Addition of Soluble Fiber in Low-Fat Purified Diets Maintains Cecal and Colonic Morphology, Modulates Bacterial Populations and Predicted Functions, and Improves Glucose Tolerance Compared with Traditional AIN Diets in Male Mice

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ABSTRACT

Background: Purified diets (PDs) contain refined ingredients with one main nutrient, allowing for greater control relative to grain-based diets (GBDs), which contain unrefined grains and animal byproducts. Traditional PDs like the American Institute of Nutrition (AIN)-76A (76A) and AIN-93G (93G) can negatively impact metabolic and gut health when fed long term, in part due to lower total fiber, no soluble fiber, and higher sucrose content.

Objective: Two studies were conducted to determine how PDs with reduced sucrose and increased fiber (soluble and insoluble) influence metabolic and gut health in mice compared with traditional AIN PDs or GBDs.

Methods: In study 1, C57Bl/6N mice (n = 75) consumed a GBD [LabDiet 5002 (5002)], 76A, 93G, or 2 PDs with reduced sucrose and higher fiber for 88 d. Body composition and metabolic parameters were assessed. In study 2, C57Bl/6N mice (n = 54) consumed either 2 GBDs (LabDiet 5001 or 5002) or PDs with different types/levels of fiber for 14 d. Microbiome alterations and predicted functional metagenomic changes were measured.

Results: The PD with 75 g cellulose and 25 g inulin per 4084 kcas marginally influenced body weight and adiposity, but improved glucose tolerance relative to 93G (P = 0.0131) and 76A (P = 0.0014). Cecal and colonic weights were lower in mice fed cellulose-based PDs compared with those fed GBDs and soluble-fiber PDs. Soluble-fiber PDs reduced alpha diversity and showed similar beta diversity, which differed from cellulose-based PDs and GBDs. Certain genera associated with improved gut health such as Bifidobacteria and Akkermansia were significantly elevated by soluble-fiber PDs (P ≤ 0.01). Metabolic pathways related to carbohydrate and fatty acid metabolism were affected by PDs.

Conclusions: PDs formulated with lower sucrose and increased fiber content, particularly soluble fiber, blunted elevations in metabolic parameters and favorably impacted the microbiota and metagenome in C57Bl/6N mice.

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Introduction

Of the many environmental variables that affect the phenotype of an animal, diet is one that can be easily controlled. Laboratory rodent diets are classified into 2 main types: grain-based diets (GBDs) or purified diets (PDs). GBDs (or cereal-based diets or natural ingredient diets) are typically closed formulas and made with grain-based ingredients and animal byproducts (1). While they provide nutrition for growth and overall health, they contain non-nutritive ingredients such as phytochemicals and potential toxins such as endotoxins, mycotoxins, and heavy metals from several ingredients, which may vary from batch to batch and potentially influence phenotype (1–3).

PDs are “open” formulas made with defined concentrations of ingredients that are highly refined, each providing 1 main nutrient (i.e.,
Sucrose is mainly carbohydrate, corn oil is mainly fat, and casein is mainly protein. Being highly refined, the nonnutrient content is minimal and the nutrient compositions of both macro- and micronutrients in PDs are well defined, limiting the variability from batch to batch (1, 4). Since each nutrient/ingredient is added individually, it also allows the researcher to selectively manipulate nutrients, thus providing a wide range of modifications (e.g., high-fat/protein, high-fructose, ketogenic) to study different phenotypes in rodents and in other animal models. The American Institute of Nutrition (AIN)-76A (76A) and AIN-93G (93G) diets (5, 6) are 2 of the most commonly used PDs, which can provide adequate growth and health of rats and mice; however, there have been reports (7, 8) of mild metabolic dysfunction (increased body weight, body fat, mild insulin resistance, hyperlipidemia, etc.) in animals consuming these diets, relative to GBD-fed animals. While several differences exist between these 2 types of diets, these perturbations may be, in part, due to certain ingredients in these diets, including the higher sucrose content (10% and 50% wt:wt in 93G and 76A, respectively) and a low amount of total and mostly nonfermentable fiber (5% cellulose [CEL]) in PDs (9). This is in stark contrast to the presence of minimal amounts of sucrose and relatively higher amounts (15–25% wt:wt) of fiber in GBDs. In addition, GBDs also contain diverse sources of fiber including soluble (beta-glucan, pectin, etc.), partially soluble (hemicellulose), and insoluble fibers (CEL, lignin, etc.) (1, 2, 10).

Metabolic differences that are observed in rodents consuming AIN PDs (or those formulated with only fiber as insoluble fiber CEL) relative to those fed GBDs may be driven, in part, by differences in sucrose and fiber—in particular, soluble fiber (8). Modifications to these PDs include replacement of sucrose with sources such as corn starch and dextrose to minimize fructose, an initiator of metabolic disease, including insulin resistance, glucose intolerance, and hyperlipidemia (11, 12). Even relatively low levels of sucrose may influence glucose tolerance over more chronic feeding periods (13). The fiber content of PDs can also be increased and, furthermore, refined soluble-fiber sources, such as fructo-oligosaccharides (FOS) or inulin (IN), which have been long known to promote metabolic health via the gut (14–16), can be added to these diets. We and others have shown that the addition of soluble fibers such as IN, in the context of a diet higher in fat, can reduce body weight, adiposity, blood and liver lipids, and inflammation, and improve glucose tolerance of rodents, perhaps in part due to elevated short chain fatty acid (SCFA) production by the gut bacteria or other factors (17–20).

The purpose of this study, therefore, was to assess whether changes to carbohydrates and fiber components of the AIN PDs could improve the metabolic health of rodents. Furthermore, we determined how changes in the type and amount of fiber affected gut health/microbiome profile. Metabolic and gut microbiome effects in mice fed PDs were compared with those fed GBDs.

**Methods**

**Dietary formulations**

**Study 1.**

In addition to the traditional AIN PDs, 76A and 93G, we used 2 modified versions of the AIN diets, referred to as the open standard diets (OSDs). The nutritional profiles of the 4 PDs utilized are presented in **Table 1**. The OSDs contained only trace levels of sucrose (in the vitamin and mineral mixes), providing around 1% of total kilocalories. The OSD D11112201 (75CEL:25IN) was formulated with 100 g of added fiber per 4084 kcal in a 3:1 ratio of CEL to IN (75 g CEL and 25 g IN per 4084 kcal; 9.3% fiber wt:wt), with IN providing approximately 1.5 kcal/g from fermentation (21). The OSD D11112202 (225CEL:25IN; 20.5% total fiber wt:wt) contained 3 times as much CEL (225 g per 4084 kcal) as 75CEL:25IN, but the same amount of IN (25 g per 4084 kcal), to be more in line with GBDs that contain higher amounts of fiber as insoluble fiber with some soluble fiber. All PDs were formulated and produced by Research Diets, Inc. These diets were compared with the GBD, LabDiet 5002 (5002). The fiber content of 5002 was analyzed by the laboratory of Dr. Kelly Swanson, Department of Animal Sciences, University of Illinois, using the method of Prosky et al. (22). The total fiber content was 23.9%. Insoluble fiber content was 18.6%, and soluble fiber content was 5.3%.

