

RESEARCH LETTER

Plant-based Enteral
Nutrition Modifies the
Gut Microbiota and
Improves Outcomes in
Murine Models of Colitis

Exclusive enteral nutrition (EEN), ie, restricting patients to commercially available liquid diets with avoidance of solid food, is an effective treatment for Crohn's disease (CD).¹ However, commercial formulas used for EEN generally have low fiber and high sugar content and contain emulsifiers that worsen colitis in animal models.² Despite its efficacy, EEN appears to accentuate microbial changes observed in CD, specifically a loss of protective anaerobes and the growth of pathogens.³ Plant-based enteral nutrition (PBEN) represents a high-fiber alternative to conventional enteral nutrition (CEN) without added sugar or artificial ingredients. The advent of commercially available PBEN is relevant to inflammatory bowel disease because diets low in fruits and vegetables represent a risk factor for CD, and because many plant-derived compounds are therapeutic in preclinical colitis models.⁴⁻⁶

We sought to determine the effects of PBEN on the microbiota and outcomes in murine colitis models. We initially observed that male C57BL/6 mice randomized to receive PBEN (Liquid Hope; Functional Formularies,

Centerville OH) experienced better outcomes in a 4% dextran sulfate sodium (DSS) (MW 40,000-50,000, Thermo Fisher Scientific, Waltham, MA) colitis model than chow (IsoPro RMH 3000; LabDiet, St Louis, MO) or 2 conventional formulas (Vital or PediaSure; Abbott Laboratories, Lake Bluff, IL). We refer to these groups as PBEN, CHOW, CEN1, and CEN2. Mice fed PBEN experienced less weight loss than CHOW, CEN1, and CEN2 mice after exposure to DSS (Figure 1, $P < .001$). At death, plasma interleukin 6, fecal lipocalin, histologic scores of inflammation, and colon length suggested less inflammation in PBEN (Supplementary Figure 1). Similar results were obtained in numerous permutations of the original experiment: females instead of males, Taconic Biosciences rather than Jackson Laboratory, 2% DSS instead of 4%, and the rectally instilled trinitrobenzene sulfonic acid colitis model (Supplementary Figure 2A). We also modified the original experiment by alternating formula feeding and 4% DSS water every 12 hours to confirm that the benefit of PBEN was not an artifact from admixing DSS and liquid formulas (Supplementary Figure 2B). Finally, to determine whether these observations extend beyond chemically induced colitis, we randomized mice in a T-cell transfer colitis model to receive CHOW, PBEN, or CEN. Improved outcomes were observed

with PBEN in all experiments (Supplementary Figure 2C).

To assess the effect of each diet on the gut microbiota, we analyzed 16S rRNA gene sequences within fecal pellets collected before and after a 7-day feeding trial. At baseline, the number of observed taxa (alpha diversity) was equal across all groups. After the feeding trial, alpha diversity was significantly decreased in CEN1/CEN2 compared with PBEN/CHOW mice (Figure 2; analysis of variance [ANOVA], Tukey honestly significant difference [HSD], $P < .001$). Similar results were seen with community composition (beta diversity; Supplementary Figure 3). Baseline samples from all groups initially clustered together in principal coordinates analysis space. After 7 days of feeding, PBEN samples clustered separately from the other groups (Supplementary Figure 3A and B; permutational multivariate analysis of variance [PERMANOVA], $P < .05$). PBEN and CHOW samples were marked by increased commensal anaerobes (Clostridiales, *Lachnospiraceae*, and *Ruminococcus*) (Supplementary Figure 3C; LefSe, $P < .05$). CEN1/CEN2 samples were remarkably similar to each other and were enriched with gram-negative pathogens from the family *Enterobacteriaceae*, a finding previously described in CD patients receiving EEN.^{3,7}

To ascertain whether the gut microbiota mediates the protective effect of PBEN, we repeated the 4% DSS colitis model in germ-free animals receiving CEN1 or PBEN for 7 days before and during DSS exposure. As shown in Supplementary Figure 2D, weight loss in the absence of a gut microbiota was severe in both the PBEN and CEN1 groups. In addition, we administered antibiotics to mice fed CEN1 or PBEN for 7 days before and during DSS exposure. Antibiotics significantly worsened outcomes in PBEN mice (Supplementary Figure 2E; $P < .05$) but did not affect outcomes in CEN1 mice. These results support the conclusion that gut microbes are not required for the onset of colitis but are indeed necessary for the protective benefit of the PBEN diet.

