Exploring changes in the human gut microbiota and microbial-derived metabolites in response to diets enriched in simple, refined, or unrefined carbohydrate-containing foods: a post hoc analysis of a randomized clinical trial

Tyler Faits,[1] Maura E Walker,[1,2] Jose Rodriguez-Morato,[3,4] Huicui Meng,[4,5] Julie E Gervis,[4] Jean M Galluccio,[4] Alice H Lichtenstein,[4] W Evan Johnson,[1] and Nirupa R Matthan[4]

[1]Division of Computational Biomedicine, Department of Medicine, Boston University School of Medicine, Boston, MA, USA; [2]Section of Preventive Medicine and Epidemiology, Department of Medicine, Boston University School of Medicine, Boston, MA, USA; [3]Pompeu Fabra University, Barcelona, Spain; [4]Cardiovascular Nutrition, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA; and [5]School of Public Health (Shenzhen), Sun Yat-sen University, Guangzhou, Guangdong Province, China

ABSTRACT

Background: Dietary carbohydrate type may influence cardiometabolic risk through alterations in the gut microbiome and microbial-derived metabolites, but evidence is limited.

Objectives: We explored the relative effects of an isocaloric exchange of dietary simple, refined, and unrefined carbohydrate on gut microbiota composition/function, and selected microbial metabolite concentrations.

Methods: Participants [$n = 11$; age: 65 ± 8 y; BMI (in kg/m^2): 29.8 ± 3.2] were provided with each of 3 diets for 4.5 wk with 2-wk washout, according to a randomized, crossover design. Diets [60% of energy (%E) carbohydrate, 15%E protein, and 25%E fat] differed in type of carbohydrate. Fecal microbial composition, metatranscriptomics, and microbial-derived SCFA and secondary bile acid (SBA) concentrations were assessed at the end of each phase and associated with cardiometabolic risk factors (CMRFs).

Results: *Roseburia* abundance was higher (11% compared with 5%) and fecal SBA concentrations were lower (lithocolic acid –50% and deoxycholic acid –64%) after consumption of the unrefined carbohydrate diet relative to the simple carbohydrate diet [false discovery rate (FDR): all $P < 0.05$), whereas *Anaerostipes* abundance was higher (0.35% compared with 0.12%; FDR: $P = 0.04$) after the simple carbohydrate diet relative to the refined carbohydrate diet. Metatranscriptomics indicated upregulation of 2 cellular stress genes (FDR: $P < 0.1$) after the unrefined carbohydrate diet compared with the simple carbohydrate or refined carbohydrate diets. The microbial expression of 3 cellular/oxidative stress and immune response genes was higher (FDR: $P < 0.1$) after the simple carbohydrate diet relative to the refined carbohydrate diet. No significant diet effect was observed in fecal SCFA concentrations. Independent of diet, we observed 16 associations (all FDR: $P < 0.1$) of taxon abundance (15 phylum and 1 genera) with serum inflammatory markers and also with fecal SCFA and SBA concentrations.

Conclusions: Consuming an unrefined carbohydrate–rich diet had a modest effect on the gut microbiome and SBAs, resulting in favorable associations with selected CMRFs. Simple carbohydrate– and refined carbohydrate–rich diets have distinctive effects on the gut microbiome, suggesting differential mechanisms mediate their effects on cardiometabolic health. This trial was registered at clinicaltrials.gov as NCT01610661. *Am J Clin Nutr* 2020;112:1631–1641.

Keywords: carbohydrate quality, microbiome, metatranscriptomics, cardiometabolic risk factors, bile acids, randomized clinical trial

Introduction

Cardiovascular disease (CVD) affects 48% of adults in the United States and is closely associated with the clustering of key risk factors (abdominal obesity, insulin resistance, hyperglycemia, dyslipidemia, and hypertension) (1). Prospective cohort studies have consistently reported favorable associations between diets rich in unrefined carbohydrate (whole grains and fiber) and rates of CVD and total mortality (2, 3). In contrast, the consumption of simple and refined carbohydrate, as part of a Western diet, is generally associated with poor cardiometabolic health (4–7). Clinical trials indicate that the impact of carbohydrate quality on CVD is mediated in part via effects on cardiometabolic risk factors (CMRFs), including inflammatory markers, blood lipid, and lipoprotein profiles (8–10). We have previously documented that a diet enriched in refined compared with simple or unrefined carbohydrate resulted in higher fasting serum LDL and non-HDL cholesterol concentrations, suggesting that refined carbohydrate may have differential effects on CMRFs distinct from simple and unrefined carbohydrate (7).

The human gut microbiome and microbial-derived metabolites have emerged as important contributors to poor metabolic health and CVD development (11). Experimental evidence suggests

that dietary carbohydrate quality and quantity can rapidly alter microbial composition and function (12, 13). Dietary fiber is a main fuel source for distinct microbial taxa (14, 15) and results in the production of SCFAs, which have a broad array of favorable physiological effects on the regulation of host metabolism, gut health, and immune responses (14, 15). Limited data suggest that dietary carbohydrate-induced changes in gut microbial composition affect the colonic bile acid pool and subsequently alter farnesoid X receptor and G protein-coupled bile acid receptor 1 (TGR5) signaling to influence host immune and metabolic signaling (16), as well as glucose homeostasis and inflammation (16, 17). Thus, modulation of gut microbiome composition and subsequent production of microbial-derived metabolites may be a potential mechanism mediating associations between carbohydrate quality and CMRFs.

In a secondary analysis of our prior work (7), we determined the effect of an isocaloric exchange of different types of carbohydrate (simple, refined, and unrefined) on gut microbial composition (16S sequencing) and function (metatranscriptomics) and concentrations of selected fecal microbial-derived metabolites [SCFAs and secondary bile acids (SBAs)]. In addition, we explored associations of microbial taxon with microbial-derived metabolites and serum CMRFs. We hypothesized that carbohydrate type would differentially affect gut microbial composition, function, and concentrations of fecal SCFAs and SBAs. In addition, we hypothesized that microbial composition would be associated with serum CMFRs.

Methods

Study design and participants

The current study is a secondary analysis of a randomized clinical trial designed to examine the effect of carbohydrate type on CMRFs. Results from the primary study have been published, including details of the study design, inclusion/exclusion criteria, recruitment, and power calculations for the primary outcomes (7). The randomization sequence for each participant was generated by the statistician before the start of the study according to a block design, and assignment was based on enrollment date and time. Investigators and laboratory personnel were blinded to the random order. The study was conducted between 2012 and 2015, in accordance with the Declaration of Helsinki guidelines and with approval of the Institutional Review Board of Tufts University and Tufts Medical Center, and registered at clinicaltrials.gov as NCT01610661 on November 7, 2011.