**Study 2.**

To further understand the role of fiber type and concentration in PDs and how they compare to GBDs, we used 6 additional versions of the OSD with either 100 or 200 g of CEL, IN, or FOS per 4084 kcal. The 6 experimental OSDs (100CEL, 200CEL, 100IN, 200IN, 100FOS, and 200FOS; Research Diets, Inc.) were compared with 2 GBDs: LabDiet 5001 (5001) and 5002 (**Table 2**). Fiber contents of 5001 and 5002 were analyzed by Covance Laboratories (Madison, WI) using AOAC method 991.43 (modified) (23) to determine total, soluble, and insoluble fiber (5001: 18.7% total, 15.9% insoluble, 2.8% soluble; 5002: 18.2% total, 14.9% insoluble and 3.3% soluble).

**Animals and study design**

**Study 1.**

This study was conducted at MuriGenics, Inc. (Vallejo, CA, USA), and was approved by the Institutional Animal Care and Use Committee of MuriGenics in conformation with the Guide for the Care and Use of Laboratory Animals from the National Research Council. Weanling male C57Bl/6N mice (n = 75) were purchased from Charles River Laboratories in conformation with the Guide for the Care and Use of Laboratory Animals from the National Research Council. Weanling male C57Bl/6N mice (n = 75) were purchased from Charles River Laboratories (Hollister, CA, USA). Mice were housed (n = 5/cage on Alpha-Dri bedding, Shepherd Specialty Papers, Inc.) in micro-isolators on a 12-h light/dark cycle and maintained on 5002. Food and water were provided ad libitum. At 4 wk of age, mouse cages were randomly assigned to 1 of the 5 treatment groups (3 cages/treatment; n = 15 mice/treatment). Mice were maintained on the experimental diets for 88 d. Food and water intakes per cage and body weights were measured weekly throughout the study.

**Study 2.**

The study was conducted at Charles River Laboratories (Wilmington, MA, USA) and was approved by the Animal Care and Use Committee of Charles River Laboratories in conformation with the Guide for the Care and Use of Laboratory Animals from the National Research Council. Male weanling C57Bl/6N mice (n = 54) from Charles River Laboratories were housed 3 per cage on Alpha-Dri bedding. Mice were fed 5001 ad libitum and housed in standard vivarium conditions on a 12-h light/dark cycle. After 2 d following receipt, 6 mice were euthanized for baseline data. At 4 wk of age, initial body-weight measurements were collected and mouse cages were randomly assigned to 1 of 8 treatment groups (2 cages/treatment, n = 6/treatment). Mice were maintained on

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**Table 1**

| Formulation | Total Fiber (g per 4084 kcal) | Soluble Fiber (g per 4084 kcal) |
|-------------|-----------------------------|---------------------------------|
| GBD         | 18.6%                       | 14.9%                           |
| 5001        | 23.9%                       | 18.7%                           |
| 5002        | 23.9%                       | 14.9%                           |

**Table 2**

| Formulation | Total Fiber (g per 4084 kcal) | Soluble Fiber (g per 4084 kcal) |
|-------------|-----------------------------|---------------------------------|
| GBD         | 18.6%                       | 14.9%                           |
| 5001        | 23.9%                       | 18.7%                           |
| 5002        | 23.9%                       | 14.9%                           |
TABLE 1 Composition of the PDs in study 1 as formulated

| Ingredient                        | D10001 76A | 76A kcal | 93G | 93G kcal | D1112201 75CEL25IN | 75CEL25IN kcal | D1112202 225CEL25IN | 225CEL25IN kcal |
|-----------------------------------|------------|---------|-----|---------|-------------------|--------------|-------------------|------------------|
| Protein                           | 20         | 21      | 20  | 20      | 19                | 20           | 17                | 20               |
| Carbohydrate                      | 66         | 68      | 64  | 64      | 63                | 65           | 42                | 65               |
| Fat                               | 5          | 12      | 7   | 16      | 7                 | 15           | 6                 | 15               |
| Total                             | 100        | 100     | 100 | 100     | 100               | 100          | 100               | 100              |
| kcal/g                            | 3.90       | 4.00    | 3.81| 3.34    | 3.81              | 3.34         |                   |                  |

The experimental diets for 14 d. Body weights were measured prior to euthanasia.

**Glucose-tolerance test**

**Study 1.**

On day 83, an oral-glucose-tolerance test was performed on all mice, except for 1 in the group 75CEL25IN, which was euthanized prior to testing (n = 74). Following 6-h food deprivation, baseline blood glucose measurements were taken. Immediately afterwards, mice were gavaged with a 20% dextrose solution to deliver 2 g glucose/kg body weight. Blood glucose measurements were collected via the tail vein at 15 min, 30 min, and at 30-min intervals over the course of 2 h. All measures were performed with a TRUEtrack blood glucose monitoring system and test strips (Trividia Health, Inc.). Food was restored upon completion of the procedure.

**Euthanasia and necropsy**

**Study 1.**

Prior to day 88, 2 mice were euthanized due to fighting (1 from 75CEL25IN and 1 from 76A). On day 88 (n = 73), mice were feed deprived for 6 h, blood samples were collected via cardiac puncture, and mice were euthanized. Serum was separated and stored at −80°C until analysis. Following blood collection, mice were perfused with saline. Carcass, liver, and select adipose deposits (mesenteric, gonadal, inguinal, retroperitoneal) were weighed. Livers were flash-frozen in liquid nitrogen and stored at −80°C for triglyceride analysis.

**Study 2.**

At the end of 14 d, mice (n = 54) were euthanized using CO2. Following CO2 asphyxiation, the cecum and colon were harvested together with their contents remaining intact. The total tissue was weighed. For the first mouse in each group, an image of the colon and cecum (attached) was taken next to a standard ruler. The colon and cecum were separated from each other (contents still intact) and each were weighed. Colon and cecum contents were collected into separate vials and placed on dry ice before being stored at −80°C. The colon and cecum were cleaned with double distilled water, blotted dry, and weighed individually. All cecum and colon weight data are rela-
TABLE 2 Composition of PDs in Study 2 as formulated¹

| Ingredient                | D11112222 | D11112223 | D11112224 | D11112225 | D11112226 | D11112227 |
|---------------------------|-----------|-----------|-----------|-----------|-----------|-----------|
| Casein                    | 200       | 800       | 200       | 800       | 200       | 800       |
| L-Cystine                 | 3         | 12        | 3         | 12        | 3         | 12        |
| Corn Starch               | 390.5     | 1562      | 315.5     | 1262      | 315.5     | 1262      |
| Maltodextrin 10           | 110       | 440       | 110       | 440       | 110       | 440       |
| Dextrose                  | 150       | 600       | 150       | 600       | 150       | 600       |
| Cellulose                 | 200       | 0         | 0         | 0         | 100       | 0         |
| Inulin                    | 0         | 200       | 300       | 0         | 0         | 100       |
| Fructooligosaccharide     | 0         | 0         | 200       | 300       | 0         | 100       |
| Soybean Oil               | 70        | 630       | 70        | 630       | 70        | 630       |
| Mineral Mix S10026        | 10        | 0         | 10        | 0         | 10        | 0         |
| Dicalcium Phosphate       | 13        | 0         | 13        | 0         | 13        | 0         |
| Calcium Carbonate         | 5.5       | 0         | 5.5       | 0         | 5.5       | 0         |
| Potassium Citrate, 1 H2O  | 16.5      | 0         | 16.5      | 0         | 16.5      | 0         |
| Vitamin Mix V10001        | 10        | 40        | 10        | 40        | 10        | 40        |
| Choline Bitartrate        | 2         | 0         | 2         | 0         | 2         | 0         |
| Yellow Dye #5, FD&C       | 0.05      | 0         | 0         | 0         | 0.025     | 0         |
| Red Dye #40, FD&C         | 0         | 0.05      | 0         | 0         | 0.025     | 0         |
| Blue Dye #1, FD&C         | 0         | 0         | 0.05      | 0         | 0         | 0.025     |
| Total                     | 1180.55   | 4084      | 1105.55   | 4084      | 1105.55   | 4084      |

¹FD&C: Food, Drug, and Cosmetic; PD, purified diet; 100CEL, open standard diet with 100 g cellulose per 4084 kcal; 200CEL, open standard diet with 200 g cellulose per 4084 kcal; 100IN, open standard diet with 100 g inulin per 4084 kcal; 200IN, open standard diet with 200 g inulin per 4084 kcal; 100FOS, open standard diet with 100 g fructo-oligosaccharides per 4084 kcal; 200FOS, open standard diet with 200 g fructo-oligosaccharides per 4084 kcal.

tive to body weight to account for variations in body weights among animals. Colonic length was recorded once the tissue was cleaned and dry.