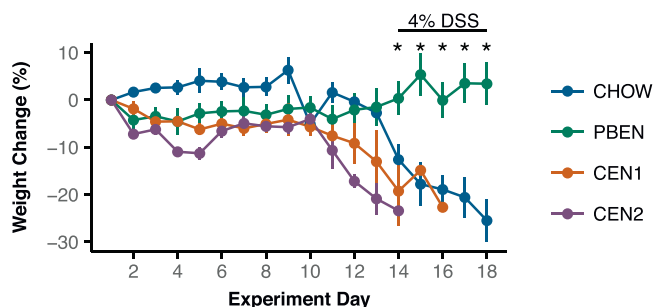


Figure 1. Effect of diet on outcomes in 4% DSS colitis model. Weight curves in mice receiving experimental diets for 7 days, followed by DSS exposure ($n = 8$ /group). CHOW mice received DSS in their drinking water. Asterisks denote significance in post hoc test between only PBEN and all other individual groups (ANOVA, Tukey HSD, $P < .05$). Error bars represent standard deviation.

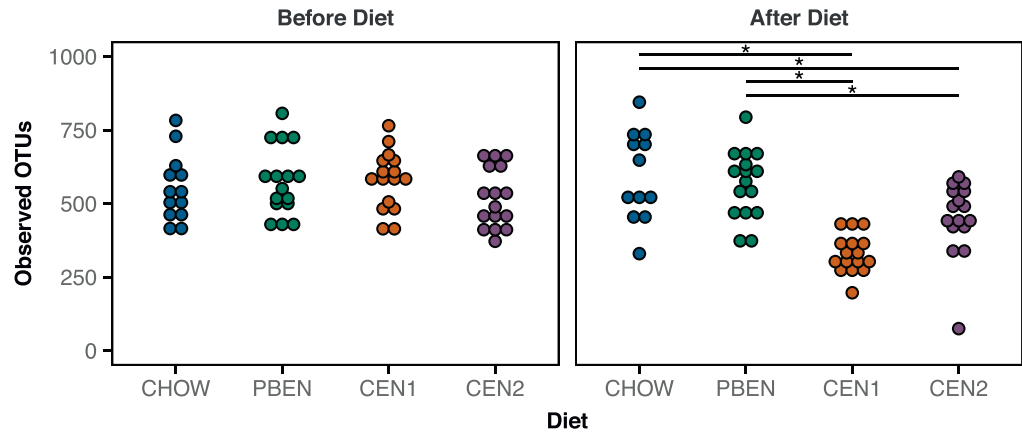


Figure 2. Effect of diet on gut microbial diversity. Alpha diversity of fecal microbial communities (observed OTUs) before and after a 7-day trial of the experimental diets. Asterisks denote significance (ANOVA, Tukey HSD, $P < .05$).

Because of the impact of the microbiota on the protective effect of PBEN, we measured the concentrations of microbial metabolites in cecal contents from mice receiving PBEN, CEN1, or CEN2 for 21 days (Supplementary Figure 4). Samples from CHOW mice were not analyzed. PBEN samples contained increased amounts ($P < .05$) of the bile acids lithocholate and tauroolithocholate and the plant-derived hydroxycinnamic acid, which have been shown to exert anti-inflammatory activity or confer protection in colitis models.^{8,9} Also, CEN1/CEN2 mice possessed higher concentrations of amino acids than PBEN ($P < .05$), consistent with a described association between fecal amino acids, gut dysbiosis, and disease activity in CD.¹⁰ Concentrations of short-chain fatty acids were not different among diets except for propionate, which was significantly increased in CEN1 compared with CEN2 and PBEN ($P < .05$).