Briefly, 11 participants (7 postmenopausal women and 4 men; 64% Caucasian) met all recruitment criteria and completed all 3 diet phases, following a randomized crossover design (**Supplemental Figure 1**). Blood samples were collected at the end of each diet phase. Three samples were collected following a 12-h fast, and 1 postprandial sample was collected 4 h following the consumption of a meal consistent with the respective diet phase.(7) Weight was maintained constant throughout the study. Average ± SD of baseline characteristics were as follows: age: 65 ± 8 y; systolic blood pressure: 123 ± 10 mm Hg; diastolic blood pressure: 71 ± 9 mm Hg; BMI (in kg/m^2): 29.8 ± 3.2; glucose: 5.6 ± 0.6 mmol/L; and LDL-C: 3.5 ± 0.7 mmol/L (7).

Study diets

Participants consumed each of 3 diets enriched in simple, refined, or unrefined carbohydrate-containing foods for 4.5 wk, with a minimum 2-wk washout period between each diet (Supplementary Figure 1). Each participant visited the Metabolic Research Unit 3 times per week to consume 1 study meal on site and received additional meals for consumption offsite. Diets for each carbohydrate phase were isocaloric, matched for macronutrients (60% energy total carbohydrate, 15% energy protein, and 25% energy total fat), and differed only in carbohydrate type. The simple carbohydrate diet contained a higher proportion of foods containing sucrose and/or high-fructose corn syrup. The refined grain carbohydrate diet included a higher proportion of foods made from refined grains, such as white rice, white bread, and white pasta. The unrefined grain carbohydrate diet contained a higher proportion of foods made from whole grains. Detailed sample study menus have previously been described (7).

Stool collection

Study participants were given stool sample collection kits after the end of the third week of each dietary intervention phase along with instructions for collection and storage during week 4 of the intervention. Stool collection kits contained an ice pack for transporting samples back to the Metabolic Research Unit at the Jean Mayer USDA Human Nutrition Research Center on Aging within 18 h of production. Samples were aliquoted upon receipt, with 1 aliquot immediately processed with a PowerMicrobiome RNA Extraction kit from MoBio in the Phoenix laboratory at Tufts Medical Center. Remaining aliquots were kept at −80°C until the extraction of bacterial DNA using Qiagen's QIAmp

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Data described in the manuscript, code book, and analytic code will be made available pending review and approval of the request by e-mailing the corresponding author.

TF and MEW contributed equally to this work.

Supplemental Figures 1–3 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

Address correspondence to NRM (e-mail: nirupa.matthan@tufts.edu).

Abbreviations used: ASV, amplicon sequence variant; CMRF, cardiometabolic risk factor; CVD, cardiovascular disease; FDR, false discovery rate; hsCRP, high-sensitivity C-reactive protein; RNA-seq, RNA sequencing; rRNA, ribosomal RNA; SBA, secondary bile acid.

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DNA Stool minikit and for SCFA analysis. Another aliquot was freeze-dried for subsequent bile acid analysis.

Sample processing

16S amplification and sequencing.

We used the 515F/806R primers described by Caporaso et al. (18) (forward primer sequence of GTGCCAGCMGCCGCGGTAA and reverse primer sequence of GGACTACHVGGGTATCTAAT) to isolate and amplify the V4 hypervariable region of the bacterial 16S ribosomal subunit gene in DNA extracted from stool samples. V4 16S amplicon molecules were sequenced using an Illumina MiSeq machine at the Tufts University Core Facility. Reads were automatically demultiplexed into individual paired forward and reverse FASTq files for each sample. Sequences with ambiguous, low-quality, mismatched, or unknown barcodes were discarded.

Metatranscriptomics ribosomal RNA-depleted RNA sequencing.

We used MoBio's PowerMicrobome RNA Extraction kit to isolate RNA from aliquots from each stool sample, and we used Illumina's TruSeq Stranded Total RNA kit with Ribo-Zero to deplete ribosomal RNA (rRNA). These rRNA-depleted complementary DNA libraries were barcoded, pooled, and run through an Illumina HiSeq 2500 with 6 samples per lane. To remove lingering rRNA and human RNA contamination, we aligned all reads to hg19 and rRNA reference libraries using Bowtie2 and removed all hits.

Fecal bile acids.

We measured fecal concentrations of bile acids in freeze-dried stool samples as described previously (19). Extraction and purification were completed using a chloroform methanol solution followed by a multistage sample purification process (19). SBAs were quantified by isotopic dilution using LC–quadrupole time-of-flight MS. Data were normalized by the dry weight of fecal samples and are expressed as micrograms per milligram.

Fecal SCFAs.

Fecal SCFAs were analyzed in frozen stool samples using the method described previously (20). Samples underwent a derivatization with 3-nitrophenylhydrazine, and SCFAs were quantified using quadrupole ion trap 5500 LC-MS/MS. The intra-assay CVs were $<12\%$.

Serum cardiometabolic risk factors.

We have previously reported assay details and the results of dietary carbohydrate type on serum concentrations of CMRFs, including lipids and lipoproteins (total cholesterol, LDL, HDL, non-HDL, VLDL, triglycerides, and nonesterified fatty acids), glycemic markers (insulin, glycated hemoglobin, and HOMA-IR), and inflammatory markers [IL-6 and high-sensitivity C-reactive protein (hsCRP)] (7). For the current analysis, the CMRF data were used to explore associations with microbial taxon.

Bioinformatics analyses

16S amplicon sequencing data analysis.

We processed demultiplexed 16S reads using Qiime2 (version 2018.4) (21). We converted the raw demultiplexed FASTq files into Qiime artifact format. We then denoised and grouped our reads into amplicon sequence variants (ASVs) using the DADA2 algorithm (built into Qiime2) (22) and selected parameters to trim 5 bp from the start of each read, but without truncation. Taxonomy was assigned to each ASV using a naive Bayes classifier built from the Greengenes database (release 13.8) (23). ASVs that the classifier failed to identify were given unique labels, marking them as unassigned but retaining them for analysis.

