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Fiber supplementation influences phylogenetic structure and functional capacity of the human intestinal microbiome: follow-up of a randomized controlled trial[1–4]

Hannah D Holscher, J Gregory Caporaso, Seema Hooda, Jennifer M Brulc, George C Fahey Jr., and Kelly S Swanson

ABSTRACT

Background: In our published randomized, double-blind, placebo-controlled, 3-period crossover trial, healthy adult men (n = 21) consumed bars containing no supplemental fiber (placebo; NFC), polydextrose (21 g/d), and soluble corn fiber (SCF; 21 g/d) for 21 d each. Fecal specimens were collected between days 16 and 21 for fermentative end-product analysis and 16S ribosomal RNA bacterial gene amplification for bacterial taxa identification. Fiber supplementation decreased fecal putrefaction compounds and shifted abundances of several bacterial taxa.

Objective: The objective was to perform whole-genome shotgun 454 pyrosequencing on the same fecal specimens collected in that clinical trial to obtain comprehensive fecal bacterial genome sequencing coverage and explore the full range of bacterial genetic information in the fecal microbiome, thereby using a systematic approach to study the impact of dietary fiber supplementation on fecal metabolites, bacterial taxa, and bacterial metagenomes.

Design: Fecal samples were subjected to whole-genome shotgun 454 pyrosequencing to identify both fecal bacterial populations present and their functional genetic capacity.

Results: Whole-genome shotgun sequencing results revealed that fiber consumption shifted the Bacteroidetes:Firmicutes ratio, increasing the relative abundance of Bacteroidetes 12 ± 2% and 13 ± 2% with polydextrose and SCF, respectively, compared with NFC. Bivariate correlations showed a positive correlation between the Bacteroidetes: Firmicutes ratio and total dietary fiber intake but not body mass index. Principal coordinates analysis of Bray-Curtis distances indicated that bacterial gene composition was more similar in participants consuming fibers (polydextrose and SCF combined) in comparison with NFC. Shifts in bacterial gene abundances after polydextrose and SCF supplementation included genes associated with carbohydrate, amino acid, and lipid metabolism, as well as metabolism of cofactors and vitamins.

Conclusion: This study conveys novel information about the impact of dietary fiber supplementation on the phylogenetic structure and functional capacity of the fecal microbiome of healthy adults. This trial was registered at clinicaltrials.gov as NCT02091349. *Am J Clin Nutr* 2015;101:55–64.

Keywords fiber, gastrointestinal microbiota, metagenome, microbiome, prebiotic

INTRODUCTION

The human gastrointestinal microbiome is estimated to contain up to 1000 different microbial strains and 100-fold more genes than the human genome. Recently, microbiome perturbations have been associated with some of the most pressing public health issues, including obesity, cardiovascular disease, and type 2 diabetes (1–3). Similarly, low dietary fiber intake is positively associated with increased risk of obesity, cardiovascular disease, and type 2 diabetes (4–7). Nearly 90% of Americans fail to meet the recommended dietary reference intake amount for fiber (38 g/d for men and 25 g/d for women), with average intakes ranging from 12 to 18 g/d (8, 9).

Microbiome diversity and composition are influenced by a number of factors. Although several studies have investigated the influences of disease, age, and geography on the gastrointestinal microbiome (10, 11), the impact of diet remains largely underinvestigated (12). Insights into how diet influences the composition and functional capacity of the gastrointestinal microbiome are emerging; however, few well-controlled clinical trials have used a systematic approach to study the impact of dietary fiber supplementation on gastrointestinal bacterial taxa, bacterial metagenomes, and metabolites. Therefore, a critical next step in this research area is to characterize how dietary fibers shift gastrointestinal microbial populations and their functional capacity in healthy adults. Our study is among the first to investigate the impact of dietary supplementation of specific fibers on the gastrointestinal bacterial populations and bacterial metagenomes of healthy adults.

Polydextrose and soluble corn fiber (SCF)[5] are soluble fibers that are poorly digested (<30% digested) by human alimentary

[1] From the Department of Animal Sciences and Division of Nutritional Sciences, University of Illinois, Urbana, IL (HDH, SH, GCF, and KSS); the Department of Biological Sciences and Computer Sciences, Northern Arizona University, Flagstaff, AZ (JGC); and General Mills Inc., Bell Institute of Health and Nutrition, Minneapolis, MN (JMB).

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[3] Supplemental Tables 1–3 and Figures 1–6 are available from the "Supplemental data" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

[4] Address correspondence to KS Swanson, 1207 West Gregory Drive, Urbana, IL 61801. E-mail: ksswanso@illinois.edu.

[5] Abbreviations used: KEGG, Kyoto Encyclopedia of Genes and Genomes; KO, Kyoto Encyclopedia of Genes and Genomes Orthology; NFC, no fiber control; OTU, operational taxonomic unit; PCoA, principal coordinates analysis; rRNA, ribosomal RNA; SCF, soluble corn fiber.

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enzymes and partially fermented by the microbiota (13, 14). Previously, our laboratory used a randomized, double-blind, placebo-controlled, crossover design to assess polydextrose and SCF utilization by gastrointestinal microbial populations. The primary outcomes were measurements of fecal fermentation end products and 16S ribosomal RNA (rRNA) gene amplification–based bacterial population analysis. That study demonstrated that polydextrose and SCF shift gastrointestinal bacterial populations at several taxonomic levels in comparison with no-fiber controls (NFCs) and that fecal putrefaction compounds, including ammonia, phenols, and indoles, were decreased with fiber supplementation (15). However, in that trial, only the V4–V6 region of the 16S rRNA gene was amplified, so the functional genetic capacity of the fecal bacterial populations was not quantified, and, thus, it was not possible to determine how microbial genes associated with these shifts in bacterial populations and fecal metabolites were affected. In this follow-up report, we subjected the same fecal samples from that clinical study to whole-genome shotgun pyrosequencing, thereby obtaining comprehensive genome coverage and allowing for exploration of the full range of bacterial genetic information, not just taxonomic identification. The objectives of the current study were to determine how polydextrose and SCF affected the bacterial community structure and functional capacity of the gastrointestinal microbiome compared with NFCs by using whole-genome shotgun sequencing technology. We hypothesized that consumption of polydextrose and SCF would beneficially shift the gastrointestinal microbiome in comparison with NFCs.

SUBJECTS AND METHODS

Experimental design, treatments, and subjects

This report is a follow-up to a previously conducted trial. Details of the design and results of the primary study, which assessed fiber utilization by gastrointestinal microbial populations by measuring fecal fermentation end products and identifying bacteria present via amplicon-based measures, were previously reported (15). Briefly, a randomized, double-blind, placebo-controlled, 3-period crossover trial was undertaken with 3 treatments: *1*) NFC, *2*) polydextrose (Litesse II; Danisco), and *3*) SCF (PROMITOR; Tate and Lyle Ingredients). Participants completed all 3 treatment arms in a randomized order (based on computer-generated codes) with no washout periods between treatments. Treatments were provided as rice crisp bars individually wrapped in coded packages. Bars were identical in appearance and manufactured by General Mills Inc. Investigators, staff, and study participants were blinded to treatment codes. Polydextrose and SCF bars were formulated to contain 7 g supplemental fiber each (15). Participants were instructed to consume 3 bars/d for a total of 21 g/d supplemental fiber (polydextrose and SCF) or 0 g/d supplemental fiber (NFC). Participants maintained dietary records and completed daily gastrointestinal tolerance and bowel function questionnaires throughout the trial. Individual weekly meetings were conducted with study participants to assess treatment bar consumption, collect dietary and tolerance records, and inquire about any other adverse effects. Dietary records were assessed by using the ESHA Food Processor SQL computer software program version 10.7.0 (ESHA Research). Experimental periods were 21 d in length. The first 16 d were considered an adaptation phase. During the last 5 d of each period, 3 fresh fecal samples were collected (within 15 min of defecation), homogenized, and processed for further analysis.