In summary, the benefits of PBEN result in part from diet-driven changes in the gut microbiota, which in turn impact bile and amino acid metabolism. We acknowledge that many components of plant-based diets likely impart health benefits independently of the microbiota. We also acknowledge that currently available formulations of CEN have yielded positive outcomes as a clinical treatment for patients with CD. We therefore conclude that further studies are indicated to determine whether PBEN can

improve outcomes even further for patients requiring supplemental enteral nutrition.

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Abbreviations used in this letter: ANOVA, analysis of variance; CD, Crohn's disease; CEN, conventional enteral nutrition; DSS, dextran sulfate sodium; EEN, exclusive enteral nutrition; HSD, honestly significant difference; PBEN, plant-based enteral nutrition; PERMANOVA, permutational multivariate analysis of variance

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Author contributions

Andrew Yeh: study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript for important intellectual content; statistical analysis. Eric M. Connors: acquisition of data; analysis and interpretation of data. Rafael Ramos-Jimenez: acquisition of data; analysis and interpretation of data. Brian Firek: acquisition of data; analysis and interpretation of data; critical

revision of the manuscript for important intellectual content. Elizabeth A. Novak: study concept and design; acquisition of data; analysis and interpretation of data. Matthew B. Rogers: analysis and interpretation of data; statistical analysis. Richard Cheek: acquisition of data. John Ozolek:

analysis and interpretation of data; technical support. Kevin Mollen: study concept and design; analysis and interpretation of data; technical, or material support. Michael J. Morowitz: study concept and design; analysis and interpretation of data; drafting of the manuscript; critical revision of the manuscript

for important intellectual content; statistical analysis; obtained funding; technical, or material support; study supervision.

Conflicts of interest

The authors disclose no conflicts.

Supplementary Methods

Animals

Animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Male C57BL/6 mice (8 weeks, 20–30 g) (Jackson Laboratories, Bar Harbor, ME and Taconic Biosciences, Hudson, NY) and BALB/c mice (4 weeks, 15–20 g) (Taconic) were used.

Diets

Mice were fed standard mouse chow (CHOW), a standard adult polymeric formula, Vital; Abbott Laboratories, Lake Bluff, IL (CEN1), a standard pediatric polymeric formula, PediaSure; Abbott Laboratories (CEN2), and a plant-based whole foods formula, Liquid Hope; Functional Formulas, Centerville, OH (PBEN). PBEN was diluted 3:4 to be isocaloric with CEN1 and CEN2.

Dextran Sulfate Sodium Colitis

C57BL/6 mice were given the experimental diets for 7 days. Mice were then introduced to DSS or control water for 4 days. Mice receiving CHOW received a water bottle with 4% (w/v) DSS. Mice receiving CEN1, CEN2, or PBEN received an equivalent of 20 mL of 4% DSS colitis or control water mixed with their liquid diets daily. Disease activity index and weight were measured daily.

An alternative DSS experiment was also performed where after 7 days of the experimental diet, mice alternated every 12 hours between exclusive access to their experimental diet and exclusive access to control or 4% DSS water.

A modified DSS experiment with antibiotics was also performed. Using previously published concentrations of antibiotics used to ablate the microbiome (vancomycin 0.5 g/L, metronidazole 1 g/L, ampicillin 1 g/L, neomycin 1 g/L), antibiotics were added daily to the liquid diets. All mice were then given DSS for 4 days in addition to continued antibiotic administration.

TNBS Colitis

BALB/c mice received experimental diets for 7 days. Before TNBS administration, mice were fasted for 24 hours. Mice were then administered a 2.5 mg dose of TNBS in 50% ethanol solution transrectally.

CD4⁺CD45⁺ RB^{high} T-Cell Transfer Model of Colitis

Colitis was induced according to the methods published in previous article with modifications.¹

Germ-free Experiment

Germ-free experiments were performed at the National Gnotobiotic Rodent Resource Center at the University of North Carolina. Germ-free C57BL/6 mice underwent the DSS experiment detailed above with PBEN and CEN1.