We performed paired differential analyses (for taxa between each pair of diets at the phylum and genus taxonomic levels) using the R package DESeq2 from Bioconductor (24), which applies a Wald test to a negative binomial generalized linear model. For microbiome data, DESeq2 has been shown to return lower false discovery rates (FDRs) compared with other methods (25), and it is particularly well suited for smaller data sets such as ours (26). Read counts were normalized using the "estimateSizeFactors()" function from DESeq2. Models to derive statistical significance included the host participant as a covariate. We performed an FDR correction, using the Benjamini–Hochberg procedure (27), on all P values within each taxonomic level independently. We considered an FDR $P \leq 0.1$ as statistically significant.

Although rarefaction of metagenomic data is not ideal for many types of analyses, it is still a useful step in generating meaningful diversity metrics and is used in Qiime2's standard operating procedures. For α- and β-diversity calculations only, we rarefied our data to a depth of 80,000 reads, a depth chosen based on DADA2's output table in order to ensure all of our samples would be included in our diversity analyses with minimal information loss. We used Qiime2's α-rarefaction tool to estimate the α-diversity of each sample using the Shannon index as our metric, iterating 10 times and taking the average value of all iterations. We used mafft, fasttree, and Qiime2's alignment mask and phylogeny midpoint-root algorithms to construct a phylogenetic tree from the ASVs generated by DADA2, which we then used to calculate the β-diversity between each pair of samples, reported as weighted UniFrac distances. We performed a principal coordinate analysis on the UniFrac distance matrix. For all statistical tests involving microbial diversity (α and β), we considered a P value ≤ 0.05 as statistically significant.

Metatranscriptomic data analysis.

To address limitations in functional annotation of microbial genes, we examined microbial gene families identified de novo by clustering genes closely related in both sequence and function into a single feature. This reduces the effective number of features under analysis (improving statistical power) and more clearly identifies changes in community function instead of community composition. All RNA-sequencing (RNA-seq) reads (after human and rRNA filtering) were pooled into a single FASTq file, which was submitted as an input to the Trinity de

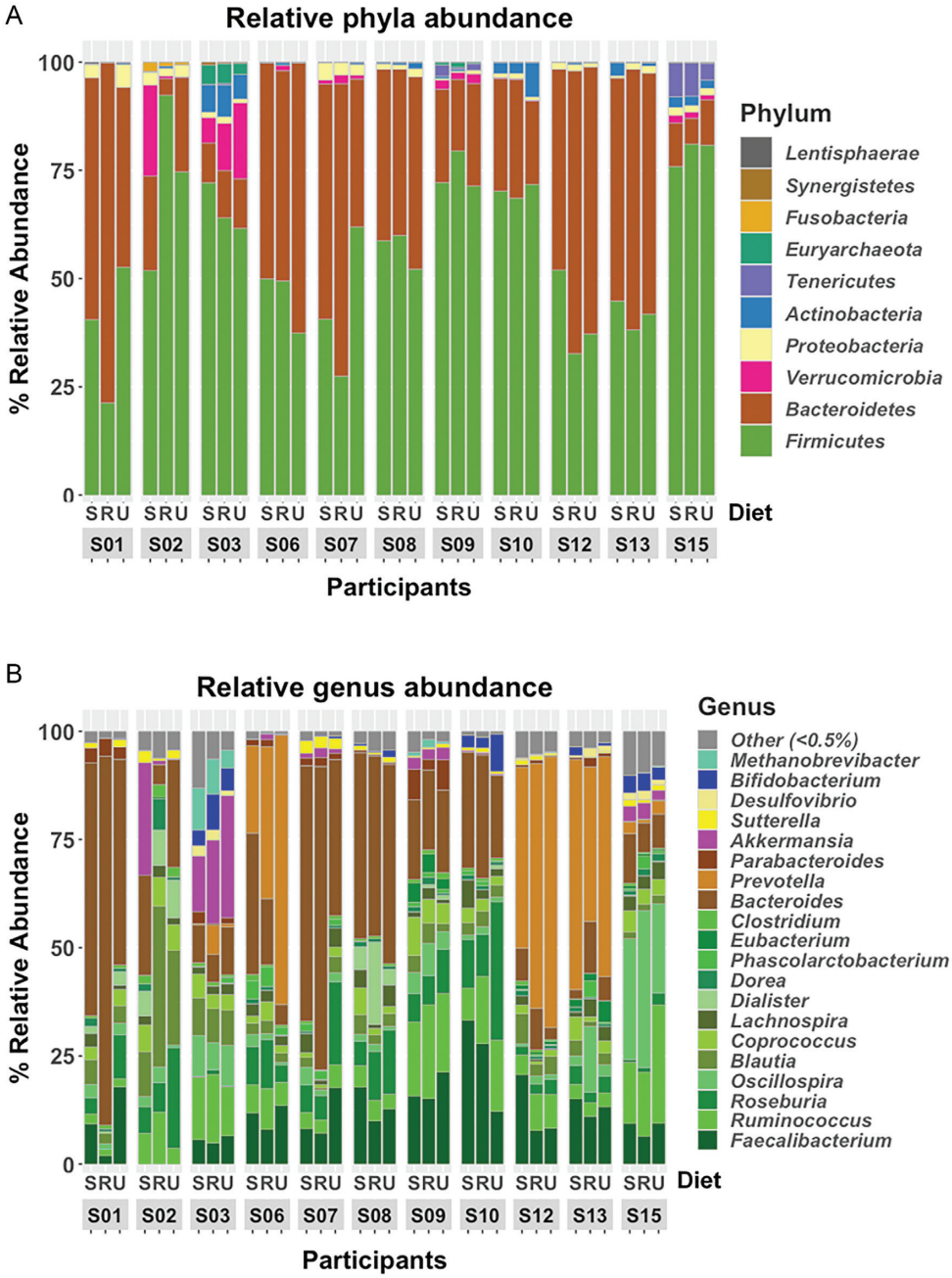

FIGURE 1 Relative abundance of phyla (A) and the 20 most abundant genera (B), based on 16S rDNA sequencing. Data are depicted as estimated percentage relative abundance grouped by study participant ($n = 11$), designated S01–S15, and ordered by carbohydrate type, with simple carbohydrate diet labeled "S," refined carbohydrate diet labeled "R," and unrefined carbohydrate diet labeled "U." Phyla and genera were assigned using Qiime2 and based on the Greengenes database. rDNA, ribosomal DNA

novo transcript assembler (28). Trinity builds contigs from input sequences and then clusters those contigs into putative genes. The filtered RNA-seq reads from each sample were aligned to the Trinity-derived transcripts using Bowtie2 (29), and gene and isoform expression values were calculated using RSEM (30). We used RSEM's expected counts output for each assembled transcript and each sample to derive an expression matrix for differential expression analysis. All differential expression calculations were performed with DESeq2 using the same models as described previously (16S amplicon sequencing data analysis). We performed pairwise differential expression between diets, and we report only data where any 1 of the 3 pairwise comparisons passed an FDR P-threshold ≤ 0.10.