Healthy adult men (n = 25) were recruited via an e-mail sent to the University of Illinois' College of Agricultural, Consumer, and Environmental Sciences students, staff, and faculty. Volunteers were screened to ensure general health. Inclusion criteria were as follows: *1*) aged 20–40 y, *2*) be free of metabolic and gastrointestinal diseases with no history of such diseases, *3*) refrain from consumption of medications known to affect gastrointestinal function, *4*) refrain from consuming probiotic and/or prebiotic supplements, *5*) avoid changes to chronic medications and vitamin and mineral supplements, *6*) maintain current level of physical activity, *7*) consume a moderate fiber diet, and *8*) agree to complete study questionnaires, maintain a dietary and stool record throughout the study, and donate fecal specimens as required. All participants voluntarily signed an informed consent as approved by the University of Illinois Institutional Review Board before study initiation. Twenty-one of the 25 enrolled participants completed the 9-wk intervention study (3 treatment periods of 3 wk each). Two discontinued because they moved out of the area, one started a medication restricted by the study, and one was removed because of aberrant fecal patterns (>3 watery stools/d). Baseline characteristics of participants are listed in **Table 1**. This study was registered with clinicaltrials.gov as NCT02081349.

Fecal DNA extraction, pyrosequencing, and bioinformatics

Fecal samples collected during the clinical trial detailed above were divided into aliquots, snap-frozen in liquid nitrogen, and stored at −80°C. To generate the novel data in this report, DNA from those fecal specimens previously collected was extracted with the QIAamp DNA stool mini kit (Qiagen) by using the bead beating plus column method (16, 17). The extracted fecal DNA was subjected to whole-genome shotgun 454 pyrosequencing, conducted at the W. M. Keck Center for Biotechnology at the University of Illinois with the 454 Genome Sequencer FLX + Titanium system to characterize both fecal microbial population and bacterial functional capacity.

Whole-genome sequence data were analyzed with QIIME 1.6.0, an open source software package for comparison and analysis of microbial communities (18). Quality filtering and demultiplexing were performed by using split_libraries.py with default parameters. Reads were subsequently mapped against the QIIME/IMG reference database (v25oct2012) with blat v. 34 by using QIIME's parallel_map_reads_to_reference.py. This resulted in a table of counts of functional genes with associated Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) categories on a per-metagenome basis. Subsequent diversity analyses were

TABLE 1
Physical characteristics of participants (n = 21)

Characteristic	Mean ± SD	Range
Age, y	27.5 ± 4.33	21–38
Body weight, kg	86.2 ± 13.48	60–110
BMI, kg/m^2	27.0 ± 4.02	20–34
Systolic blood pressure, mm Hg	124.2 ± 14.46	99–148
Diastolic blood pressure, mm Hg	74.5 ± 8.28	54–87

performed on collapsed KO category data on a per-metagenome basis. Bray-Curtis distances were computed between all samples at all KO levels at an even sampling depth of 8462 sequences per sample, resulting in distance matrices between all samples for each level in the KO hierarchy. All other analyses were performed by using all sequences but filtering samples that contained fewer than 8462 sequences.

Reprocessing of amplicon data also was performed with QIIME 1.6.0. Read quality was found to drop considerably at 420 bases, so all 454 amplicon reads were truncated after position 420. Sequences were subsequently demultiplexed and quality filtered with split_libraries.py by using default parameters. The resulting sequences were clustered into operational taxonomic units (OTUs) with an open-reference OTU picking protocol, where reads were first clustered against the Greengenes 12_10 reference OTUs (97% similarity threshold against the Greengenes 97% OTUs), and reads that failed to hit the reference database were clustered de novo. This was performed with pick_subsampled_reference_otus_through_otu_table.py. Weighted and unweighted UniFrac distances were computed between all pairs of samples at an even sampling depth of 2893 sequences per sample. All other analyses were performed by using all sequences but filtering samples that contained fewer than 2893 sequences. Although very low-abundance OTUs may be important, they may not be observed due to limited sampling depth (19).

Statistics

Bacterial taxa data generated from whole-genome shotgun sequencing accounting for ≥0.1% of total sequences for each respective taxonomic hierarchical level were analyzed by using the mixed-models procedure of SAS, whereby treatment was a fixed effect, and participant and period were random effects. Post hoc Tukey adjustments were used to control for multiple comparison. Data normality was tested with the UNIVARIATE procedure and the Shapiro-Wilk statistic; log transformations were used as needed. Bivariate correlations (Pearson's r) between bacterial phyla, total dietary fiber (dietary fiber plus 21 g SCF/d, 21 g polydextrose/d, and 0 g NCF/d), and BMI were also assessed. A probability of $P < 0.05$ was accepted as statistically significant, and a probability of $P < 0.10$ was considered a trend.

Shotgun and amplicon sequence compositional comparisons were performed with pairwise 2-tailed, 2-sample Monte Carlo t tests with 1000 iterations. All comparisons were made by comparing the distribution of within- and between-category differences. For example, to determine whether NFC, polydextrose, and SCF were significantly different in their L4 KO composition, we compared the distribution of all within-sample type distances (i.e., distances from NFC samples to other NFC samples, polydextrose samples to other polydextrose samples, and SCF samples to other SCF samples) with the distribution of between-sample type distances (i.e., distances from NFC samples to polydextrose samples, NFC samples to SCF samples, and polydextrose to SCF samples). For each Monte Carlo iteration, the sample type labels were shuffled, and the P value was computed as the fraction of t statistics based on shuffled labels that were more extreme than the actual t statistic. The false discovery rate correction was used to correct for multiple comparisons within the metagenomics data analysis, and all P values presented are corrected for multiple comparisons. Our significance was set at $q = 0.05$. Our false discovery rate–corrected P values are referred to as q values throughout the text.

RESULTS

Fecal bacterial DNA extracted from the 60 fecal specimens was subjected to whole-genome shotgun pyrosequencing on a Roche GS FLX+ platform for metagenomic analysis. A total of 19,866,919 sequences were generated, with a mean ± SD of 228,536 ± 53,698 reads per sample. Whole-genome shotgun pyrosequencing identified 28 bacterial phyla, 279 families, and 715 genera in the adult male fecal samples. Although a number of taxa were identified at each taxonomic level, only a few accounted for the majority—more than 80% of the sequences at each National Center for Biotechnology Information taxonomic hierarchy were made up of 2 phyla, 10 families, and 16 genera, respectively. Conversely, 20 phyla, 191 families, and 443 genera made up less than 1% of total sequences at those taxonomic levels.