Assessment of Disease Activity

To assess colitis severity, a composite score of weight loss, stool consistency, and blood in the stool was determined. Weight loss was scored on the basis of a percentage from baseline before starting DSS or TNBS (0 = <1%, 1 = 1%–5%, 2 = 5%–10%, 3 = 10%–15%, 4 = >15%). Stool consistency was scored on the following scale: 0 = formed and hard, 1 = formed but soft, 2 = loose stools, 3 = mild diarrhea, 4 = gross diarrhea. Blood in the stool was scored on the following scale: 0 = absence of blood, 2 = no gross blood but positive fecal occult blood test, 4 = gross blood.

Histology

Colons were fixed in 4% paraformaldehyde and fixed in paraffin. Cryostat sections of colon were prepared and stained with hematoxylin-eosin. Degree of inflammation (none = 0, mild = 1, moderate = 2, severe = 3) was multiplied by the estimated percent involvement of the total colon.

Multiplex Cytokine Assay

A multiplex cytokine assay (M60009RDPD; Bio-Rad, Hercules, CA) was completed in accordance with the manufacturer's instructions using the Luminex 200 (Luminex, Austin, TX).

Fecal Lipocalin-2 Assay

Total protein concentration in stool was determined for normalization by using a bicinchoninic acid assay (Sigma-Aldrich, St. Louis, MO). Lipocalin-2 concentrations were determined by using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN).

Fecal Microbiome Sequence Processing and Analysis

Microbial DNA was extracted from stool samples by using the QIAamp PowerFecal DNA Isolation kit (Qiagen, Venlo, Netherlands). Bacterial 16S rRNA gene sequences were amplified and sequenced on the Illumina MiSeq platform. Amplicons were produced by using primers that target the 16S V4 region (515F and 806R primers).

Sequence data were analyzed by using Quantitative Insights into Microbial Ecology (QIIME). Samples were rarefied to 5000 sequences. Alpha and beta diversity were calculated using QIIME. Significant differences were assessed by using ANOVA and post hoc Tukey HSD test where appropriate. Variations in beta diversity were assessed with PERMANOVA and PERMDISP algorithms in QIIME. Linear discriminant analysis effect size (LEfSe) was used to determine differentially abundant taxa across groups of samples.²

Fecal Metabolomic Processing and Analysis

Sample processing was performed at the University of Hawaii Cancer Center Metabolomics Shared Resource. Sample preparation for bile acid analysis was performed.^{3,4}

Ultra-performance liquid chromatography coupled to tandem mass spectrometry was used to quantify bile acids.

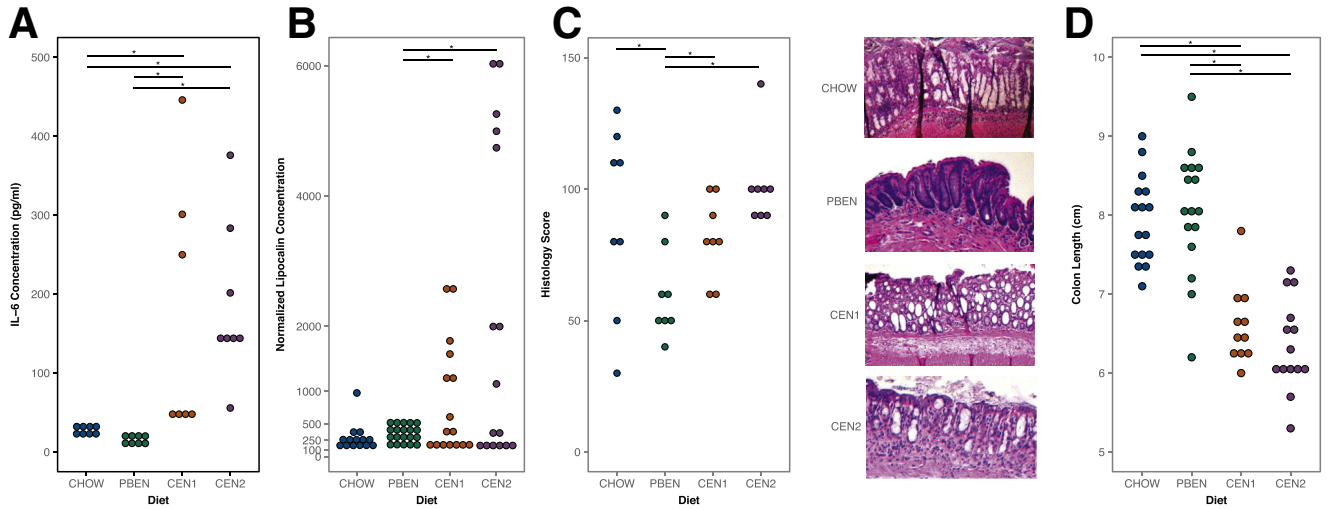
A total of 145 representative compounds were used as standards for the remaining metabolites. Serial dilutions of the working standard solutions were used to generate the calibration curves. Cecal sample preparation for remaining metabolite analysis was performed by using a previously published protocol.⁵ Gas chromatography coupled to time-of-flight mass spectrometry was used to quantify gut microbial metabolites.