Additional statistical analysis

We used a repeated-measures ANOVA model (PROC MIXED), built in SAS for Windows (version 9.4; SAS

Institute), to test potential differences in fecal SBAs and SCFA concentrations between diet phases. The model used study participants as a random effect, with main effects including diet phase, diet sequence, age, BMI, and sex. We applied the Tukey–Kramer method as a post hoc analysis whenever our ANOVA model resulted in P values ≤ 0.05. All SCFA and SBA concentrations were log-transformed to normalize their distributions prior to analyses. We considered a P value ≤ 0.05 as statistically significant.

In an exploratory analysis, we assessed the associations of relative taxon abundances with concentrations of fecal SBAs, fecal SCFAs, and serum CMRF markers. Due to the compositional nature of microbe abundance data, we used the additive log ratio transform of our relative abundance measurements in our regression models (31). In order to test the significance of each pairwise association, we performed a series of mixed-effects linear regressions between the z scores

$$Z = \frac{x - \bar{x}}{sd} \quad (1)$$

of each measurement. R package lme4 (32), available through CRAN, was used to produce a mixed model for every taxon–marker pair, with the abundance of the taxon as a fixed effect and participant as a random effect. Taxa were only included in this analysis if they had a mean relative abundance of at least 0.1% across all samples. We performed FDR correction on P values derived from these mixed models, and we report the β coefficients for all models with an FDR P value ≤ 0.1.

Results

Impact of carbohydrate type on microbial composition and diversity

We obtained 330,000–545,000 16S rRNA reads from each sample after quality control filtering. Of these, Qiime2 was able to assign between 12,472 and 139,194 reads per sample to ASVs with phylum-level (or better) taxonomy annotations (median = 104,117); 8,511–128,610 of those reads per sample were annotated all the way to the genus level (median = 87,638). We found 11 distinct phyla and 73 distinct genera with a minimum relative abundance of at least 0.01% in at least 1 sample. The 2 most dominant phyla, Bacteroidetes and Firmicutes, represented 93.4% of all estimated abundance (**Figure 1**A). *Bacteroides* and *Prevotella* were the 2 most abundant genera, collectively representing 37.8% of all estimated abundance (Figure 1B).

The abundance of 2 genera was significantly altered by carbohydrate type

Roseburia had a higher abundance after consumption of the unrefined carbohydrate diet (11.3%) compared with the simple carbohydrate diet (5.1%) (FDR: $P = 0.04$; **Figure 2**A), but due to larger variance, it was not significantly different from the refined carbohydrate diet (5.3%). *Anaerostipes* had a higher abundance after the simple (0.35%) and unrefined carbohydrate diet (0.27%) compared with the refined carbohydrate diet (0.12%) (Figure 2B). However, only the difference in abundance between the simple and refined carbohydrate diets was significant (FDR: $P = 0.04$).

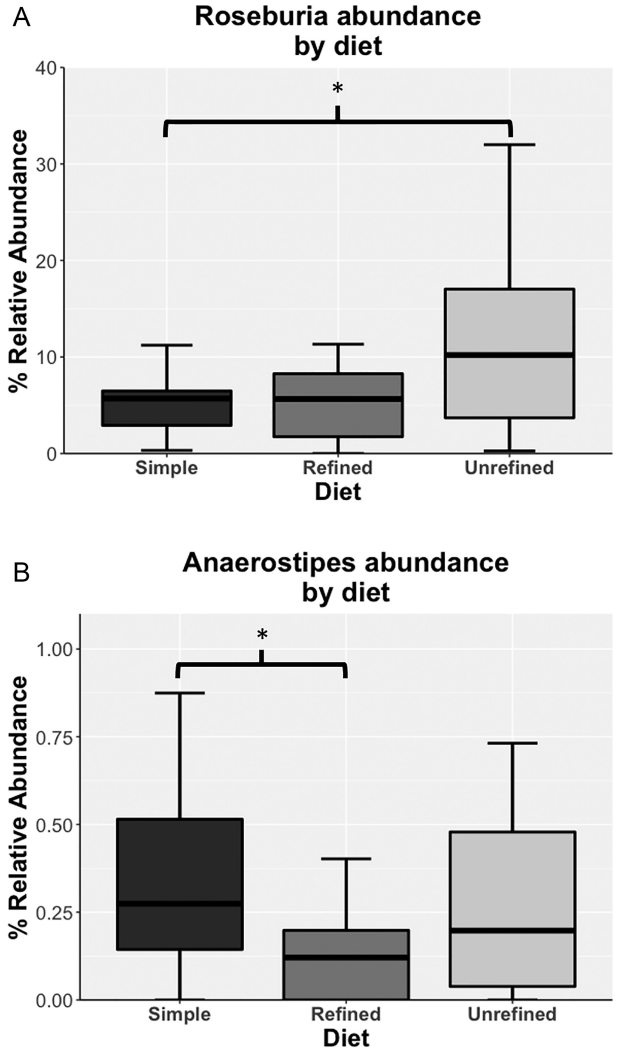

FIGURE 2 Relative abundance of the genera *Roseburia* (A) and *Anaerostipes* (B) in response to dietary carbohydrate type at the end of the study ($n = 11$ for each diet). Data are depicted as box-and-whisker plots that display the IQR (box) and extremities (whiskers) and percentage relative abundance. Differential abundance between dietary carbohydrate type was estimated using a negative binomial model including adjustment for participant. Statistical significance was determined using a false discovery rate P value ≤ 0.1.

Abundance of all microbial taxa examined by carbohydrate type and participants is presented in the supplementary data link.

We calculated the Shannon index of α-diversity H' within each sample (range: 3.33–6.63; **Figure 3**A) and found no significant differences in α-diversity between diets ($P = 0.37$). However, we observed a broad range of stability in α-diversity scores; the value of H' for several participants remained within a 0.5 window for all 3 diets, whereas that for other participants increased or decreased by as much as 1.8 between diets (Figure 3B).