Polydextrose and SCF supplementation shifted the relative abundance of the 2 dominant phyla, the Bacteroidetes and the Firmicutes. The abundance of the Firmicutes phylum was lower ($P < 0.001$) when men consumed polydextrose (56%) and SCF (55%) than when they consumed NFC (68%) (**Table 2**). There was a subsequent increase ($P < 0.001$) in the relative abundance of fecal Bacteroidetes for polydextrose and SCF (36% and 38%, respectively) compared with NFC (25%). Bivariate correlations showed a significant positive correlation between the Bacteroidetes:Firmicutes ratio and total dietary fiber intake ($r = 0.45$, $P = 0.003$; **Figure 1A**). This correlation was maintained when adjustments were made for caloric intake; total fiber per kilocalorie was positively correlated with the Bacteroidetes:Firmicutes ratio ($r = 0.39$, $P = 0.0023$; Figure 1B). The Bacteroidetes:Firmicutes ratio was not significantly related to BMI ($r = 0.06$, $P = 0.64$; Figure 1C).

Information on predominant families within the 6 representative bacterial phyla is also presented in Table 2. Among Firmicutes, the Clostridia class constituted ~90% of sequences, being dominated by Ruminococcaceae, Lachnospiraceae, Eubacteriaceae, and Clostridiaceae families. The proportions of Ruminococcaceae, Lachnospiraceae, and Eubacteriaceae decreased ($P < 0.05$) with polydextrose and SCF supplementation. Within Bacteroidetes, the Bacteroidia class constituted ~98% of sequences, being dominated by Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, and Rikenellaceae families. Polydextrose and SCF supplementation increased ($P < 0.001$) the proportion of Porphyromonadaceae. Among Actinobacteria, Bifidobacteriaceae and Coriobacteriaceae families dominated. Fecal Coriobacteriaceae was lower ($P < 0.05$) with fiber supplementation.

A number of shifts also were present at the genus level (**Table 3**). Most notably, fiber supplementation resulted in a 4.9 ± 1% and 5.3 ± 1% decrease ($P < 0.05$) in *Eubacterium* and a compensatory increase ($P < 0.05$) of 5.2 ± 1% and 6.3 ± 1% in the proportion of *Parabacteroides* with polydextrose and SCF, respectively. *Roseburia*, *Ruminococcus*, *Dorea*, and *Lachnospiraceae* also were significantly reduced ($P < 0.05$) after fiber supplementation.

Principal coordinates analysis (PCoA) of weighted UniFrac distances between samples based on their 97% OTU composition and abundances (**Figure 2A**) indicated that gut microbial communities were more similar within individuals consuming fiber

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TABLE 2
Predominant fecal bacterial phyla and families present in adult men[1]

	Treatment, % of sequences			
Phylum and family	NFC	PDX	SCF	Pooled SEM
Firmicutes	68.2[a]	56.3[b]	55.4[b]	2.05
Ruminococcaceae	26.3[a]	22.8[ab]	22.0[b]	1.21
Lachnospiraceae	14.7[a]	10.2[b]	10.3[b]	0.70
Eubacteriaceae	11.3[a]	6.42[b]	6.04[b]	1.27
Clostridiaceae	7.25	7.97	7.57	0.45
Veillonellaceae	1.13	1.43	1.89	0.42
Acidaminococcaceae	1.32	1.50	1.71	0.24
Erysipelotrichaceae	1.23	1.09	1.07	0.13
Clostridiales unclassified	0.95	0.87	0.90	0.06
Bacteroidetes	24.7[b]	36.3[a]	38.0[a]	2.13
Bacteroidaceae	15.9	20.3	18.3	1.95
Porphyromonadaceae	2.10[b]	7.44[a]	8.48[a]	0.77
Prevotellaceae	2.86	4.61	7.10	1.70
Rikenellaceae	3.21	3.19	3.23	0.61
Flavobacteriaceae	0.39	0.48	0.50	0.08
Proteobacteria	2.34	2.33	2.75	0.25
Enterobacteriaceae	0.42	0.25	0.62	0.18
Actinobacteria	2.50	1.93	1.85	0.28
Bifidobacteriaceae	1.09	0.89	0.89	0.22
Coriobacteriaceae	1.16[a]	0.79[b]	0.72[b]	0.11
Verrucomicrobia	0.67	1.29	0.40	0.45
Verrucomicrobiaceae	0.66	1.26	0.38	0.44
Fusobacteria	0.18[x]	0.18[x]	0.16[y]	0.01
Fusobacteriaceae	0.15	0.14	0.12	0.01

[1]Values are least squares means and pooled SEMs determined by using ANOVA with post hoc Tukey adjustments, $n = 21$. [a,b]Means in a row without a common superscript letter differ, $P < 0.05$. [x,y]Means in a row without a common superscript letter tend to differ, $P < 0.08$. NFC, nonfiber control; PDX, polydextrose; SCF, soluble corn fiber.

(i.e., polydextrose and SCF combined) or control (NFC) diet categories ($t = -3.5$; $P < 0.001$, 2-tailed, 2-sample Monte Carlo t test with 1000 iterations) than across categories. Alternatively, when the abundance of bacterial species was not accounted for, as was the case in the unweighted UniFrac PCoA in Figure 2B, distances between samples based on their 97% OTU composition indicated that OTU composition was not more similar within diet categories than across categories ($t = 0.7$; $P = 0.534$, 2-tailed, 2-sample Monte Carlo t test with 1000 iterations).

Shotgun metagenomics was next applied to characterize the impact of fiber supplementation on the metabolic capacity of the human gastrointestinal microbiota. Microbial gene content was determined by mapped sequencing reads to the KEGG database. Through use of this method, bacterial enzymes were mapped onto known metabolic pathways. Of the 53% of sequences that were mapped to the reference database, 20% contained a KO annotation. At the level 2 orthology, the most represented functional categories were in the metabolism and environmental informational processing lineages (**Supplemental Table 1**). At level 2, 14% of the reads were attributed to carbohydrate metabolism, 9% to amino acid metabolism, 4% to metabolism of cofactors and vitamins, and 2% to lipid metabolism. Five level 2 KEGG pathways were significantly affected by dietary treatments ($P \leq 0.007$; $q < 0.05$). In the metabolism category, fiber supplementation had the highest abundance of genes in xenobiotic biodegradation (mean polydextrose: 0.17%; mean SCF: 0.22%; mean NFC: 0.14%; $q = 0.0108$, $P = 0.0004$) and enzyme families (mean polydextrose: 3.5%; mean SCF: 3.7%; mean NFC: 3.3%; $q = 0.014$, $P = 0.0015$). Energy metabolism genes were enriched with polydextrose supplementation (mean polydextrose: 4.9%; mean SCF: 4.6%; mean NFC: 4.7%; $q = 0.038$, $P = 0.0054$). The proportion of genes classified within cellular processing and signaling was present in the highest abundance in the 2 fiber groups (mean polydextrose: 7.7%; mean SCF: 7.8%; mean NFC: 7.3%; $q = 0.0398$, $P = 0.007$). Membrane transport represented the greatest proportion of identified genes within the environmental information-processing lineage. We observed that individuals consuming supplemental fiber had a reduced number of microbial genes in the membrane transport lineage (mean polydextrose: 9.0%; mean SCF: 8.9%; mean NFC: 9.9%; $q = 0.011$, $P = 0.0008$).