Statistics

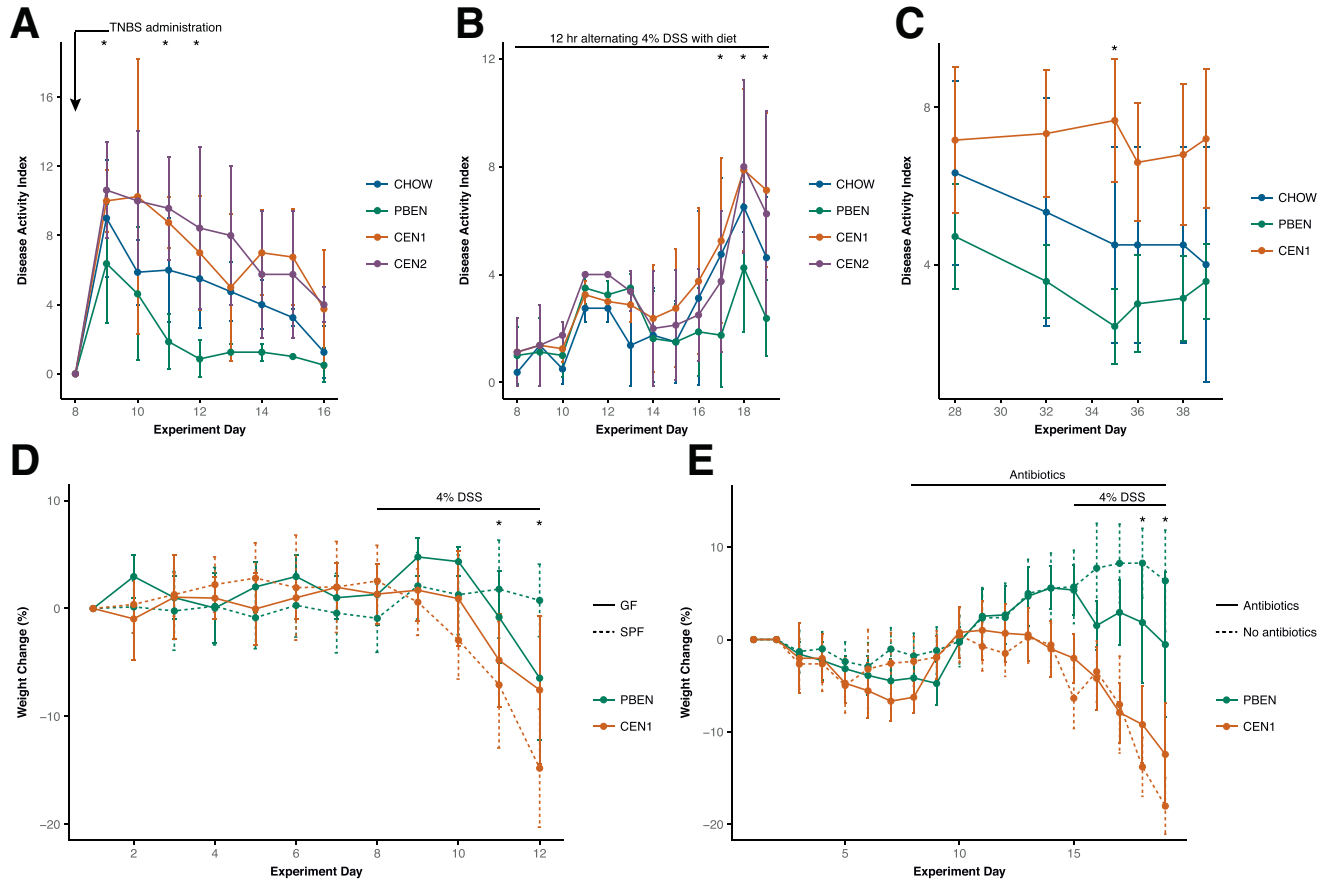
A *P* value of .05 was used to determine significance in all statistical tests.