Serum biochemistry and liver triglyceride analysis

**Study 1.**

Serum from each mouse (n = 73) was analyzed for fasting blood glucose, leptin, triglycerides, and total cholesterol at MuriGenics (Vallejo, CA, USA). Insulin was measured using an ELISA (Crystal Chem). Serum triglycerides, cholesterol, and leptin were measured on a Heska Element (dry chemistry) analyzer (Heska Corp.). Insulin samples were assayed for triglyceride content by Vascular Strategies LLC (Wynnewood, PA, USA). Lipid was extracted from livers using the method of Bligh and Dyer (24) and triglyceride mass (milligrams) was assayed using the Wako TG-M microplate method and then reported per gram of liver weight.

Microbiome analysis

**Sequencing QC and analysis.**

16S rRNA sequencing was performed on the cecum and colon content samples (n = 54) at the Argonne National Laboratory (Argonne, IL, USA). Briefly, total DNA was extracted from the samples, and the V3–V4 regions of the 16S rRNA were amplified using polymerase chain reaction and sequenced using their MG-RAST (Metagenomic Rapid Annotations using Subsystems Technology) pipeline. Sequence data from this pipeline were transferred to Diversigen (Minneapolis, MN, USA) for subsequent analysis. Raw FASTQs for all samples were run through the QC pipeline at Diversigen. Briefly, this involves trimming of adapter sequences (if present) using the program cutadapt followed by filtering of reads to remove any with a mean Q-score of <30. Next, FASTQ files were run through their standard dada2 pipeline to produce the raw Amplicon Sequence Variant (ASV) count table. The raw table was then filtered to remove any ASVs at <0.0001% sum relative abundance across all samples. Finally, we used the filtered ASV table as input to PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) to infer functional metagenomic content of all samples. The outputs of this were an abundance table of predicted Enzyme Commission (EC) numbers, as well as an abundance table of the predicted Metacyc Pathways derived from the EC abundance data.

**Taxa and functional summary plots.**

The filtered ASV table was used to generate summary stacked bar plots of relative abundances per sample at the phylum and ASV levels for both cecums and colons. Comparisons between phyla and genera by diet and tissue type can be found in the Supplemental Tables 1–4. For visualization purposes, only the top 20 most abundant genera are plotted in the genus-level plot, with the rest allocated to an “other” category. At the phylum level, the ratio of Firmicutes:Bacteroidota was calculated per sample by dividing the relative abundances for each of these. The results of these ratios were then plotted across all diet groups. To assess whether the Firmicutes:Bacteroidota ratio differed across diet groups, we used a 1-factor ANOVA across treatment groups followed by Tukey’s honestly
significant difference (HSD). To examine broad patterns of predicted functional genomic content across diet groups, the predicted EC count table was used as an input. EC numbers in this table were then grouped according to 6 broad functional categories as defined in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database: 1) Carbohydrate Degradation and Absorption, 2) Fructose and Mannose Metabolism, 3) Galactose Metabolism, 4) Starch and Sucrose Metabolism, 5) Fatty Acid Metabolism, and 6) Fatty Acid Biosynthesis. Counts for ECs found within each of these categories were then aggregated per category, and the resulting count data were plotted as box plots. To test whether counts differed across diet groups within these categories, we used a 1-factor ANOVA across all treatment groups followed by Tukey’s HSD.

**Alpha- and beta-diversity analyses.**

Using the ASV, EC, and Pathways tables, we rarefied each table to the sample with the lowest mapped counts. The range of mapped reads per sample was 11,721 to 105,613, with an average of 48,417. We then calculated 3 alpha-diversity metrics for each of the 3 feature tables: the Shannon index, the Chao1 index, and Observed Features. To examine all pairwise comparisons of each treatment group to every other group, we used a 1-factor ANOVA across all treatment groups followed by Tukey’s HSD. All results were then plotted as box plots with strip charts overlaid to show all data points. To assess differences in between-sample (i.e., beta) diversity, we calculated distance matrices for all 3 rarefied feature tables using the Bray-Curtis Dissimilarity metric. Next, we used Permutational Multivariate Analysis of Variance (PERMANOVA) from the R package vegan (The R Foundation for Statistical Computing) to assess differences in beta diversity between treatment groups. The results were then plotted using principal coordinates analysis (PCoA) plots with samples colored by treatment group. We also determined the beta-diversity variability within each treatment group by calculating the distance-to-centroid for every sample within its group. We then used the same statistical methodologies used for alpha diversity to determine if beta-diversity variability is different between any treatment groups. All results of statistical testing are included with individual plots.

**Differential abundance analysis.**

Finally, we assessed whether any taxa—phylum or genus level or functions (Metacyc Pathways-level)—differed in abundance between the treatment groups in the study. Due to the compositional nature of microbiome abundance data, we utilized the R package ALDEx2. Briefly, this package performs differential abundance testing of count data across samples between 2 experimental groups by starting with zero estimation for any features with zero abundance in some samples but not others. Next, per-feature technical variation for each feature was estimated for each sample using Monte-Carlo sampling from a Dirichlet distribution. Each instance of Monte-Carlo sampling was then transformed using the centered log-ratio (CLR) transformation, at which point pairwise statistical testing was performed between experimental groups using the CLR-transformed abundance values. This process was repeated for each instance, and results of statistical testing were aggregated yielding adjusted P values (Benjamini-Hochberg corrected) for each feature in the differential abundance test of interest. We used the above procedure to test for differential abundance between every pairwise comparison of treatment groups. Individual P-value tables of the results of statistical testing for every feature in a given pairwise comparison are presented in Supplemental Tables 1–4. For all statistical tests, an adjusted P value of <0.05 was used as a threshold for significance.

**Statistical analysis**

For study 1 and study 2 (non-microbial data), all data were analyzed by 1-factor ANOVA with GraphPad Prism (GraphPad Software, Inc.). Post hoc analyses were performed if P < 0.05 using Tukey’s HSD for comparisons among groups (both studies). Homogeneity of variance was tested using Bartlett’s and Brown-Forsythe tests and normality was tested using Shapiro-Wilk and Kolmogorov-Smirnov tests. For study 2 microbial data, 1-factor ANOVA followed by Tukey’s HSD was employed using R as presented in the section above. An adjusted P value <0.05 was used as a threshold for significance for all statistical tests.