At level 3, 27 pathways were affected by dietary treatment ($P < 0.008$; $q < 0.05$) (**Supplemental Table 2**). Primary pathways continued to cluster in the metabolism and environmental information-processing lineages. Level 3 pathways affected by diet also were classified within genetic information-processing and unclassified level 1 orthologies. Membrane transporters within the environmental information-processing lineage accounted for the largest proportion of level 3 genes significantly shifted by dietary treatment (mean polydextrose: 8.4%; mean SCF: 8.2%; mean NFC: 9.0%; $q = 0.029$, $P = 0.0026$). Amino acid metabolism, including peptidases, accounted for the largest significant enrichment in the metabolic pathway genes after fiber supplementation (mean polydextrose: 2.9%; mean SCF: 3.1%; mean NFC: 2.7%; $q = 0.0004$, $P < 0.0001$). Four level 3 amino acid metabolism categories (Supplemental Table 2) were significantly ($P < 0.008$, $q < 0.05$) affected by treatment: glycine, serine and threonine metabolism, and phenylalanine metabolism were increased with fiber supplementation; histidine metabolism genes were depleted after polydextrose and enriched with SCF compared with NFC; and tryptophan metabolism genes were depleted after fiber supplementation (Supplemental Table 2). Within carbohydrate metabolism, butanoate metabolism was depleted 15% and 20% with polydextrose and SCF, respectively, compared with NFC, and the difference in the means was statistically significant (polydextrose: 0.41%; SCF: 0.37%; NFC: 0.47%; $P = 0.004$, $q = 0.032$).

Level 4 KO analysis identified 183 enzyme function groups that were different ($q < 0.05$) when participants consumed fibers (polydextrose and SCF combined) compared with NFC. PCoA of Bray-Curtis distances between samples based on their L4 KO composition (**Supplemental Figure 1**) indicated that level 4 KO composition was more similar in participants consuming fiber supplementation (polydextrose and SCF combined) compared with NFC ($t = -5.5$; $P < 0.001$, 2-tailed, 2-sample Monte Carlo t test with 1000 iterations). When all 3 groups were compared, 87 KOs were different ($P < 0.05$; $q < 0.05$) (**Supplemental Table 3**). PCoA of Bray-Curtis distances between samples based on their level 4 KO composition (**Figure 3**) indicated that KO composition is more similar within diet categories (i.e., NFC or polydextrose or SCF) ($t = -4.3$; $P < 0.001$, 2-tailed, 2-sample Monte Carlo t test with 1000 iterations). Of those, 19 clustered within general metabolic pathways, 10 in microbial metabolism in diverse environments, 6 in biosynthesis of secondary metabolites, 5 in ABC transporters, 4 in 2-component systems, 4 in starch and sucrose metabolism, 4 in purine metabolism, and 3 in sulfur metabolism. Protein metabolism pathways included those

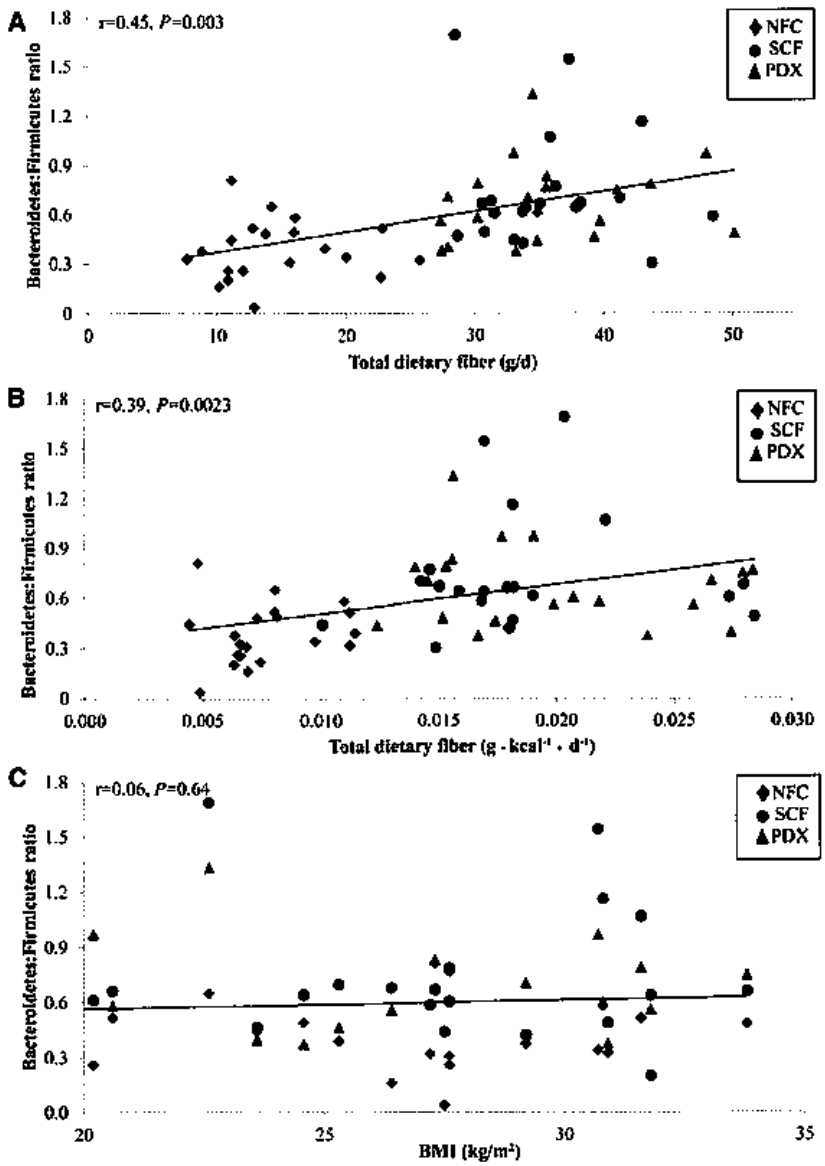

FIGURE 1 Scatterplots depicting relationships between the Bacteroidetes:Firmicutes ratio and total dietary fiber intake (dietary fiber + 21 g PDX/d, 21 g SCF/d, or 0 g NFC/d) (A), total dietary fiber intake per kilocalorie per day (B), and BMI (C). Statistical relationships were determined by using bivariate correlations (Pearson's *r*), and a probability of $P < 0.05$ was accepted as statistically significant; $n = 21$. NFC, no fiber control; PDX, polydextrose; SCF, soluble corn fiber.

for protein digestion and absorption; histidine, tyrosine, valine, leucine, and isoleucine degradation; glycine, serine, and threonine metabolism; cysteine and methionine metabolism; and biosynthesis of amino acids. Vitamin B metabolic pathways also were represented, including pantothenate and coenzyme A biosynthesis, as well as pyrimidine and thiamine metabolism.