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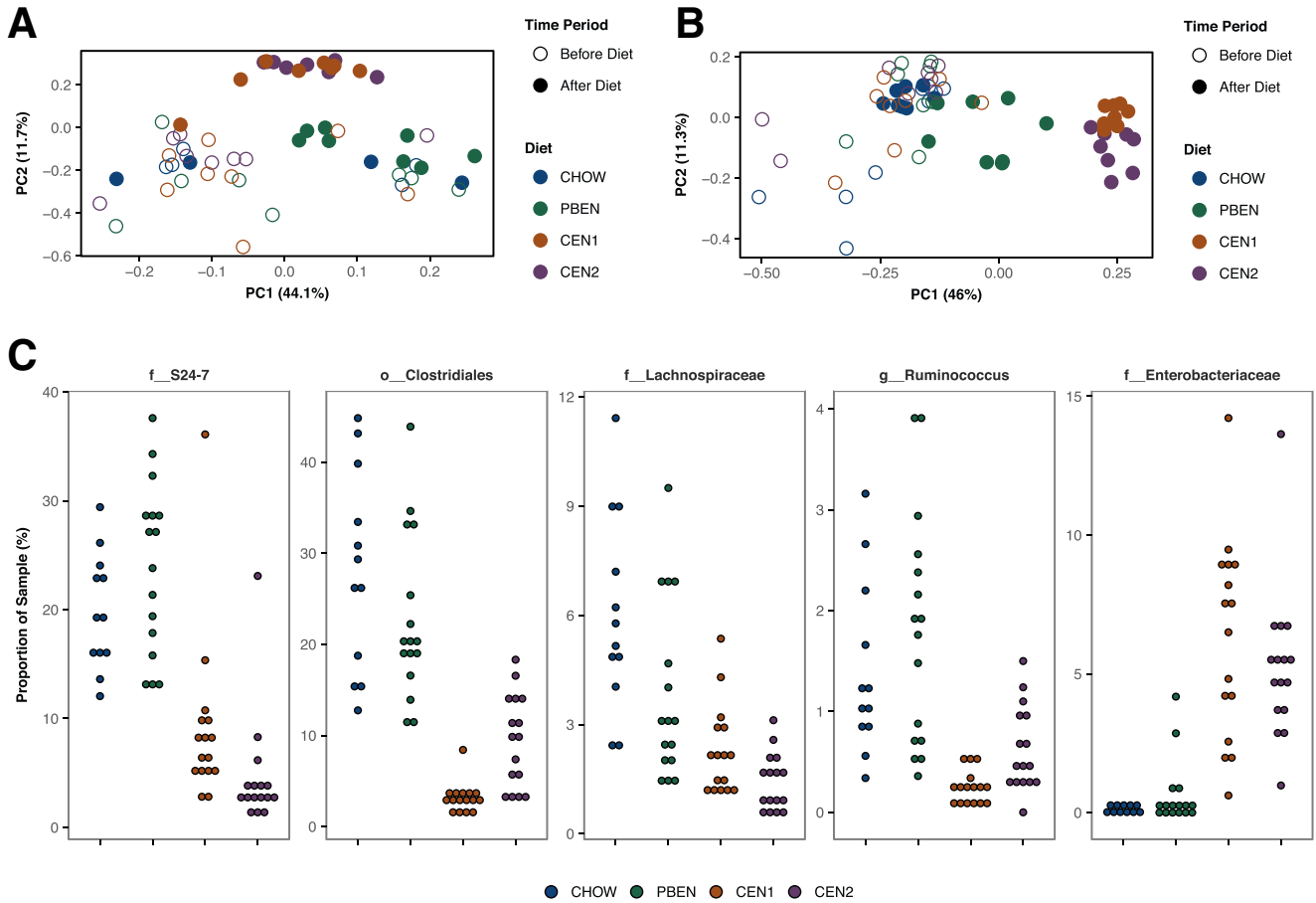
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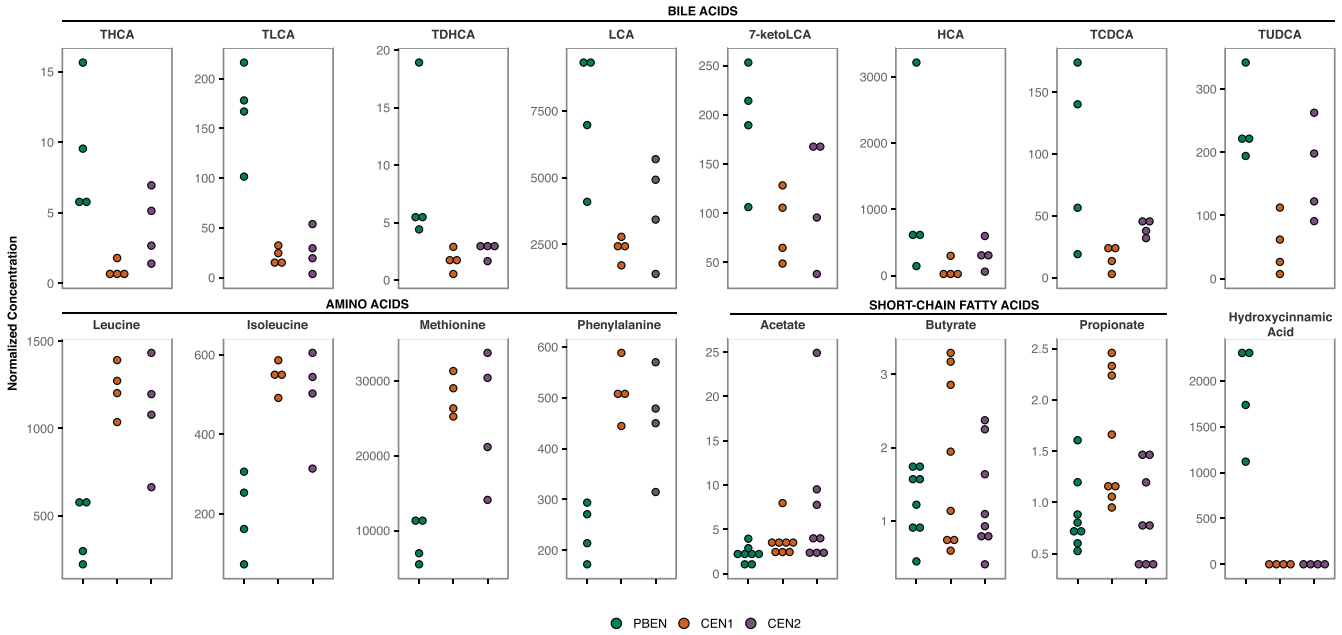
Supplementary Figure 1. Effect of diet on gut inflammation in DSS colitis models. Plasma interleukin 6 measurements (n = 8/group) (A), normalized fecal lipocalin concentrations (n = 14-20/group) (B), histologic scores of mucosal injury (n = 8/group) (C), and colon length (n = 11-16/group) (D) in mice receiving CHOW, CEN1, CEN2, or PBEN for 7 days, followed by 4% DSS mixed into the diets. CHOW animals were exposed to the same concentration of DSS mixed in water. Asterisks denote significance (ANOVA, Tukey HSD, *P* < .05). Lipocalin concentration (pg/ μ L) was normalized to total protein concentration (μ g/ μ L) in stool. (C) Representative slides of H&E staining of colonic mucosal tissue from each diet group.