**Results**

**Study 1: Body composition and metabolic assessment**

Over time, the average body weights of 75CEL25IN and 93G treatment groups were slightly higher (by a mean of 2.3 g) compared with those fed the 225CEL25IN, 76A, and 5002 diets (Figure 1). Terminal body weights were similar for all groups, although 75CEL25IN was statistically higher than 76A (P = 0.0102), but similar to 5002 (P = 0.163) and 93G (P = 0.8223), and additional fiber as CEL in the 225CEL25IN group blunted this effect. All individual fat pad (mesenteric, gonadal, retroperitoneal, and inguinal) weights were generally similar among groups. The 75CEL25IN-fed mice had significantly heavier gonadal fat pads (P = 0.0312) and total fat (P = 0.0479) compared with 5002-fed mice and significantly heavier inguinal (subcutaneous) fat pads (P = 0.0036) compared with 93G-fed mice (Table 3); this difference was blunted by the addition of extra CEL in the 225CEL25IN group. The carcass weights were generally similar across groups, although, in the 75CEL25IN group, the carcass weight was significantly higher than in the 76A group (P = 0.019). Adiposity index (g total fat/100 g carcass) was also similar among groups, although it tended to be higher in 75CEL25IN- than in 5002-fed mice (P = 0.0548), but significantly greater in 75CEL25IN compared with 93G-fed mice (P = 0.0372), due mainly to a lower inguinal fat pad weight in the latter group. The adiposity index and in the 225CEL25IN group blunted the adiposity index and resulted in similar levels relative to all groups (Table 3). The liver weights were also not significantly different among the 5 groups (data not shown).

**Serum biochemistry and liver triglycerides**

Glucose homeostasis was assessed prior to study termination with an oral-glucose-tolerance test (Figure 2). All groups had similar 6-h fasting blood glucose concentrations (Figure 2A). Glucose tolerance was significantly reduced in 93G and 76A mice (as shown by a greater area under the curve, AUC) compared with both 5002 [vs. 93G (P = 0.0399); vs. 76A (P = 0.005)] and 75CEL25IN mice [vs. 93G (P = 0.0131); vs. 76A (P = 0.0014)] (Figure 2B). 225CEL25IN was intermediate and similar to all groups. Additional measurements of 6-h fasting serum glucose and insulin measurements were made at study termination (Table 4). Serum cholesterol was significantly higher in all PD groups compared with 5002 (75CEL25IN, P = 0.0037; 225CEL25IN, P = 0.0005; 76A, P = 0.001) and was highest in the 93G group (P < 0.0001) (Table 4). Serum triglycerides were similar among groups, but were
The body weights of mice in all groups were similar at the end of the 2-wk experimental period. Body weight and weight gain in only the 200FOS group were significantly lower compared with the GBD groups (5001, \( P = 0.0498 \); 5002, \( P = 0.0191 \)) (Table 5). Despite minimal differences in weight gain, rapid changes to lower intestinal morphology were observed after 2 wk on certain diets. Representative pictures of ceca and colons from each group indicate that, regardless of fiber amount, the CEL-supplemented PDs yielded smaller ceca and shorter colons compared with soluble fiber–supplemented PDs or the GBDs (Figure 3). As suggested by the differences in the photos, statistical analysis of the organ weights and lengths showed similar trends and significant differences between groups (Table 5). Significantly shorter colons were observed in the 100CEL (\( P = 0.0045 \)) and 100FOS (\( P = 0.0123 \)) groups compared with the 5002 group, but both PD groups were similar to 5001; however, all other PD groups were statistically similar to both GBD groups (5001 and 5002). All cecum and colon weight data are relative to body weight to account for variations in body weights among animals. Cecum plus colon wall weights for the 200IN and 200FOS groups were similar to one another but significantly elevated compared with all other treatments (200IN and 200FOS vs. most groups, \( P < 0.0001 \); 200FOS vs. 5002, \( P = 0.0008 \); 100CEL had significantly lower cecum plus colon weights than both GBDs [vs. 5001 (\( P = 0.0253 \)); vs. 5002 (\( P < 0.0252 \)] and the 2 IN groups and 200FOS (100CEL vs. 100IN, \( P = 0.0255 \); 100CEL vs. 200IN and 200FOS, \( P < 0.0001 \)), while 200CEL increased weights slightly and was similar to 100IN, 100FOS, and GBD groups. The 100IN group maintained similar cecum plus colon weights as GBDs. Cecum weights of the different groups followed similar trends.
as cecum plus colon weights, with 200IN and 200FOS being similar to one another but higher compared with all other groups (200IN vs. all groups, \( P < 0.0001 \); 200FOS vs. 5001, \( P = 0.0011 \); 200FOS vs. 5002, \( P = 0.0006 \); 200FOS vs. 100 CEL and 200 CEL, \( P < 0.0001 \); 200FOS vs. 100 IN, \( P = 0.0031 \); 200FOS vs. 100 FOS, \( P = 0.0009 \)). Cecum weight in 100 IN tended to be higher than those of 100 CEL (\( P = 0.074 \)), but 200 IN had significantly higher cecum weights than 200 CEL (\( P < 0.0001 \)). The 100 IN group allowed for similar cecum weights as for those fed GBDs. Colon weights alone followed a similar trend and were more varied. The 200 IN group tended to have higher colon weights than those fed 200 CEL, but this was not statistically higher (\( P = 0.0533 \)) and the addition of more IN or FOS also tended to increase colon weights. Notably, the 100 CEL group demonstrated significantly reduced colon weight compared with the GBD groups (5001, \( P = 0.048 \); 5002, \( P = 0.0186 \)). In contrast, 200 CEL maintained colonic weights similar to GBD groups.

**Changes to the prominent microbial taxa after 14 d on the dietary treatments**

**Alpha- and beta-diversity measures.**

To assess the response of the microbiome to the different dietary treatments, 16S rRNA sequencing was performed on the cecum and colon contents of each mouse. When examining differential abundance for both taxa and functions across treatment groups, we found a large number of features at all levels examined that differed between at least 1 (and usually more) pairwise comparison of treatment groups. In the cecum samples, 168 ASVs (50 genera, 25 families, and 7 phyla), 1342 EC numbers, and 282 Metacyc Pathways were differentially expressed. In the colon samples, 165 ASVs (51 genera, 24 families, and 7 phyla), 1322 EC numbers, and 279 Metacyc Pathways were differentially expressed. Globally, although the diets 5001 and 5002 have slightly different composition, there were no noteworthy or significant changes to report between these 2 groups. With respect to alpha diversity, the soluble fiber diets (IN and FOS) were similar and both soluble fibers significantly reduced species richness compared with the GBDs and CEL-based diets in cecums and colons for Chao1, Observed, and Shannon diversity indices (Figure 4). Dietary soluble-fiber treatments significantly influenced alpha-diversity metrics for both sites (\( P < 0.001 \) for all analyses). The GBDs were able to support the greatest number of species in both tissue types regardless of the diversity metric used and, in most cases, both the 100 CEL and 200 CEL groups were also able to maintain a statistically similar number of species as GBDs. Overall, fiber dose did not have a significant impact on alpha diversity. In terms of beta diversity, PCoA plots depicting the Bray-Curtis dissimilarity of ASVs for cecums