Heatmaps were created based on the 25 (**Figure 4**), 50 (**Supplemental Figure 2**), and 100 (**Supplemental Figure 3**) level 4 KOs that were most significantly different across diet categories by using ANOVA. Heatmaps were presented on an individual basis based on the 25 (**Supplemental Figure 4**), 50 (**Supplemental Figure 5**), and 100 (**Supplemental Figure 6**) level 4 KOs that were most significantly different across diet categories by using ANOVA. Metabolism pathway genes accounted for the largest proportion (45%) of the top 100 level 4 KO gene shifts after dietary treatments, with carbohydrate metabolism (11), enzyme families (11), nucleotide metabolism (4), metabolism of cofactors and vitamins categories (4), amino acid metabolism (3), lipid (2), and energy (4) accounting for a total of 39 of all significant differences. Environmental information-processing level 1 categories also had a number of significant shifts (12) after fiber supplementation, including transporters (6) and 2-component systems (4). Twelve genetic information-processing differences were observed with transcriptional (3) and translational (3) genes accounting for most changes in this category. Eleven KOs were categorized as poorly characterized or function unknown.

Assessing the level 4 genes based on the differences in relative abundance of gene counts across diet categories revealed that when all 3 treatment groups were compared, glycine betaine/proline transport system permease protein (K02001), an ABC transporter, demonstrated the greatest enrichment after fiber supplementation. There was an 18-fold increase with polydextrose supplementation

TABLE 3
Predominant fecal bacterial phyla and genera present in adult men[1]

Phylum and genus	Treatment, % of sequences			
	NFC	PDX	SCF	Pooled SEM
Firmicutes				
Faecalibacterium	16.6	14.8	14.6	1.06
Eubacterium	11.2[a]	6.31[b]	5.94[b]	1.28
Clostridium	7.16	7.88	7.50	0.44
Ruminococcus	8.87[a]	6.65[b]	6.06[b]	0.53
Roseburia	3.80[a]	2.33[b]	2.40[b]	0.41
Subdoligranulum	2.42	1.73	1.77	0.29
Lachnospiraceae bacterium	2.09[a]	1.54[b]	1.83[a,b]	0.11
Coprococcus	1.70	1.34	1.15	0.20
Phascolarctobacterium	1.02	1.17	1.36	0.23
Dialister	0.71	0.88	1.32	0.42
Dorea	1.32[a]	0.81[b]	0.82[b]	0.12
Undefined Clostridiales	0.92	0.83	0.88	0.06
Blautia	0.59	0.54	0.58	0.03
Butyrivibrio	0.56	0.39	0.56	0.11
Anaerotruncus	0.47	0.57	0.49	0.06
Marvinbryantia	0.47	0.45	0.45	0.02
Eubacterium	0.36	0.33	0.35	0.08
Undefined Lachnospiraceae	0.40[a]	0.33[a,b]	0.31[b]	0.02
Bacillus	0.25	0.25	0.26	0.02
Lactobacillus	0.20	0.18	0.18	0.01
Bacteroidetes				
Bacteroides	15.9	20.3	18.3	1.95
Parabacteroides	1.66[b]	6.83[a]	7.94[a]	0.77
Prevotella	2.70	4.41	6.91	1.68
Alistipes	3.21	3.19	3.23	0.61
Proteobacteria				
Escherichia	0.30	0.13	0.46	0.16
Actinobacteria				
Bifidobacterium	1.08	0.88	0.88	0.22
Collinsella[2]	0.74	0.43	0.43	0.10
Verrucomicrobia				
Akkermansia	0.65	1.25	0.38	0.44
Fusobacteria				
Fusobacterium	0.11	0.12	0.11	0.00

[1]Values are least squares means and pooled SEMs determined by using ANOVA with post hoc Tukey adjustments, $n = 21$. Means in a row without a common superscript letter differ, $P < 0.001$. NFC, nonfiber control; PDX, polydextrose; SCF, soluble corn fiber.

[2]NFC tended to be greater than PDX and SCF ($P < 0.08$).

and a 13-fold increase with SCF compared with NFC, and the difference in the means was statistically significant (polydextrose: 0.021%, SCF: 0.015%, NFC: 0.001%; $P = 0.001$, $q = 0.021$). In addition, the ABC transporter molybdate transport system ATP-binding protein (K05776) was increased 3-fold with fiber supplementation (polydextrose: 0.026%, SCF: 0.028%, NFC: 0.009%; $P = 0.001$, $q = 0.0235$). Conversely, 2 sulfate transporter protein genes were present in the lowest abundances in the NFC, and the difference in the means was statistically significant for each transporter (K02047 polydextrose: 0.020%, SCF: 0.018%, NFC: 0.039%; $P = 0.001$, $q = 0.042$; K02048 polydextrose: 0.007%, SCF: 0.009%, NFC: 0.021%; $P = 0.001$, $q = 0.017$).

In the metabolism-related pathways, trehalose 6-phosphate synthase (K00697), an enzyme in the starch and sucrose metabolism pathways, was increased by 6- and 10- fold for polydextrose and SCF, respectively (polydextrose: 0.015%, SCF: 0.025%, NFC: 0.002%; $P = 0.001$, $q = 0.027$). Conversely, an endoglucanase (K01179) also classified in starch and sucrose metabolism was reduced 1.6-fold with fiber supplementation (polydextrose: 0.079%, SCF: 0.079%, NFC: 0.130%; $P = 0.001$, $q = 0.407$). N-acylglucosamine 2-epimerase (K01787), an enzyme involved in amino sugar and nucleotide sugar metabolism, also was increased 4-fold with fiber supplementation ($q = 0.0412$). Glyoxylate reductase (K00015), which is involved in carbohydrate metabolism—specifically, glyoxylate and dicarboxylate metabolism—was increased by 3- and 4-fold for polydextrose and SCF, respectively ($q = 0.0307$). There also was a 1.8- and 2.5-fold increase in K00239, a tricarboxylic acid cycle gene involved in succinate dehydrogenase regulation, with SCF and polydextrose, respectively (polydextrose: 0.037%, SCF: 0.051%, NFC: 0.021%; $P = 0.001$, $q = 0.446$). In addition, in the metabolism pathway, a number of peptidases were enriched ($q < 0.05$) 2- to 9-fold with fiber supplementation (K01278, K03592, K03568, K01284, K01372, and K07263).

Conversely, several genes involved in carbohydrate metabolism demonstrated significant depletion with fiber supplementation with statistically significant differences in the means. We observed a 1.7-fold reduction of a protein involved in trehalose biosynthesis (K02438: polydextrose: 0.035%, SCF: 0.033%, NFC: 0.058%; $P = 0.001$, $q = 0.0403$) and a 3.3-fold reduction in a sugar fermentation protein (K06206: polydextrose: 0.010%, SCF: 0.016%, NFC: 0.033%; $P = 0.001$, $q = 0.0401$) with fiber supplementation. polydextrose and SCF supplementation also resulted in a 2.3- and 2.0-fold reduction in a fructose-specific transporter gene (K02770), respectively (polydextrose: 0.044%, SCF: 0.050%, NFC: 0.099%; $P = 0.001$, $q = 0.0118$). In the amino acid related enzymes, a transfer RNA synthase (K01876) was depleted by 19% and 38% with polydextrose and SCF, respectively (polydextrose: 0.142%, SCF: 0.109%, NFC: 0.175%; $P = 0.001$, $q = 0.0123$).