We calculated β-diversity metrics among samples using weighted UniFrac distances, which indicate that most of the participants' microbiomes remained relatively stable across diets and show no evidence of structural ecological shifts in response to carbohydrate type ($P = 0.81$; Figure 3C). Principal coordinate analysis (Figure 3D, **Supplemental Figures 2**

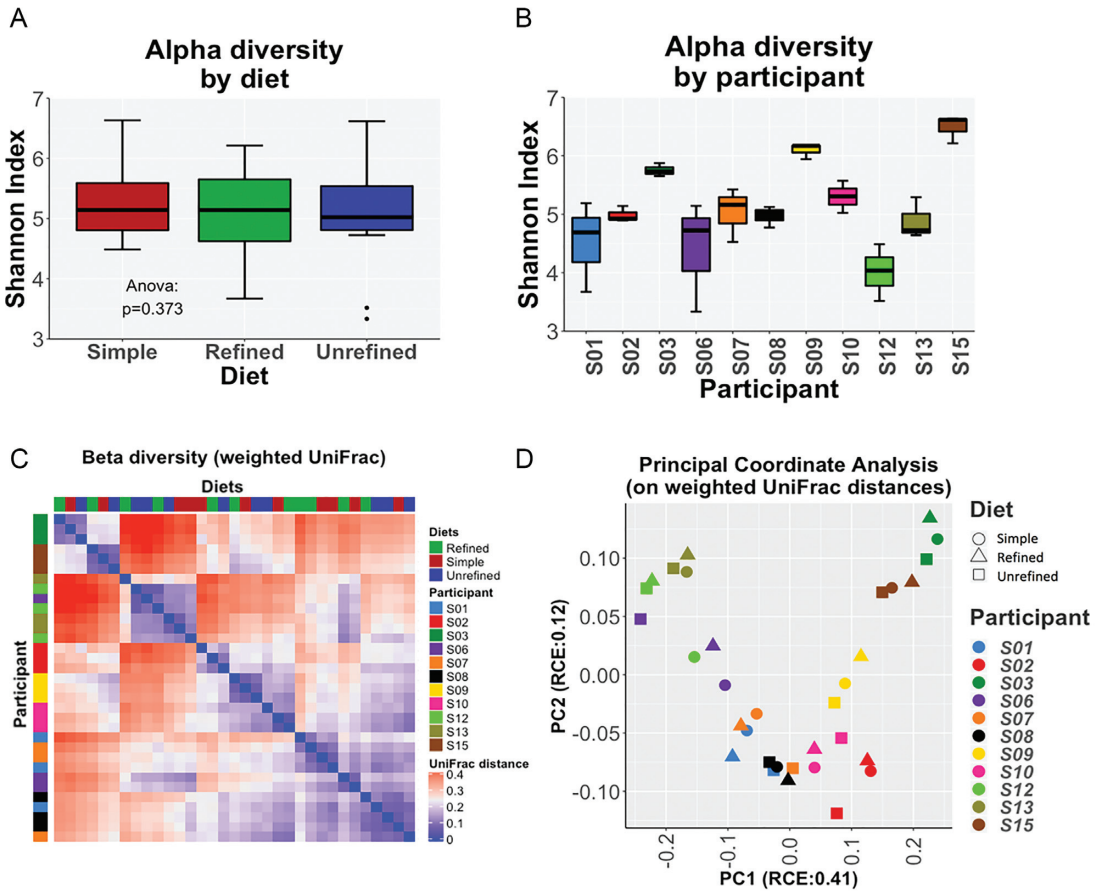

FIGURE 3 α-Diversity, within samples grouped by diet ($n = 11$ for each diet) (A) and each study participant ($n = 3$ for each participant) (B). α-Diversity was calculated as the Shannon index, calculated with a rarefaction depth of 80,000 sequencing reads. Data are depicted as box-and-whisker plots that display the IQR (box) and extremities (whiskers) of α-diversity. (C) β-Diversity between samples, depicted in a heatmap, was calculated as the weighted UniFrac distance between each pair of samples. (D) The top 2 principal coordinate axes (PC1 and PC2) based on a principal coordinate analysis of the weighted UniFrac distance matrix depicting the bacterial community by participant and carbohydrate type ($n = 33$). Each participant is represented by a different color, and the diets are represented as a circle for simple carbohydrate, triangle for refined carbohydrate, and square for unrefined carbohydrate. The RCE of each principal coordinate axis indicates the relative portion of total variance within the data set is captured by that axis. Statistical significance was determined using a P value ≤ 0.05. RCE, relative corrected eigenvalue.

and **3**) indicated that samples largely clustered by participants rather than distinguishing among dietary carbohydrate type.

Impact of carbohydrate type on microbial metatranscriptomics

We obtained between 15,041,570 and 45,692,257 rRNA-depleted RNA-seq reads per sample after quality control filtering. Total reads (769,040,612) across all samples were assembled into 5,124,369 unique bacterial transcripts and subsequently clustered into 504,115 bacterial gene families, which we used as a reference library for sequence alignment. As a minimum threshold for further analysis, we considered only assembled transcript clusters to which at least 100 reads aligned across all samples, yielding 79,467 assembled transcript clusters with such coverage. After filtering out human RNA and rRNA, each sample had between 358,314 and 4,963,992 reads align to the assembled transcript clusters.

We performed differential expression on the 79,467 assembled transcript families between each pair of diets. Following FDR correction, 9 transcript families were found to be significantly differentially expressed by carbohydrate type (all FDR: $P \leq 0.10$: **Figure 4**A–E). BLASTn searches on these 9 transcripts revealed that 4 of them have no known homologs in the National Center for Biotechnology Information's nucleotide database (33). Of the remaining 5 genes, 3 (heat shock protein 60/chaperonin GroEl, Clp protease, and flavin adenine dinucleotide–containing oxidoreductase) had higher expression after consumption of the simple compared with the refined carbohydrate diet (all FDR: $P \leq 0.1$). Notably, these 3 genes also tended to have higher expression with the simple carbohydrate diet compared with the unrefined carbohydrate diet, but this was not statistically significant (Figure 4A–C). Expression of heat shock protein 20/α-crystalin was higher after consumption of the unrefined carbohydrate diet relative to both refined and simple carbohydrate diets. However, only the difference between the unrefined and simple diets was statistically significant (FDR: $P = 0.03$). Last, expression of CsbD-like protein was higher after consumption of the unrefined compared with the refined carbohydrate diet (FDR: $P = 0.08$).