**TABLE 4**  Biochemical measures of serum and liver of the mice at the end of study 1 (day 88)\(^1\)

|                  | 75CEL25IN | 225CEL25IN | 93G | 76A | 5002 | \( P \)  |
|------------------|-----------|------------|-----|-----|------|--------|
| Serum insulin, ng/mL | 4.6 (0.6) | 3.4 (0.4)  | 5.2 (0.6) | 3.3 (0.6) | 3.5 (0.5) | 0.0378 |
| Serum glucose, mg/dL  | 267.9 (16.4) | 244.1 (12.9) | 254.7 (8.9) | 251.0 (15.9) | 236.0 (11.3) | 0.5248 |
| Serum triglyceride, mg/dL | 89.4 (6.0)\(^{a,b}\) | 92.2 (2.7)\(^{b}\) | 92.7 (3.2)\(^{a,b}\) | 89.9 (0.05)\(^{b}\) | 102.7 (3.2)\(^{a}\) | <0.0001 |
| Serum cholesterol, mg/dL | 166.7 (10.2)\(^{a}\) | 171.9 (5.3)\(^{a}\) | 223.5 (6.2)\(^{c}\) | 170.6 (9.0)\(^{b}\) | 129.7 (2.6)\(^{b}\) | <0.0001 |
| Serum leptin, ng/mL  | 34.3 (1.2)\(^{a}\) | 23.5 (2.6)\(^{b}\) | 35.6 (2.4)\(^{a}\) | 26.2 (3.0)\(^{a,b}\) | 22.2 (2.6)\(^{b}\) | 0.0002 |
| Liver weight, g       | 1.55 (0.10) | 1.39 (0.08) | 1.36 (0.08) | 1.36 (0.06) | 1.57 (0.04) | 0.1096 |
| Liver triglyceride, mg/g | 10.1 (1.0)\(^{a,b}\) | 8.2 (0.9)\(^{b,c}\) | 10.5 (1.3)\(^{a}\) | 6.5 (1.0)\(^{b,c}\) | 4.5 (0.3)\(^{c}\) | <0.0001 |

\(^1\)Values are means (SEM); \( n = 15 \)/group. Different letters across rows indicate significantly different values, \( P < 0.05 \). 76A, AIN-76A rodent diet; 93G, AIN-93G rodent diet; 5002, LabDiet 5002; 75CEL25IN, open standard diet D11112201; 225CEL25IN, open standard diet D111112202.
and colons (Figure 5) indicated that treatment groups were quite distinct from one another. In fact, 3 significantly different clusters of microbial communities were observed in both cecums (P = 0.001) and colons (P = 0.001). Once again, dietary fiber types appeared to be the primary differentiating factor, with the 3 distinct clusters consisting of soluble-fiber–based diets (both IN and FOS diets clustered together), CEL-based diets (100CEL, 200CEL), or GBDs (5001, 5002). Fiber dose did not appear to influence beta diversity, as the low-dose and high-dose treatments for each fiber type were clustered together in both sites of sampling.

Phylum changes.

When looking more closely at the relative proportions of taxa, shifts in relative abundance of certain microbes were observed at the phyla and genus level (Figure 6) and associated P values for individual microbial taxa differences can be found in Supplemental Tables 1–4. At the phylum level, Firmicutes was the dominant phylum found in the GBD treatment groups (5001, 5002), followed by Bacteroidota for cecum samples (Figure 6A). The opposite trend was observed in the colon samples, where Bacteroidota was the dominant phylum (Figure 6B). Interestingly, all of the soluble fiber diets (100IN, 200IN, 100FOS, 200FOS) significantly reduced the Firmicutes:Bacteroidota ratio in the cecum samples (Supplemental Figure 1A) compared with the GBDs (5001, 5002); however, in the colon, it was not significant (except for the 200IN group vs. 5002) (Supplemental Figure 1B). The addition of CEL to the diets led to minor phylum shifts, such as increased abundance of Deferribacterota in the cecums. This shift was significant for 100CEL (P = 0.0226) and 200CEL (P = 0.0183) groups compared with the 5002 group (Supplemental Table 1). No differences were observed between the CEL diets (100CEL, 200CEL) and the GBDs (5001, 5002) at the phylum level in the colon samples. In contrast, major phylum-level shifts were observed in both tissue types for the mice fed the soluble-fiber diets. The soluble-fiber diets generally reduced the abundance of Firmicutes in both cecum and colon, reaching significance for the 200IN, 100IN, and 200FOS groups compared with both GBDs (P values < 0.039 in each case for both tissue sites) (Supplemental Tables 1 and 2). In the cecums, marked reductions in Firmicutes abundance alongside elevations in Verrucomicrobiota were observed, particularly for the GBD treatment groups. In fact, Verrucomicrobiota abundance was significantly higher for 100FOS compared with 5001 (P = 0.0137), while the 200FOS group demonstrated significantly lower Firmicutes abundance compared with 5001 (P = 0.012) and 5002 (P = 0.0176) (Supplemental Table 1). Specifically in the colon, Verrucomicrobiota abundance was generally elevated compared with the GBDs, reaching significance for 200FOS (P = 0.0133), 100FOS (P = 0.0106), and 100IN (P = 0.0245) compared with 5001, and for 100FOS (P = 0.0411) compared with 5002. Both FOS groups also demonstrated significant elevations in the phylum Actinobacteriota compared with both GBDs (Supplemental Table 1). Similar trends were observed in the colon samples. Colon Actinobacteriota populations were also significantly increased by both GBD treatment groups compared with both GBDs (P values < 0.026) (Supplemental Table 2).

Genus-level changes.

Notable shifts at the genus level were also observed due to the different dietary treatments. In the cecum samples (Figure 6C), prominent

| TABLE 5 | Body weights, colon lengths, and cecum/colon weights of the mice at the end of study 2 (day 14) |
|---------|-----------------------------------------------|
|         | Day 1 body weight, g | Day 14 body weight, g | % Change in body weight | Colon length, cm | Cecum + colon weight, g/100 g BW | Cecum weight, g/100 g BW | Colon weight, g/100 g BW |
| 5001    | 15.2 (0.3)          | 23.2 (0.4)          | 52 (17)                     | 8.7 (0.3)               | 0.67 (0.02)               | 0.8 (0.1)               |
| 5002    | 15.3 (0.4)          | 23.5 (0.6)          | 53 (19)                     | 8.7 (0.3)               | 0.69 (0.02)               | 0.8 (0.1)               |
| 100IN   | 15.3 (0.2)          | 23.5 (0.4)          | 50 (17)                     | 8.5 (0.3)               | 0.67 (0.02)               | 0.8 (0.1)               |
| 100FOS  | 15.3 (0.4)          | 23.5 (0.6)          | 54 (18)                     | 8.7 (0.3)               | 0.67 (0.02)               | 0.8 (0.1)               |
| 200IN   | 15.3 (0.2)          | 23.5 (0.4)          | 50 (17)                     | 8.7 (0.3)               | 0.67 (0.02)               | 0.8 (0.1)               |
| 200FOS  | 15.3 (0.4)          | 23.5 (0.6)          | 54 (18)                     | 8.7 (0.3)               | 0.67 (0.02)               | 0.8 (0.1)               |

1Values are means (SEM); n = 6/group. Different letters across rows indicate significantly different values, P < 0.05. BW, body weight; 100CEL, open standard diet with 100 g cellulose per 4084 kcal; 200CEL, open standard diet with 200 g cellulose per 4084 kcal; 100IN, open standard diet with 100 g inulin per 4084 kcal; 200IN, open standard diet with 200 g inulin per 4084 kcal; 100FOS, open standard diet with 100 g fructo-oligosaccharides per 4084 kcal; 200FOS, open standard diet with 200 g fructo-oligosaccharides per 4084 kcal; 5001, LabDiet 5001; 5002, LabDiet 5002.