When polydextrose and SCF treatments were combined and compared with NFC, similar changes were noted in level 4 KOs. However, some gene enrichments after fiber supplementation were detected only when the 2 fiber groups were combined: *1*) a transcarbamylase (K13043) involved in arginine and proline metabolism increased 13-fold ($q = 0.0457$); *2*) fumarate hydratase (K01679), a tricarboxylic acid cycle enzyme, increased 3.7-fold ($q = 0.0262$); *3*) an ABC transporter, glycine betaine/proline transport system permease protein (K02002), increased 3.7-fold ($q = 0.0352$); and *4*) a membrane dipeptidase (K01273) was enriched 3.6-fold ($q = 0.0431$).

DISCUSSION

Whole-genome shotgun sequencing data demonstrated that dietary fiber supplementation induced changes in the gastrointestinal microbiome of healthy adults. A shift in the Bacteroidetes:Firmicutes ratio was observed when participants consumed polydextrose and SCF, and changes in bacterial populations were associated with shifts in the bacterial metagenome. Notably, we detected separations of treatment groups when visualized with PCoA plots when individuals consuming 21 g polydextrose or SCF per day were compared with those consuming NFC with both bacterial population-based analyses and bacterial metagenome-based analyses.

Phylogenic composition was compared by using UniFrac, a β-diversity measure (20). The PCoA of weighted UniFrac distances, which accounts for the differences in abundance of taxa, between samples based on their 97% OTU composition indicated

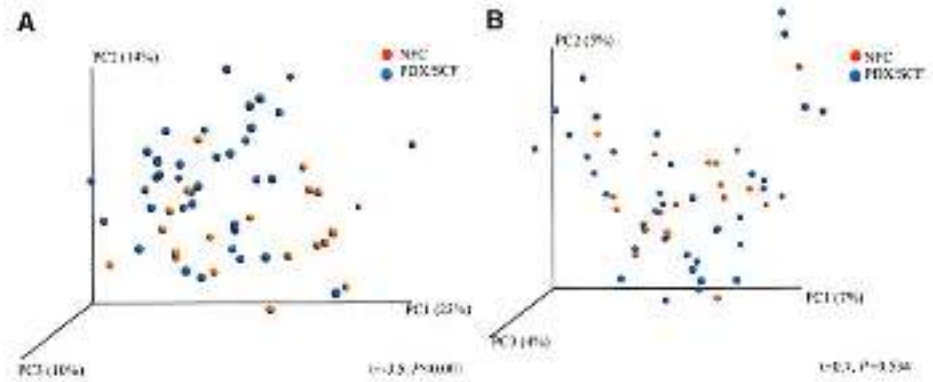

FIGURE 2 PCoA of weighted UniFrac distances between samples (n = 21/group) based on their 97% OTU composition and relative abundances (A). Points that are closer in space are more similar in their 97% OTU composition and relative abundances. This plot indicates that 97% OTU composition (i.e., phylogenetic) and relative abundances are more similar within diet categories (i.e., NFC or PDX/SCF combined) (t = −3.5; P < 0.01, 2-tailed, 2-sample Monte Carlo t test with 1000 iterations). B: PCoA of unweighted UniFrac distances between samples (n = 21/group) based on their 97% OTU composition. Points that are closer in space are more similar in their 97% OTU composition. This plot indicates that 97% OTU composition is not more similar within diet categories (i.e., NFC or PDX/SCF combined) (t = 0.7; P = 0.534, 2-tailed, 2-sample Monte Carlo t test with 1000 iterations). NFC, no fiber control; OTU, operational taxonomic unit; PC, principal coordinate; PCoA, principal coordinates analysis; PDX, polydextrose; SCF, soluble corn fiber.

that polydextrose and SCF samples were more similar to each other than to NFC, indicating that the added fiber diets have a similar effect on the gut microbiome. With unweighted UniFrac analysis, which accounts for the presence or absence of taxa but not their abundances, no group separation occurred. Taken together, these findings suggest that polydextrose and SCF primarily altered the community structure (i.e., the abundance of community members who are already there) rather than changing the community composition (i.e., introducing new or removing existing community members).

In the present report, whole-genome shotgun pyrosequencing was used so that bacterial taxa and genetic capacity could be assessed. A challenge to testing the impact of fiber on the human gastrointestinal microbiome is known primer bias and underestimation of certain taxa by using 16S rRNA gene-based approaches with certain primers (21). In our previous report, our laboratory used 16S rRNA gene amplification to characterize the fecal bacterial populations of the same fecal specimens that were subjected to whole-genome shotgun sequencing in this report; in that report, we acknowledged the primer bias against Bacteroidetes (16). The current report, using whole-genome shotgun sequencing, revealed a less biased characterization of bacterial populations in the Bacteroidetes phylum because 16S primers were not required. Specifically, the 16S gene-based approach utilized in our previous report revealed Bacteroidetes sequence abundances accounting for <1% of total bacterial sequences; in the present report, novel whole-genome shotgun data revealed that Bacteroidetes accounted for nearly 40% of the total bacterial sequences. Proportions of the top bacterial phyla identified by whole-genome shotgun sequencing in this report were similar to those typically reported in gastrointestinal and fecal samples (22–25). With the exception to the Bacteroidetes phylum, taxa identified in the present report by whole-genome shotgun sequencing and shifts after treatments were similar to our previous findings, which used 16S rRNA gene-based amplification methods (16). We acknowledge, however, that other sources of bias related to databases, analytic methods, and so on also exist in studies using whole-genome sequencing technologies.

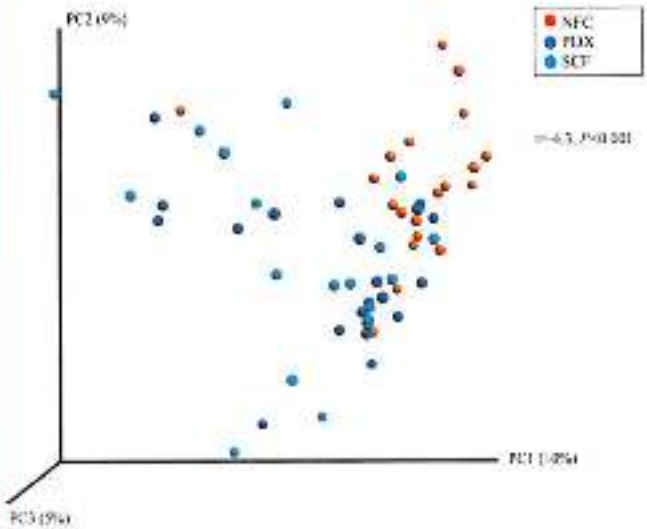

FIGURE 3 PCoA of Bray-Curtis distances between samples (n = 21/group) based on their level 4 KO composition comparing PDX fiber addition, SCF fiber addition, and no fiber addition. Points that are closer in space are more similar in their level 4 KO (i.e., functional gene) composition. This plot indicates that KO composition is more similar within diet categories (i.e., NFC or PDX or SCF) (t = −6.3; P < 0.001, 2-tailed, 2-sample Monte Carlo t test with 1000 iterations). KO, Kyoto Encyclopedia of Genes and Genomes Orthology; NFC, no fiber control; PC, principal coordinate; PCoA, principal coordinates analysis; PDX, polydextrose; SCF, soluble corn fiber.