Supplementary Figure 2. Effect of diet on outcomes in alternative colitis models. (A) Disease Activity Index (DAI) in mice fed experimental diets for 7 days, followed by a single dose of TNBS administered by enema on day 8. Asterisks denote where mean DAI was significantly lower in mice fed PBEN compared with CHOW, CEN1, and CEN2 in post hoc testing ($n = 8/\text{group}$; ANOVA, Tukey HSD, $P < .05$). (B) DAI in mice fed experimental diets for 7 days, followed by water only, alternating every 12 hours with the experimental diet mixed with 4% DSS (day 8 to day 19). Asterisks denote where mean DAI was significantly lower in mice fed PBEN compared with CHOW, CEN1, and CEN2 in post hoc testing ($n = 8/\text{group}$; ANOVA, Tukey HSD, $P < .05$). (C) DAI in mice undergoing T-cell transfer (day 8) fed experimental diets for 40 days. DAI measurements started on day 28. Asterisks denote where mean DAI was significantly lower in mice fed PBEN compared with CHOW, CEN1, and CEN2 in post hoc testing ($n = 8/\text{group}$; ANOVA, Tukey HSD, $P < .05$). (D) Weight curve in germ-free (GF) mice fed CEN1 or PBEN for 7 days, followed by 4% DSS exposure ($n = 9/\text{group}$), compared with specific pathogen-free mice (SPF, $n = 28/\text{group}$). Asterisks denote significance between the PBEN-GF group compared with the PBEN-SPF group (ANOVA, Tukey HSD, $P < .05$). (E) Weight curves in mice fed CEN1 and PBEN receiving enteral antibiotics throughout a 7-day feeding period, followed by 4% DSS exposure ($n = 8/\text{group}$). Asterisks denote significance between mice fed PBEN with antibiotics and mice fed PBEN without antibiotics (ANOVA, Tukey HSD, $P < .05$). Error bars represent standard deviation.