2Values for individual microbial values are presented in Supplemental Tables 1–4.
genera present in the GBDs (5001, 5002) included the Lachnospiraceae NK4A136 group and an unclassified genus from the family Muribaculaceae. Interestingly, the Lachnospiraceae NK4A136 group was significantly higher in abundance for both GBDs compared with the CEL- and FOS-based diets, and tended to be higher than 200IN (5001 vs. 200IN, \( P = 0.062 \); 5002 vs. 200IN, \( P = 0.093 \)), but not different from those fed 100IN (5001 vs. 100IN, \( P = 0.53 \); 5002 vs. 100IN, \( P = 0.57 \)). The family Oscillospiraceae (undefined genus) was significantly increased by CEL groups compared with both GBDs and most of the soluble-fiber groups, except where 100CEL was not significantly different from 100IN (\( P = 0.14 \)). Alistipes was significantly elevated by 200CEL relative to what was found in both GBDs (vs. 5001, \( P = 0.035 \); vs. 5002, \( P = 0.009 \)) and compared with all soluble-fiber groups (\( P < 0.02 \)). All of the soluble-fiber diets at both doses, relative to GBDs, significantly increased the relative abundance of Bifidobacteria, Akkermansia, and Faecalibaculum (GBDs 5001 and 5002 vs. all soluble-fiber diets, \( P < 0.01 \)), while decreasing Roseburia (GBDs 5001 and 5002 vs. 200IN or 200FOS diets, \( P < 0.01 \); vs. 100IN or 100FOS, \( P < 0.05 \)); similar trends were also observed relative to CEL-based diets 100CEL and 200CEL (Bifidobacteria, Akkermansia, and Faecalibaculum, \( P = 0.01 \); for Roseburia, \( P < 0.02 \)). Almost all PDs caused a significant increase in the relative abundance of the genus Bacteroides (\( P < 0.03 \)), except for 5002 relative to 100CEL (\( P = 0.13 \)), and all PDs had more of the family Tannerellaceae (genus undefined) compared with GBDs (\( P < 0.01 \)) (Figure 6C, Supplemental Table 3). Bacteroides was significantly increased by soluble-fiber diets at lower fiber doses, but not at higher doses compared with the CEL diets at the same dose (100IN and 100FOS vs. 100CEL, \( P = 0.01 \); 200IN and 200FOS vs. 200CEL, \( P > 0.09 \)). Dose of fiber (regardless of type of fiber) had no significant influence on any of the above-mentioned genera.

Similar trends in relative abundance at the genera level were observed in the colon samples (Figure 6D). Not surprisingly, the soluble-fiber–based diets (100IN, 200IN, 100FOS, 200FOS) significantly increased the abundance of Bifidobacteria, Akkermansia, and Faecalibaculum compared with CEL and GBDs (5001, 5002). Unlike in the cecums, Roseburia abundance was significantly increased for 100IN (\( P = 0.0194 \)), 200IN (\( P = 0.0105 \)), and 200FOS (\( P = 0.011 \)) relative to 5002; however, there were no differences compared with the 5001 group for any PDs (Supplemental Table 4). Similar to the cecum samples, all PD groups increased the relative abundance of the genera Bacteroides (except for 100CEL vs. 5002, \( P = 0.06 \)) and Prevotella_UGC-001 and the Tannerellaceae family (undefined genus) compared with GBDs. The family Tannerellaceae (undefined genus) was also significantly higher for 100CEL compared with 100IN (\( P = 0.02 \)). Bacteroides was significantly increased by 100IN and 100FOS relative to 100CEL (\( P = 0.01 \)) and for 200FOS relative to 200CEL (\( P = 0.045 \), but was not
FIGURE 4 Study 2. Alpha-diversity measurements for Shannon index (A/D), Chao1 index (B/E), and observed ASVs (C/F) by dietary treatment group for colon (A–C) and cecum (D–F) samples. Data are expressed as means ± SEM for each treatment group. Groups with different letters represent significantly different results by 1-factor ANOVA with Tukey's post hoc analysis (P < 0.05) after 14 d fed either a GBD (5001 or 5002) or high-fiber PDs (100CEL, 100IN, 100FOS, 200CEL, 200IN, 200FOS), with n = 6/group. ASV, Amplicon Sequence Variant; GBD, grain-based diet; PD, purified diet; 100CEL, open standard diet with 100 g cellulose per 4084 kcal; 200CEL, open standard diet with 200 g cellulose per 4084 kcal; 100IN, open standard diet with 100 g inulin per 4084 kcal; 200IN, open standard diet with 200 g inulin per 4084 kcal; 100FOS, open standard diet with 100 g fructo-oligosaccharides per 4084 kcal; 200FOS, open standard diet with 200 g fructo-oligosaccharides per 4084 kcal; 5001, LabDiet 5001; 5002, LabDiet 5002.

changed by fiber dose. In contrast, an undefined genus in the family Muribaculaceae was significantly reduced by 100IN and 100FOS relative to 100CEL (P ≤ 0.03) and 200FOS vs. 200CEL (P = 0.012). Collectively, these differences clearly demonstrate that different fiber types support the growth of different microbes present in the gastrointestinal tract and that both soluble fibers tended to support similar microbial growth.

**Predicted metabolic functions of microbiota**

The predicted function of the gut microbiota with respect to metabolism in response to the dietary treatments was evaluated using the **PICRUSt** program. When examining the gene counts for specific categories of Metacyc Pathways for cecum (Figure 7) and colon (Supplemental Figure 2) samples, key differences between treatment groups were observed. These pathways were related to carbohydrate digestion and absorption, fructose and mannose metabolism, galactose metabolism, starch and sucrose metabolism, fatty acid metabolism, and fatty acid biosynthesis (Figure 7). Generally speaking, the PDs had higher gene counts than GBDs for all the 6 tested pathways in the cecal samples, but only the 200CEL and 200IN treatments significantly elevated the gene counts for each of these pathway groupings compared with both the GBDs. Only the CEL groups produced a significant effect of fiber dose on pathway upregulation, as the 200CEL group demonstrated higher gene counts than the 100CEL group for all pathways except for carbohydrate degradation and absorption, which failed to reach significance. Unlike IN or CEL, neither of the FOS diets significantly affected the metabolic pathways compared with GBDs regardless of the dose. Fewer differences were observed in terms of metabolic pathways in the colon samples (Supplemental Figure 2). PDs demonstrated higher gene counts related to carbohydrate degradation and absorption, with the 200CEL, 200IN, and the 200FOS groups reaching significantly higher levels compared with the GBDs. Galactose metabolism was affected by 200CEL and 200FOS relative to GBDs. In the case of fructose and mannose metabolism, starch and sucrose metabolism, fatty acid metabolism, and fatty acid biosynthesis, only the 200CEL dietary treatment produced significantly higher gene counts compared with all other groups. As in the cecum, the 200CEL group increased gene counts compared with the 100CEL group in most pathways except for carbohydrate degradation and absorption, which failed to reach significance. The soluble-fiber groups, regardless of dose, did not significantly modulate metabolic pathway gene counts in the colon.
**Effect of fiber type on gut and metabolic health**

**FIGURE 5**  Study 2. Bray-Curtis Dissimilarity plots for colon (A) and cecum (B) samples after 14 d of either a GBD (5001 or 5002) or high-fiber PDs (100CEL, 100IN, 200CEL, 200IN, 200FOS), with \( n = 6 \)/group. GBD, grain-based diet; PD, purified diet; PERMANOVA, Permutational Multivariate Analysis of Variance; 100CEL, open standard diet with 100 g cellulose per 4084 kcal; 200CEL, open standard diet with 200 g cellulose per 4084 kcal; 100IN, open standard diet with 100 g inulin per 4084 kcal; 200IN, open standard diet with 200 g inulin per 4084 kcal; 100FOS, open standard diet with 100 g fructo-oligosaccharides per 4084 kcal; 200FOS, open standard diet with 200 g fructo-oligosaccharides per 4084 kcal; 5001, LabDiet 5001; 5002, LabDiet 5002.