To our knowledge, this is the first RCT to report a shift in the Bacteroidetes:Firmicutes ratio in healthy adults consuming fiber supplements. Several studies have noted shifts in the Bacteroidetes:Firmicutes ratios in lean compared with obese individuals; however, few have investigated the impact of fiber supplementation during weight maintenance diets in healthy populations. Lean mice have increased Bacteroidetes:Firmicutes ratios compared with their obese counterparts, and transplantation of an obese microbiome into a germ-free animal confers an obese phenotype to the recipient animal (26, 27). In humans, an increase in Bacteroidetes was demonstrated after weight loss (28), and lean twins had increased proportions of Bacteroidetes compared with their obese counterparts (22). Herein, we demonstrated an increased Bacteroidetes:Firmicutes ratio with fiber supplementation, independent of caloric restriction. Bivariate correlations

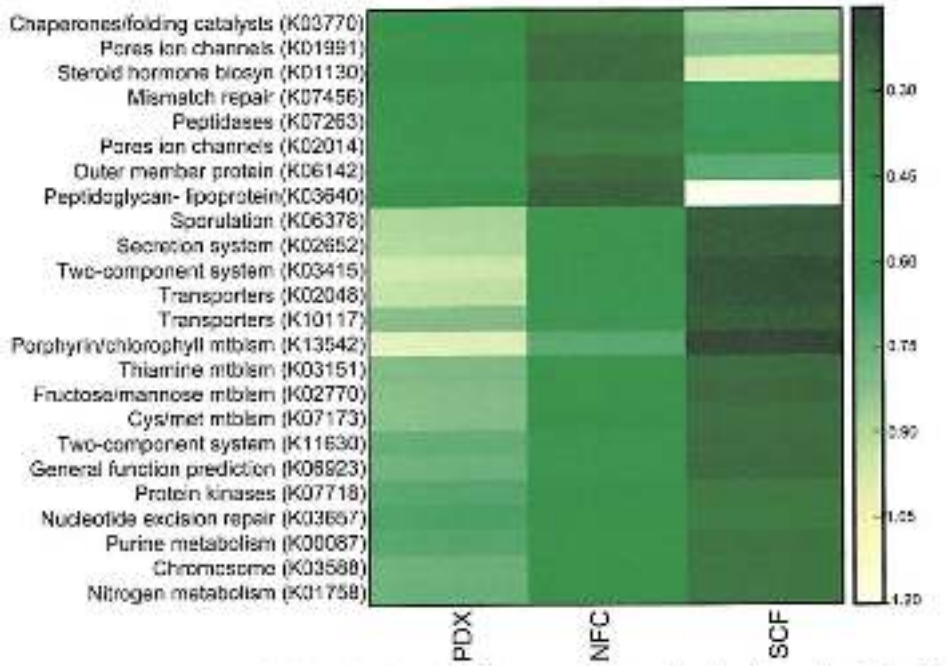

FIGURE 4 The 25 KO categories that were most significant, based on ANOVA, across diet categories when all samples within a diet category are grouped ($n = 21$/group). Colors indicate the relative abundance of each KO on a per-category basis, and clustering of KOs on the vertical axis was performed to group KOs that were most similar in relative abundance. biosyn, biosynthesis; Cys/met, cysteine and methionine; KO, Kyoto Encyclopedia of Genes and Genomes Orthology; mtblsm, metabolism; NFC, no fiber control; PDX, polydextrose; SCF, soluble corn fiber.

revealed that the shift in the ratio of these dominant phyla was associated with total fiber intake but not BMI. Changes in Firmicutes and Bacteroidetes may be related to bile acid sequestering by soluble fibers (29), their ability to use soluble carbohydrates (30), or the physical properties of polydextrose and SCF that may have reduced macronutrient absorption in the small intestine, resulting in an increased substrate delivery to the distal intestine (31–33).

Closer examination of the families and genera contributing to the shift in the Bacteroidetes:Firmicutes ratio with polydextrose and SCF consumption revealed that the primary contributor was the 5–6% increase in the Porphyromonadaceae family (*Parabacteroides* genus). Significant reductions in the relative abundances of Firmicutes after fiber supplementation included the genera *Eubacterium*, *Dorea*, and *Roseburia*.

Similar to our findings, supplementing 25 g SCF/d for 7 d enriched fecal *Parabacteroides* in healthy adults (34, 35). *Parabacteroides* possess α- and β-glucosidases with acetic and succinic acids as primary fermentation end products (36). Because *Parabacteroides* prefer to perform saccharolytic, instead of proteolytic, activity, its enrichment may be beneficial (e.g., a prebiotic effect) (36, 37). Fecal fermentation data previously published (15) from the same fecal samples analyzed in this report also suggested benefits, including reduced fecal pH and putrefactive compounds (e.g., ammonia, phenol, indole, and branched-chain fatty acids) after polydextrose and SCF. The depletion of bacterial tryptophan metabolism gene abundances revealed by the novel whole-genome sequencing in this report is in agreement with our previously published fecal fermentation profile findings (15).

Conversely, several taxonomic shifts would argue against benefits of polydextrose and SCF, including reduced *Eubacterium*, *Dorea*, and *Roseburia*, which are known butyrate producers. Reduced fecal butyrate concentrations after polydextrose and SCF reported previously (15) coincide with the novel findings (e.g., reduced *Eubacterium*, *Dorea*, and *Roseburia*), as do the novel metagenomics data in this report. Specifically, by using whole-genome sequencing, we observed depleted bacterial butyrate metabolism gene abundances with polydextrose and SCF.

Under the auspice of the current prebiotic definition, these results present conflicting data on the prebiotic effects of polydextrose and SCF. Clearly, additional research is needed on these fibers, as is further discussion about prebiotic claims that move beyond frequently targeted commensal bacteria (e.g., bifidobacteria, lactobacilli) and use methods that characterize the entire microbiota community. Our results highlight the importance of a systematic approach that characterizes the gastrointestinal microbiome by measuring bacterial taxa, genes, and metabolites.

Metagenomic analysis revealed 119 differences ($q < 0.05$) between control and fiber-containing treatments in the level 2 (5 categories), level 3 (27 categories), and level 4 (87 categories) KEGG categories. A false discovery rate q value threshold of 0.05 was used because of the novelty of this research instead of a highly conservative approach such as the Bonferroni correction (22, 38, 39). When collapsed to level 4 KO composition, PCoA of Bray-Curtis distances between samples indicated KO composition was more similar within diet categories (e.g., NFC clusters separated from polydextrose and SCF). Whole-genome shotgun sequencing revealed that several amino acid metabolism genes, peptidases, and transporters were shifted after fiber supplementation, which may provide an explanation for the significant reductions in fecal putrefactive compounds we previously reported with polydextrose and SCF supplementation (15). Bacterial genes involved in starch and sucrose metabolism were enriched 6- to 10-fold in the polydextrose and SCF groups compared with NFC; however, an endoglucanase involved in starch and sucrose metabolism was depleted 1.6-fold after fiber supplementation.