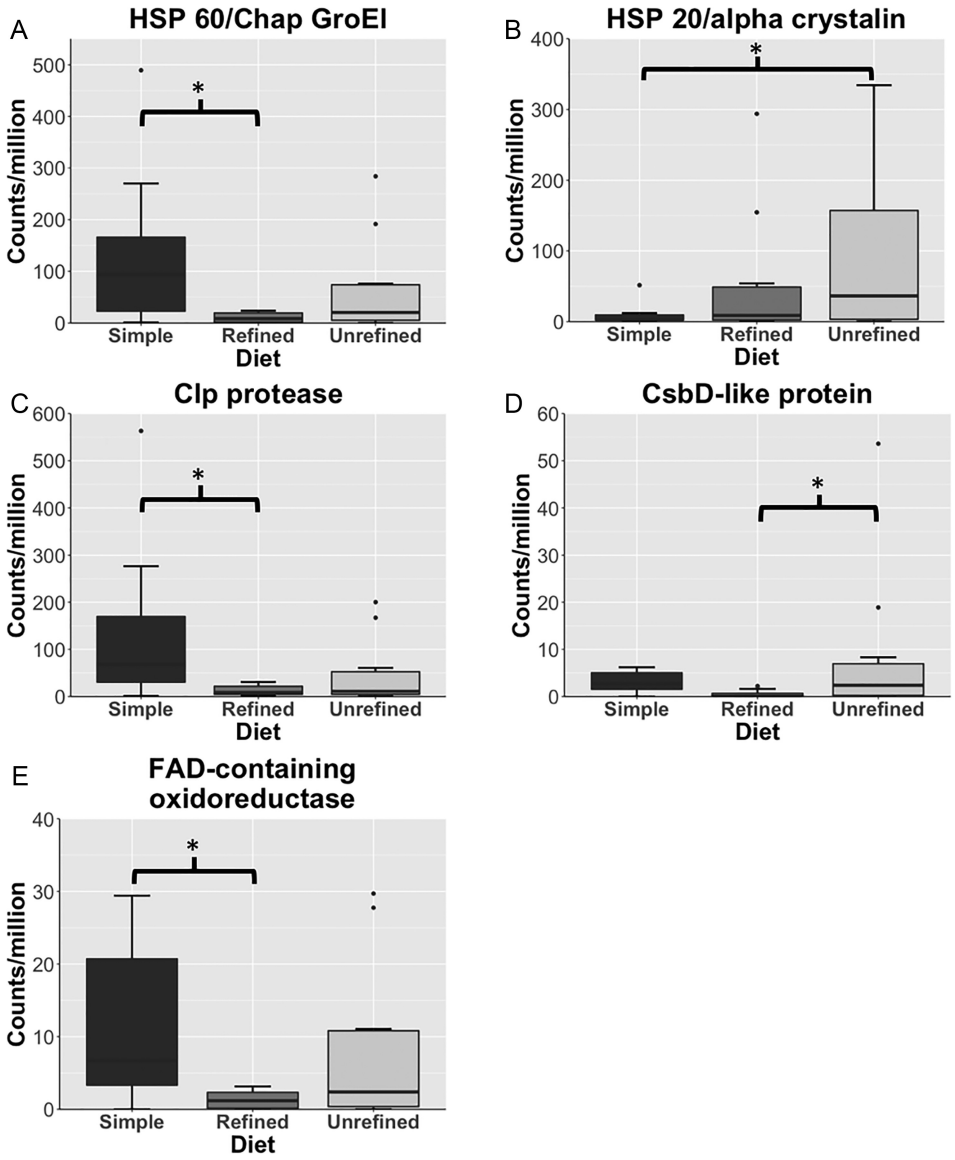

FIGURE 4 Differentially expressed bacterial gene families: (A) heat shock protein 60/chaperon groEl, (B) heat shock protein 20/α-crystalin, (C) Clp protease, (D) CsbD-like protein, and (E) FAD-containing oxidoreductase. Data are depicted as box-and-whisker plots that display the IQR (box) and extremities (whiskers) and bacterial gene family expression (read counts per million). Differential expression between dietary carbohydrate type was estimated using a negative binomial model including adjustment for participant ($n = 11$ for each diet). Statistical significance was determined using a false discovery rate P value ≤ 0.1. Asterisk (∗) indicates a significant difference between diets. Points over the individual box plots indicate participant data above the third quartile.

Impact of carbohydrate type on fecal concentrations of SBAs and SCFAs

Table 1 displays concentrations of CMRFs, as previously reported (7), and fecal concentrations of microbial-derived metabolites. Fecal concentrations of SBAs were significantly lower at the end of the unrefined carbohydrate diet phase compared with the simple carbohydrate diet phase, with intermediate concentrations at the end of the refined carbohydrate diet (both $P \leq 0.05$; Table 1). Fecal concentrations of lithocholic acid were 50% lower after the unrefined compared with the simple carbohydrate diet (2.4 compared with 4.8 μmol/g; $P \leq 0.01$), and deoxycholic acid concentrations were 64% lower (2.0 compared with 5.6 μmol/g; $P = 0.03$). No significant differences were observed in fecal concentrations of SCFAs among the 3 carbohydrate diets (Table 1).

Associations of microbial composition with microbial-derived metabolites and cardiometabolic risk factors

All associations of microbial composition with microbial-derived metabolites and CMRFs are displayed in the supplementary data link. We observed 15 phylum-level and 1 genus-level (*Acidaminococcus*) associations with serum concentrations of CMRFs and fecal concentrations of microbial-derived metabolites that remained significant after FDR correction (all

TABLE 1 Adjusted least-squares means of microbial-derived lipid metabolites and serum cardiometabolic risk factors at the end of each diet phase[1]