**Discussion**

PDs are an essential element of nutrition research, and it is important to consider the effect that background diet may have on the expressed phenotype. Since the AIN diets were designed 4 decades ago, we have learned more about how different nutrients alter the metabolic profile of mice and rats and this knowledge can be applied to improve these diets for future studies. The AIN Committee did allude to using a different source of carbohydrate in lieu of sucrose due to potential influences of this ingredient on metabolic disorders, but they did not make any specific recommendations on adjusting the fiber levels in these diets (25). The current recommendation for 5% CEL, an insoluble fiber, provides little fermentable dietary substrate accessible to the intestinal microbiota. Current data clearly show that this lack of soluble fiber leads to dramatic effects on gut and metabolic health in mice (8, 17). We therefore wished to further understand how revised versions of the AIN diets with reduced sucrose and higher amounts of total fiber, including soluble fiber, affected metabolic and gut health compared with GBDs.

In our chronic feeding study, fiber and sucrose alterations in the 2 OSDs used in this study did not result in significant changes to overall adiposity index (combining all adipose depots relative to carcass weight) relative to the GBD. Leptin was elevated in mice fed either the 93G or 75CEL25IN compared with those fed the GBD, suggesting that the reduced sucrose and fiber change had little impact on these parameters. However, the addition of more fiber as CEL suppressed this effect and these mice had similar levels as those fed the GBD. While it is unknown how added CEL led to a reduced leptin level, a trend for reduced adipose depots in these mice relative to other PDs may have partially accounted for this difference. Furthermore, there was also a slight but significant increase in liver triglycerides in the 75CEL25IN-fed mice compared with those fed the GBD, while those fed the higher-fiber 225CEL25IN diet had similar levels as those fed GBD. Overall, these changes suggested that total fiber content is more important to maintaining these static biochemical parameters. However, our data suggested that the addition of IN was key to maintaining glucose tolerance and both 75CEL25IN and 225CEL25IN groups had similar glucose tolerance compared with those fed the GBD. In addition, replacement of sucrose with glucose-derived carbohydrates may also have benefited these mice fed 75CEL25IN as sucrose may induce metabolic disease in rats and mice (26, 27). While sucrose levels were reduced to 10% in the 93G diet relative to 50% in the 76A diet, it was not completely removed due to pelleting and palatability concerns by the AIN Committee (28). However, even relatively lower levels of sucrose may elicit changes in glucose tolerance over a chronic feeding period, which is due to the fructose component of this carbohydrate (29).

Closer examination of how the type and amount of fiber in PDs influenced gut health (study 2) indicated that a replacement of CEL with soluble-fiber–based PD prevented the rapid cecum and colon weight...
loss associated with traditional CEL-based PDs. Both types of soluble fiber at low and high doses were capable of maintaining cecum and colon weights relative to GBD-fed mice. This phenomenon has also been observed previously in the context of a high-fat diet, where an IN-based high-fat PD helped to maintain cecum and colon weight changes compared with a CEL-based high-fat PD. This effect was attributed to the microbiota as inulin's ability to promote colon and cecum mass was completely absent in germ-free mice (17). Indeed, further work suggested that inulin increased enterocyte proliferation and mucosal defense along with reduced microbial encroachment on the mucosal lining and intestinal inflammation. Ultimately, these changes by inulin also required the presence of the microbiome and led to reduced metabolic syndrome in mice fed high-fat diets (18). Alongside changes to organ morphology, soluble-fiber–based PDs caused rapid and dramatic changes to the microbiota in contrast to GBDs, regardless of fiber content, including reduced species richness (alpha diversity) in both cecum and colon tissues. This may be, in part, due to the differences in fiber diversity among these diets as GBDs contain diverse fiber contents, which may support the growth of a more diverse species population, leading to greater alpha-diversity values (30). Moreover, dietary intervention studies in humans in which only 1 fiber type is added to the diet typically do not translate to increased alpha-diversity metrics (31). Still, our results indicate that CEL alone was able to support greater species richness in a PD compared with IN and FOS. This could be partially because the fiber types present in GBDs are predominantly insoluble types, like CEL (4). Previous data suggest that CEL is important for age-related diversification of the intestinal microbiota (32), and thus our results indicate that it may be better to continue including CEL in future designs of PDs. In fact, a recent study suggested that adding IN to a lower-fat diet containing CEL tended to increase alpha diversity relative to CEL alone, suggesting the importance of maintaining CEL to preserve species richness (33). Beta-diversity analysis indicated that each fiber type (GBDs, CEL based, or soluble-fiber based) supported distinctly different communities of bacteria, regardless of fiber dose. These data also suggested that both GBDs supported growth of similar microbial communities, which may not be surprising given that they contain similar ingredients and also insoluble- and soluble-fiber contents. This has been documented in other studies and, together, these findings indicate that single sources of purified fibers as part of a PD, even at higher concentrations, may not support similar microbial species as do the GBDs. It is likely the case that multiple purified fibers used in combination would optimally support the microbiome and thus are necessary to achieve closer similarity to GBDs, regardless of the dose.

Although switching from a GBD to PDs generally led to reduced species richness and supported different microbial species compared with GBDs, some of the changes to dominant microbial taxa could be...
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A

D

B

E

C

F

FIGURE 7 Study 2. Predicted carbohydrate and lipid metabolism of bacterial communities in the cecum using the PICRUSt program after 14 d on either a GBD (5001 or 5002) or high-fiber PDs (100CEL, 100IN, 100FOS, 200CEL, 200IN, 200FOS), with n = 6/group. (A) Carbohydrate digestion and absorption; (B) fructose and mannose metabolism; (C) galactose metabolism; (D) starch and sucrose metabolism; (E) fatty acid metabolism; (F) fatty acid biosynthesis. Data are expressed as means ± SEM for each treatment group. Groups with different letters represent significantly different results by 1-factor ANOVA with Tukey’s HSD post hoc analysis (P < 0.05). GBD, grain-based diet; HSD, honestly significant difference; PD, purified diet; 100CEL, open standard diet with 100 g cellulose per 4084 kcal; 200CEL, open standard diet with 200 g cellulose per 4084 kcal; 100IN, open standard diet with 100 g inulin per 4084 kcal; 200IN, open standard diet with 200 g inulin per 4084 kcal; 100FOS, open standard diet with 100 g fructo-oligosaccharides per 4084 kcal; 200FOS, open standard diet with 200 g fructo-oligosaccharides per 4084 kcal; 5001, LabDiet 5001; 5002, LabDiet 5002.

beneficial and ultimately yield better health outcomes in rodents, especially if soluble fibers were to be added to a PD alongside CEL in the future. The role of the Firmicutes to Bacteriodota ratio in the onset of obesity itself remains controversial (34, 35). However, increased relative abundance of Bacteriodota and other major phyla have demonstrated positive health effects beyond obesity remediation. For example, a study by Rabot et al. (35) indicated that an increased abundance of Bacteriodota was associated with improved glucose tolerance in a cohort of mice fed a high-fat diet. Moreover, it has been demonstrated that inclusion of prebiotic fibers, like IN and FOS, in the diet are linked with higher circulating concentrations of glucagon-like peptide 1 (GLP-1), a metabolic hormone with antidiabetic effects (36, 37). Together, these observations could partially explain why adding soluble fiber such as IN to PDs is associated with improved metabolic health compared with the insoluble-fiber, CEL-based, low-fiber, high-sucrose AIN diets as seen from results of our first study.