The shifts in starch and sucrose metabolism genes may be attributed to the fibers' chemical properties. Polydextrose is a highly branched polysaccharide with glucose units linked by α- and β-linked 1,2, 1,3, 1,4, and 1,6 glycosidic linkages (40). SCF is rich in α-1,6-glycosidic bonds (41). These findings suggest the fibers mostly escape digestion and are fermented by the microbiota in the distal gastrointestinal tract.

Study strengths included use of fecal specimens from a double-blind, placebo-controlled, crossover trial previously conducted in our laboratory, providing a systematic report of the impact of dietary fiber supplementation on gastrointestinal bacterial taxa, bacterial metagenomes, and metabolites; the number of study participants ($n = 21$ per group); the collection of detailed dietary records; the assessment of fecal fermentation profiles; and the use of state-of-the-art sequencing and bioinformatics technologies. Potential limitations included the use of fecal samples that are primarily indicative of distal colonic activity and the biases and limitations of the reference databases (e.g., Greengenes and KEGG), including the large number of unknown genes.

Next steps should include research determining whether causality exists between supplementation of polydextrose and SCF, shifts in the Bacteroidetes:Firmicutes ratio, and weight loss, as well as assessing bacterial activity by measuring messenger RNA or protein expression because the current study provides only a catalog of the bacterial genetic potential. Further assessment of bacterial metabolism and metabolites would also enhance our understanding of gastrointestinal microbiome functions.

The authors' responsibilities were as follows—JMB, GCF, and KSS: designed the research; JMB: provided essential research materials; SH: performed the experiments; HDH and JGC: analyzed data, performed the statistical analysis, and wrote the manuscript; and KSS: had primary responsibility for the final content. All authors read and approved the final version of the manuscript. JMB is an employee of General Mills Inc. None of the other authors reported a conflict of interest related to the study.

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See corresponding article on page 55.

Fiber man meets microbial man[1–3]

Fergus Shanahan

What a privilege it was to have attended a lecture by the late Denis Burkitt when audiences were fed large boluses of wit and wisdom. Even skeptics were impressed by his passion and persuasiveness. Tony Epstein found inspiration when he attended a seminar by Burkitt and credited it as a turning point in the discovery of the Epstein-Barr virus (1). The genius of this great Irishman included more than his rigorous epidemiologic insights into the lymphoma now bearing his name; he will probably always be linked with dietary fiber (2). Despite limitations, Burkitt's epidemiology was more penetrating than simple risk factor epidemiology because he attempted a rapprochement of observations with disease mechanisms. However, great minds occasionally get things wrong, or so it seemed with Burkitt, when revisionists reassessed the role of dietary fiber in certain disorders of the digestive tract. For a time, it seemed fair game to criticize Burkitt's health claims for fiber. In one critical commentary (3), the authors concluded that "fiber may appear in decline as a factor in a multitude of diseases, but do not count it out yet." Curiously, a comprehensive understanding of the influence of dietary fiber on host pathophysiology has still not been achieved. Mechanistic explanations of the apparent health benefits of fiber have been simplistic, but perhaps we have been looking in the wrong place or at the wrong read-outs.

The article by Holscher et al. (4) in this issue of the Journal is welcome, in part because it represents a well-executed, appropriately controlled intervention with 2 forms of fiber (polydextrose and soluble corn fiber) in humans and because it looks at the microbiota beyond a description of composition toward functional capacity, showing the complexity of fiber effects, and has implications for the interpretation of previous studies. The study challenges the concept and definition of what constitutes a prebiotic effect. To their credit, the authors do not emphasize the term "prebiotic," the definition of which is outdated and too narrow because it fails to encompass the effects of fiber on microbial composition and function that are not limited to lactobacilli and bifidobacteria (5).

Whole-genome shotgun sequencing showed that dietary fiber shifted the Bacteroidetes:Firmicutes ratio toward that previously associated with healthy lean rather than overweight subjects, and this was independent of caloric restriction. Both forms of added fiber altered microbial community structure rather than introducing or removing existing community members. As expected, enhanced metabolic function, particularly related to carbohydrate metabolism, was evident in the fiber-supplemented groups. Less predictable were the changes in genes involved in lipid and B-vitamin metabolism in addition to amino acid metabolism, including peptidases and transporters, which represented the largest enrichment in metabolic pathway genes after fiber supplementation. This provides a plausible explanation for the reduction in putrefactive compounds associated with fiber intake. Of course, the changes in functional capacity of the microbiome are complex and not all are seemingly beneficial. For example, the data of Holscher et al. (4) show a significant depletion of bacterial butyrate metabolism gene abundances in those individuals supplemented with both forms of fiber. This is again at odds with simplistic, unidimensional concepts of prebiotics.

Research on the human microbiome is growing rapidly, with contributions by investigators across disparate traditional disciplines. This enhances the collaborative potential and impact of sharing data but requires a level of standardization and consistency to ensure reproducibility of data. Hence, recommendations with regard to the adherence to fundamental methodology are timely (6). Accurate interpretation and avoidance of overstatement of data from microbiome studies also require an awareness of the limitations of current analytic technology. For example, Holscher et al. observed strikingly different results in quantifying the Bacteroidetes phylum when comparing their earlier 16S gene–based approach with their current whole-genome shotgun sequencing on the same samples (4). The investigators acknowledge the sources of bias in their own work and in metagenomic studies in general, which include not only methodologic variations but also an inherent potential bias in databases and analytic methods. At a clinical level, comparative and collaborative studies of the microbiome using samples from different populations will have to control for many environmental or lifestyle variables, the most important of which seems to be dietary. For example, the interpretation of a microbiome study in elderly humans in different living conditions would have been seriously confounded, and the dominant effect of food diversity would have been missed, if the details of dietary intake had not been recorded (7).

[1] From the Department of Medicine and Alimentary Pharmabiotic Centre, University College Cork, National University of Ireland, Cork, Ireland.

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[3] Address correspondence to F Shanahan, Alimentary Pharmabiotic Centre, University College Cork, National University of Ireland. E-mail: f.shanahan@ucc.ie.

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The functional impact of dietary fiber on the human microbiome joins a growing list of diet-microbe-host interactions and underscores the intersection of microbe-host metabolic cascades. Of course, important questions remain. How much fiber is optimal? Fiber is no panacea; it adversely affects the symptoms of many patients with irritable bowel syndrome (3). Is there a clue here, and how may this be reconciled with fiber-induced functional changes to the microbiota? Does the functional effect of fiber vary depending on the age of the host? Although the 2 forms of fiber studied by Holscher et al. were similar in effect, variations in the fine structure of dietary fiber molecules may influence the evolution of host-microbe interactions and determine the diversity of the developing gut microbiome (8, 9). Limitations of many diet-microbiome studies include an overreliance on fecal microbiota; fewer data are available on the microbiota at proximal sites. Finally, it may be informative to address the functional impact of fiber at a metabolomics level in conjunction with the whole-genome sequencing and compositional data.

It is time to re-examine Burkitt's claims with the full range of modern technology, moving from descriptive assessments to functional analysis of the microbiota. In addition to discarding simplistic concepts of the mechanism of fiber-related health benefits, it is wise to resist becoming captive to restrictive language and outdated terms like prebiotics. Burkitt would have approved the new science of the human microbiome. Although he maintained that in science, as in life, "the heart takes precedence over the head" (1), he might now claim that the gut rules the heart and the head.

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