Variables	Simple carbohydrate	Refined carbohydrate	Unrefined carbohydrate	*P* value[2]
Fecal microbial metabolites				
Bile acids[3] , μmol/g fecal weight				
Lithocholic acid	4.8 (3.5, 6.1)[a]	3.5 (2.1, 4.8)[a,b]	2.4 (1.1, 3.7)[b]	0.005
Deoxycholic acid	5.6 (3.6, 7.6)[a]	3.8 (1.8, 5.9)[a,b]	2.0 (0.03, 4.1)[b]	0.03
SCFAs, μmol/g fecal weight				
Acetate	12.7 (9.8, 15.6)	10.8 (7.9, 13.7)	10.4 (7.5, 13.3)	0.81
Propionate	4.5 (3.4, 5.6)	3.8 (2.7, 4.9)	3.7 (2.6, 4.8)	0.74
Butyrate	4.1 (2.9, 5.3)	3.5 (2.29, 4.7)	3.2 (2.0, 4.4)	0.95
Isobutyrate	1.1 (0.7, 1.4)	0.9 (0.5, 1.3)	0.7 (0.3, 1.1)	0.43
2-Methylbutyrate	0.8 (0.5, 1.1)	0.7 (0.4, 1.0)	0.5 (0.2, 0.8)	0.53
Valerate	0.9 (0.7, 1.1)	0.8 (0.6, 1.0)	0.8 (0.6, 1.0)	0.89
Isovalerate	0.5 (0.3, 0.7)	0.4 (0.2, 0.6)	0.3 (0.1, 0.5)	0.49
3-Methylvalerate	0.03 (0.02, 0.04)	0.03 (0.01, 0.04)	0.02 (0.01, 0.04)	0.80
Isocaproate	0.04 (0.02, 0.06)	0.03 (0.01, 0.05)	0.03 (0.01, 0.05)	0.26
Caproate	0.3 (0.1, 0.5)	0.3 (0.2, 0.5)	0.3 (0.1, 0.5)	0.25
Serum cardiometabolic risk factors				
Glycemic				
Glucose, mmol/L	5.3 (5.0, 5.6)	5.2 (5.0, 5.5)	5.1 (4.8, 5.4)	0.52
Insulin, mU/L	12.4 (9.3, 16.5)	12.0 (9.0, 16.0)	10.7 (8.0, 14.3)	0.36
Glycated hemoglobin, %	5.7 (4.9, 6.6)	5.8 (5.0, 6.7)	5.7 (4.9, 5.7)	0.65
HOMA-IR	2.9 (2.1, 4.0)	2.8 (2.0, 3.9)	2.4 (1.7, 3.4)	0.36
Blood lipids, mmol/L				
Total cholesterol	5.2 (3.8, 7.2)[b]	5.5 (4.0, 7.6)[a]	5.2 (3.8, 7.1)[b]	<0.01
LDL cholesterol	3.2 (2.2, 4.5)[b]	3.4 (2.4, 4.8)[a]	3.1 (2.2, 4.4)[b]	<0.01
Non-HDL cholesterol	4.0 (2.8, 5.7)[b]	4.3 (3.0, 6.1)[a]	4.0 (2.8, 5.6)[b]	<0.01
HDL cholesterol	1.2 (1.0, 1.5)	1.2 (1.0, 1.5)	1.2 (1.0, 1.5)	0.32
VLDL cholesterol	0.8 (0.5, 1.3)	0.8 (0.5, 1.4)	0.8 (0.5, 1.3)	0.17
Total cholesterol: HDL cholesterol	4.3 (3.6, 5.1)[b]	4.5 (3.8, 5.4)[a]	4.3 (3.6, 5.2)[b]	<0.01
LDL cholesterol: HDL cholesterol	2.6 (2.1, 3.2)[b]	2.8 (2.2, 3.4)[a]	2.6 (2.1, 3.2)[b]	0.01
Triglyceride	1.7 (1.0, 2.9)	1.9 (1.1, 3.1)	1.7 (1.0, 2.9)	0.19
Inflammatory markers				
hcCRP, mg/L	1.9 (0.6, 4.4)	2.0 (0.6, 4.6)	2.1 (0.7, 4.7)	0.84
IL-6, pg/mL	0.6 (0.4, 0.7)	0.6 (0.4, 0.8)	0.6 (0.4, 0.8)	0.77

[1] Values are least-squares means and 95% CIs from the repeated-measures ANOVA model, with the main effect of diet and covariates (phase, sequence, age, BMI, and sex) and random effect of participant. Bile acids and SCFAs were log-transformed to normalize their distributions. When a diet effect was significant at $P \leq 0.05$, multiple comparisons were carried out with the Tukey–Kramer method. Least-squares means with different letters were significantly different from each other. hsCRP, high-sensitivity C-reactive protein.

[2] *P* values for repeated-measures ANOVA.

[3] Bile acid analyses were completed on a sample of $n = 10$.

FDR: $P \leq 0.1$; **Table 2**). The phylum Verrucomicrobia had a negative association with fecal concentrations of deoxycholic acid. There was a positive association between the phylum Lentisphaerae and fecal concentrations of lithocholic acid. The phyla Lentisphaerae and Cyanobacteria both had positive associations with individual SCFA concentrations. A negative association was observed between the phylum Actinobacteria and serum concentrations of the proinflammatory cytokine IL-6, and the genus *Acidaminococcus* was positively associated with serum concentrations of hsCRP. No significant associations were observed among the other CMRFs (blood lipids and glycemic markers) and microbial taxon abundance (see the supplementary data link).

Discussion

The current study provides novel information about the impact of dietary carbohydrate type (simple, refined, and unrefined) on the gut microbiome phylogenetic structure and functional capacity. In this secondary analysis of a clinical trial with 3 isocaloric dietary intervention phases, we document that different types of carbohydrate have modest but distinctive effects on the human gut microbiome (composition and function) and fecal SBA but not SCFA fecal concentrations. These results suggest that the favorable effects of diets enriched in unrefined carbohydrate could be mediated, in part, by the gut microbiome. Interestingly, consistent with the effects on CMRFs (7), the diet enriched in simple carbohydrate had unique effects on human gut microbiota composition and gene expression that were distinct from refined carbohydrate.

A majority of intervention studies on carbohydrate quality and gut microbial composition have compared diets enriched in whole grains to refined grains (34–39). However, simple and refined carbohydrate may have differential effects on cardiometabolic health that are mediated by the gut microbiota. Similar to the current work, some clinical trials comparing

TABLE 2 Associations of microbial taxon abundance with fecal secondary bile acids, fecal SCFAs, and serum inflammatory markers[1]

Variables	Taxon	β coefficient[2]	SE of β	FDR P value[3]
Bile acids, μmol/g fecal weight				
Lithocholic acid	Lentisphaerae	0.28	0.17	0.09
Deoxycholic acid	Verrucomicrobia	−0.09	0.19	0.03
SCFAs, μmol/g fecal weight				
Propionate	Cyanobacteria	0.47	0.15	0.03
Isobutyrate	Cyanobacteria	0.29	0.17	0.03
	Lentisphaerae	0.56	0.15	<0.001
2-Methylbutyrate	Cyanobacteria	0.29	0.17	0.03
	Lentisphaerae	0.60	0.14	<0.001
Valerate	Cyanobacteria	0.41	0.16	0.03
	Lentisphaerae	0.31	0.17	0.03
Isovalerate	Cyanobacteria	0.25	0.18	0.08
	Lentisphaerae	0.63	0.14	<0.001
3-Methylvalerate	Cyanobacteria	0.28	0.10	<0.01
	Lentisphaerae	0.28	0.13	<0.001
Isocaproate	Cyanobacteria	0.58	0.13	<0.01
Inflammatory markers				
hcCRP, mg/L	Acidaminococcus	0.21	0.15	0.03
IL-6, pg/mL	Actinobacteria	−0.20	0.14	0.10

[1]Linear mixed effects models to examine the associations of taxon abundance with concentrations of serum cardiometabolic risk indicators, fecal secondary bile acids, and fecal SCFAs. All variables were assessed as standardized z scores. Taxon abundance was modeled as the fixed effect and participant as a random effect. An FDR P value $\leq$0.1 was considered statistically significant. FDR, false discovery rate, hsCRP, high-sensitivity C-reactive protein.