There is a growing body of evidence suggesting that certain microbial genera positively influence metabolic health. Akkermansia spp., which belong to the Verrucomicrobia phylum (both of which were elevated due to the inclusion of soluble fiber in the diet), have demonstrated the ability to maintain gut barrier integrity. As such, it is suspected that Akkermansia spp. may exert anti-inflammatory properties, which, in turn, could contribute to overall metabolic health, given that chronic inflammation is associated with insulin resistance and diabetes (38, 39). Other studies have indicated that administration of FOS in rodent diets appears to increase Akkermansia abundance in the gut (doses as low as 0.3 g/d, which translate to ~60 g FOS/kg diet), whereas diet-induced obesity appears to consistently reduce the abundance of this genus (40, 41). Our study suggests that IN and FOS may also increase the relative abundance of this metabolically active genus. Bifidobacteria, which are a subspecies of Actinobacteria, have historically been categorized as health-promoting microbes (42). It is believed that these bacteria play a role in stimulating host innate immunity, and may also enhance the ability of Bacteroides to metabolize carbohydrates (39, 42). Bifidobacteria also appear to reduce gut permeability, similarly to Akkermansia, and reductions in Bifidobacteria populations are found in diet-induced obese rodents (43). The results from our study indicated that the addition of soluble fiber to a PD can increase the abundance of Bifidobacteria relative to a GBD, and both CEL and soluble fibers may increase Bacteroides. Another microbial genus of interest is Roseburia, which is one of the most abundant genera of the Firmicutes phylum. Despite general observations that overall Firmicutes abundance is reduced in lean individuals relative to obese individuals, Roseburia spp. tend to be more abundant in lean individuals (44). They also gener-
ally increase in relative abundance in response to diets rich in FOS and IN (45). However, in our study, all of the soluble-fiber–based diets decreased *Roseburia* relative abundance compared with both GBDs and CEL-based diets in the cecum and colon. While these data are contrary to other reported findings (45), they do support the notion that multiple fiber types may be necessary to support the optimal growth of a consortium of beneficial bacteria. *Roseburia* spp. are well known for their ability to produce high concentrations of butyrate, a potent SCFA that is known to promote colon health (46). Production of this metabolite, by *Roseburia* or other genera, would certainly be indicative of the diet supporting a healthy gut. While SCFAs were not measured in this study, previous collaborative studies suggested that replacement of CEL with approximately 5% IN in the context of a PD with low-fat contents (similar to AIN and OSD) increased levels of fecal SCFAs in male C57BL/6 mice to a similar level as those fed a GBD (17).

Further insights regarding the functional capacity of the microbiome in response to the dietary treatments were explored using the PICRUSt program. Despite the limitation that this software can only predict functional differences in the microbiota using marker–gene sequencing techniques (47), several interesting trends with respect to the treatments were observed. First, it was evident from the metabolic pathways analysis (Figure 7 and Supplemental Figure 2) that the dietary fibers—in particular, the higher dose IN group—had a greater impact on genes related to metabolism in the cecums compared with the colons. The only group with significantly higher metabolic pathway gene counts in both cecum and colon was the 200CEL group. This could partially be explained by the fact that the cecum is the primary site of fermentation in mice, so much of the IN may have been rapidly fermented in the cecum, leaving little to no substrate available for use by microbes in the latter portions of the gut (48). However, significant differences in carbohydrate degradation and absorption were still found for IN and FOS PDs in the colon, suggesting that there was still enough remaining fructans from these sources for microbes residing in the latter portions of the gut. Another partial explanation for the high-dose CEL group (200CEL) being the only treatment group significantly influencing colonic microbial metabolism is the fact that CEL is poorly fermented by nonruminant mammals such as mice and humans (49). Unlike the fast-fermenting soluble fibers, it was likely that still some undigested CEL passed into the colon, which could be utilized by the microbes residing there. With respect to the functionality of the gut microbiome in response to the dietary treatments, it is also worth noting that there appeared to be some dose-related effects, which were not observed when looking at microbial abundance and diversity. Higher doses of CEL and IN tended to increase gene counts in specific pathways to a greater extent than low-dose counterparts in the cecum samples. In other words, greater substrate availability (i.e., higher dosage) translated to greater potential metabolic activity in these tissues. Taken together, all of these findings indicate that CEL likely plays some role in the metabolic functionality of the gut microbiome—in part, by maintaining microbial richness similar to GBDs—but soluble fiber may help to support normal colon physiology and also promote the growth of health beneficial microbial genera, which lead to improved gut morphology relative to CEL alone. Overall, higher concentrations of fiber support greater microbial activity compared with the AIN-recommended dose of 5% in a rodent diet.

In summary, it is clear that these changes to the formulation of traditional AIN PDs (increased amount of total fiber and addition of soluble fiber with replacement of sucrose with glucose-derived carbohydrate sources) provide certain improvements to metabolic health, which may have been, in part, due to changes in the gut microbiota profile. However, to more closely mimic gut microbiota in mice fed GBDs, the addition of multiple, diverse fiber sources will likely be required (4). Our results suggest that a mixture of soluble and insoluble fiber types, present at higher concentrations than the AIN formulations and without the addition of sucrose, may help to maintain microbial richness similar to a GBD while also supporting greater functionality of the microbiome. However, it is difficult to say whether one type of soluble fiber is more beneficial than the other and whether a change in fiber in a PD would allow for a similar microbiome relative to all GBDs. Thus, a PD containing multiple sources of soluble fiber including those not used in this study (e.g., mannan, beta-glucan, pectin, etc.) may need to be developed. Future efforts should be directed towards determining the optimal ratios of soluble and insoluble fibers in PDs, as well as exploring how these changes to the gut microbiome may influence animal health in longer-term studies. It is also critical to focus not only on relative microbiome shifts and predicted functionality but also to examine concentrations of circulating microbial metabolites such as SCFAs to better understand the potential benefits for metabolic health. Given the differences between PDs and GBDs, it should be clear that these 2 diet types should not be compared against each other while determining dietary effects on a given phenotype. This is particularly apparent when determining the theoretical underpinnings of how dietary effects on gut health and the microbiome drive changes in metabolic health outcomes, as suggested previously (17, 50, 51). Unfortunately, GBDs are frequently used as controls for many experimental studies testing effects of a PD (e.g., high-fat-diet studies), which leads to misinterpretation of results. While having a matched PD allows one to compare how a given change in diet is altering the rodent phenotype, one particular concern from the research community is that rodents consuming control PDs, although lower in fat, are typically not as metabolically healthy as those fed a GBD. Thus, it is imperative that the research community focus on improving the formulation to mitigate some of the adverse changes associated with consumption of PDs. A metabolically healthy control PD would greatly help the research community to decipher nutrient-related phenotypic differences in a wide range of scientific domains. While we mainly focused on sucrose levels and the type/concentration of the fiber, future studies should also examine the type and level of fat in order to optimize the formulation of a metabolically healthy PD, as recently discussed regarding the AIN series formulas (8). While we understand that the current study is limited to certain metabolic parameters and the use of prediction software to assess microbial functionality, this study reinforces the notion that laboratory animal diets must be formulated and selected with utmost care, as the gut microbiome is easily influenced by diet and shifts in the populations of microbes may impact study outcomes.

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Data Availability

Data described in the article, code book, and analytic code will be made available upon request.

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