and poultry (94)) with higher baseline levels of proportional acetate. Future prebiotic trials are needed to determine the generality of our observed association between habitual diet and SCFA production

Conclusions

Our study provides evidence that the responsiveness of an individual to prebiotic treatment may be predictable from diet and baseline concentrations of SCFA in stool. We emphasize that we do not believe these predictions should be used to modify the diets of individuals who normally consume high levels of fiber or have higher concentrations of SCFA in stool. Ample evidence supports the benefits of both a high fiber diet and adequate microbial SCFA production (70, 95, 96). Rather, we argue our findings support strategies for personalizing microbiota therapy that focus on individual selection. Ongoing prioritization efforts are being used to identify patients most likely to respond to microbiota-directed and microbiota-dependent disease therapies (97-100), and are also being explored to enhance prebiotic therapy (30, 69, 90, 91). Our study here suggests that such efforts to personalize prebiotic supplementation can rely

on dietary history and fecal metabolite levels as informative and non-invasive biomarkers of treatment outcome.

Ethics approval and consent to participate

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

Competing interests

The authors declare that they have no competing interests

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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.

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Figures

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

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

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

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

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

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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 ($\Delta\%$ butyrate) was negatively correlated with habitual dietary fiber consumption ($\rho = -0.40$, $p = 0.046$; Spearman correlation). (B) Average $\Delta\%$ 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).

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 1 and 2 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.

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

Supplementary figures and tables

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

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

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

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

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

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

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