[2]β estimates represent the difference in the respective risk marker z score per SD unit increase in the abundance of the respective taxon.

[3]FDR P value for linear mixed effect model.

whole to refined grains have found modest changes (≥2 taxa) in gut microbial composition (34, 35), although other studies have found no significant changes (36–39). We observed that the abundance of *Roseburia*, a butyrate producing genera, was higher after consumption of the unrefined carbohydrate diet compared with the simple carbohydrate diet. Results were similar but nonsignificant when comparing the unrefined carbohydrate diet with the refined carbohydrate diet. A similar trend of higher abundance of *Roseburia* in participants fed whole grain compared with a refined grain enriched diet has been reported (35). *Roseburia* is a dietary fiber fermenting genus that increases in abundance in response to dietary fiber (17, 40). We observed that the abundance of the genera *Anaerostipes* was lowest after participants consumed the refined carbohydrate diet compared with the simple carbohydrate and unrefined carbohydrate diets, although the latter did not reach statistical significance. *Anaerostipes* is also a butyrate producer; thus, low abundance after consumption of the refined carbohydrate diet may contribute to the unfavorable effects of diets rich in refined carbohydrate.

Our finding that carbohydrate type had no significant impact on gut microbial α-diversity (within participants) and β-diversity (among participants) is in agreement with most (35, 38, 39) but not all prior work (34). Notably, we observed 2 distinct patterns of Shannon index shift among participants, suggesting individual differences in diet induced shifts in microbial diversity. In addition, we found that the fecal microbial composition at the end of each intervention phase clustered by participant rather than carbohydrate type. These results support prior evidence (41) suggesting that diet-induced intraindividual variation in gut microbial composition may be insufficient to overcome inherent interindividual variation and long-term dietary habits.

In addition to using 16S amplicon sequencing to measure the relative abundances of organisms in microbial communities, we utilized metatranscriptomics to examine microbial genes differentially expressed by dietary carbohydrate type. Metatranscriptomics based on RNA-seq allows us to accurately measure the expression levels of living bacteria, viruses, and fungi and may capture the relative abundance of both living and dead (or quiescent) cells. The 3 annotated genes (heat shock protein 60/chaperonin GroEl, Clp protease, and flavin adenine dinucleotide–containing oxidoreductase) with higher expression after participants consumed the simple compared with the refined carbohydrate diet have been implicated in stress response and oxidative stress. Particularly, bacterial heat shock protein 60 has been implicated in immune response and inflammatory bowel disease (42, 43). The 2 genes with higher expression levels after the unrefined compared with refined (csbD-like protein) or simple (heat shock protein 20/α-crystalin) carbohydrate diets have also been implicated in bacterial stress response. However, the expression of csbD-like protein was relatively low in all groups. Hence, significant differences may be due to the higher variation in expression with the unrefined carbohydrate diet.

Favorable associations between carbohydrate quality and chronic disease could be mediated in part by the production of microbial-derived metabolites. Conjugated primary bile acids (cholic and chenodeoxycholic acids) are synthesized in the liver and secreted into the intestine with the release of bile. In the intestine, bile acids that are not reabsorbed may undergo microbial deconjugation and dehydroxylation to form SBAs, which have been considered inflammatory and carcinogenic (44). Our findings indicate that concentrations of fecal SBAs (lithocholic and deoxycholic acid) were lowest after participants consumed the unrefined carbohydrate diet. Consistent with prior work (45, 46), these data suggest a protective effect that supports

previous observations of reduced risk for colon cancer by whole grain–rich and high-fiber diets (47). Despite our finding that the abundance of butyrate-producing genera (*Roseburia* and *Anaerostipes*) was altered by the dietary carbohydrate type, there was no significant effect of carbohydrate type on fecal SCFA concentrations or associations between abundance of these genera and SCFAs. Similar findings have been reported with some (34, 39) but not all studies that examined diets enriched in either whole or refined grains (35). The phylum Actinobacteria includes the genus *Bifidobacterium*, which has been shown to influence systemic concentrations of IL-6 and TNF-α (48). *Bifidobacterium* has been reported to inhibit lipopolysaccharide-induced activation of NF-κB and subsequent secretion of inflammatory cytokines (49). It has been suggested that *Bifidobacterium* may influence inflammation by accumulating SBAs to reduce colonic concentrations (50), consistent with our observation that abundance of the phylum Actinobacteria was inversely associated with serum concentrations of IL-6. Although the associations among other microbial taxa with CMRFs and microbial-derived metabolites are intriguing, they warrant further investigation because these taxa were present in low abundance.

The current study has several strengths. Foremost, we conducted a well-controlled dietary intervention, in which all meals were provided to the study participants. In addition, we used a crossover study design to reduce interindividual variation, which is of particular importance in studies of gut microbiota. We collected a broad array of data on microbial composition and function and microbial-derived metabolites, targeted due to prior associations with dietary carbohydrate type. Limitations include a modest sample size that was originally determined to examine the effect of carbohydrate type on CMRFs. A larger study, based on the primary outcome of changes in gut microbial composition and metabolome, is needed to confirm our findings. Although metatranscripomics provides novel insight into differentially expressed microbial genes, current limitations in gene annotation may limit the clinical interpretation of results. Last, assessment of the gut microbiome at baseline and postintervention would allow for a better inference of interindividual variation.

In summary, we observed modest differential effects of carbohydrate type on microbial composition and function and fecal concentrations of SBAs. A diet enriched in unrefined carbohydrate had favorable effects, including higher abundance of the butyrate-producing genus *Roseburia* and lowered SBA fecal concentrations. Although exploratory in nature, our results suggest distinct effects of simple and refined carbohydrate on gut microbial composition and function. Last, we noted that the effect of our carbohydrate intervention was insufficient to overcome a high degree of synergy within participants' gut microbial communities. These findings provide novel information on potential mechanisms linking carbohydrate quality to human health and warrant replication in a larger study.

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