Supplementary Figure 3. Effect of diets on composition of gut microbial communities. Beta diversity comparisons of microbial communities in the stool between mice before and 7 days after starting the diets (n = 8/group). Shown is the principal coordinates analysis of weighted Unifrac (A) and abundance Jaccard distances (B). Axis labels indicate the proportion of variance explained by each principal coordinate. PBEN samples after 7 days of feeding clustered separately from CHOW and CEN1/CEN2 samples (PERMANOVA, $P < .05$). (C) Relative abundance of bacteria taxa that were differentially abundant in cecal contents of PBEN mice after a 7-day feeding trial. Relative abundances of S24-7, Clostridiales, *Lachnospiraceae*, and *Ruminococcus* were statistically significantly higher in mice fed PBEN and CHOW compared with CEN1 and CEN2 (LEfSe, $P < .05$). Relative abundance of *Enterobacteriaceae* was elevated in mice fed CEN1 and CEN2 compared with CHOW and PBEN (LEfSe, $P < .05$).



Supplementary Figure 4. Concentration of cecal metabolites in mice fed PBEN, CEN1, and CEN2 for 21 days. Concentrations of secondary bile acids (nmol/L per mg of cecal contents) 7-ketolithocholic acid (7-ketoLCA), γ -muricholic acid (HCA), lithocholic acid (LCA), taurochenodeoxycholic acid (TCDCA), taurodeoxyhyocholic acid (TDHCA), taurohyocholic acid (THCA), tauroolithocholic acid (TLCA), and tauroursodeoxycholic acid (TUDCA) were elevated in mice fed PBEN compared with CEN1 and CEN2 ($n = 4/\text{group}$; ANOVA, Student t test with Bonferroni correction, $P < .05$). Concentration of amino acids (ng/mL per mg of cecal contents) isoleucine, leucine, methionine, and phenylalanine were increased in mice fed CEN1 and CEN2 compared with PBEN (ANOVA, Student t test with Bonferroni correction, $P < .05$). Concentration of short-chain fatty acids (normalized concentration to an internal standard) acetate and butyrate were not significantly different between groups. Propionate was significantly increased in CEN1 compared with PBEN and CEN2 (ANOVA, Student t test with Bonferroni correction, $P < .05$).