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Treatment of Active Crohn's Disease With an Ordinary Food-based Diet That Replicates Exclusive Enteral Nutrition

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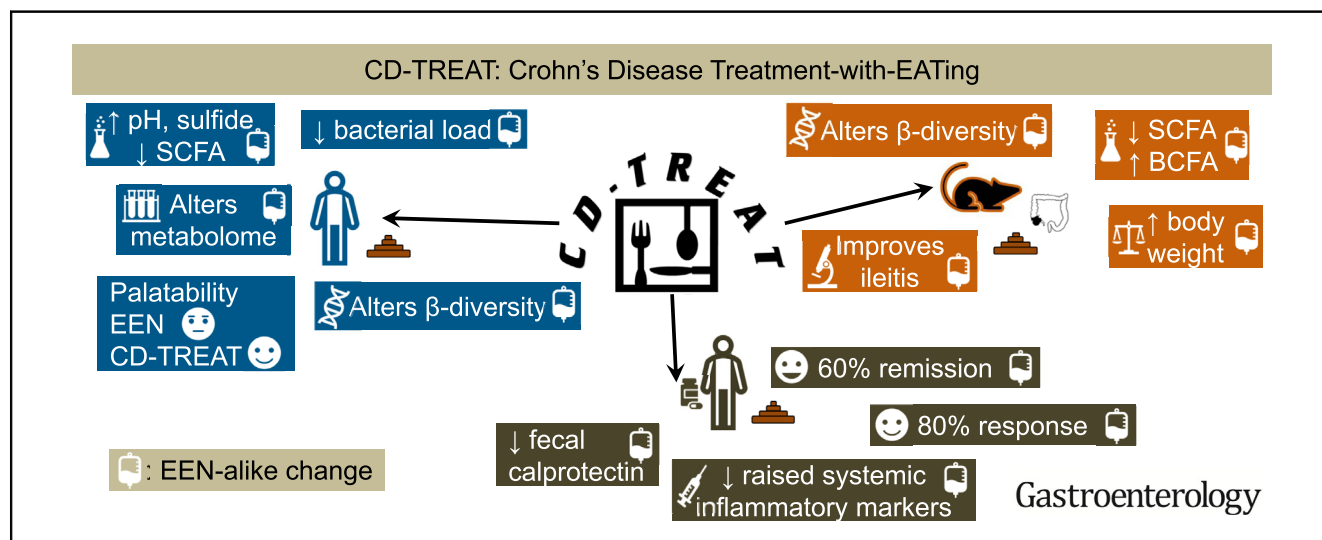
BACKGROUND & AIMS: Exclusive enteral nutrition (EEN) is the only established dietary treatment for Crohn's disease (CD), but its acceptability is limited. There is a need for novel dietary treatments for CD. **METHODS:** We evaluated the effects of an individualized food-based diet (CD-TREAT), with similar composition to EEN, on the gut microbiome, inflammation, and clinical response in a rat model, healthy adults, and children with relapsing CD. Twenty-five healthy adults randomly received EEN or CD-TREAT for 7 days, followed by a 14-day washout period, followed by the alternate diet. Fecal microbiome and metabolome were assessed before and after each diet. HLA-B7 and HLA-B27 transgenic rats with gut inflammation received EEN, CD-TREAT, or standard chow for 4 weeks. Fecal, luminal, and tissue microbiome, fecal metabolites, and gut inflammation were assessed. Five children with active CD activity received CD-TREAT and their clinical activity and calprotectin were evaluated after 8 weeks of treatment. **RESULTS:** For healthy adults, CD-TREAT was easier to comply with and more acceptable than EEN. CD-TREAT induced similar effects to EEN (EEN vs CD-TREAT) on fecal microbiome composition, metabolome, mean total sulfide (increase 133.0 ± 80.5 vs 54.3 ± 47.0 nmol/g), pH (increase 1.3 ± 0.5 vs 0.9 ± 0.6), and the short-chain fatty acids (μmol/g) acetate (decrease 27.4 ± 22.6 vs 21.6 ± 20.4), propionate (decrease 5.7 ± 7.8 vs 5.2 ± 7.9), and butyrate (decrease 7.0 ± 7.4 vs 10.2 ± 8.5). In the rat model, CD-TREAT and EEN produced similar changes in bacterial load (decrease 0.3 ± 0.3 $\log_{10}$ 16S rRNA gene copies per gram), short-chain fatty acids, microbiome, and ileitis severity (mean histopathology score decreases of 1.25 for EEN [$P = .015$] and 1.0 for CD-TREAT [$P = .044$] vs chow). In children

receiving CD-TREAT, 4 (80%) had a clinical response and 3 (60%) entered remission, with significant concurrent decreases in fecal calprotectin (mean decrease 918 ± 555 mg/kg; $P =$ .002). **CONCLUSION:** CD-TREAT replicates EEN changes in the microbiome, decreases gut inflammation, is well tolerated, and is potentially effective in patients with active CD. Clinical-Trials.gov, numbers NCT02426567 and NCT03171246

Crohn's disease (CD) is associated with high morbidity and increased health expenditure. The current paradigm of CD pathogenesis suggests an interaction between environmental factors and the gut microbiome in people with genetic susceptibility to the illness.[1]

Steroids have been the mainstay therapy in CD, particularly in adults. Although effective, prolonged use of steroids is associated with significant side effects and higher mortality compared with anti–tumor necrosis factor (TNF) agents.[2] Given the cost and side effects inherent in biological therapy, there is a need to develop novel, safe, and efficacious therapies.

Diet is important in the etiology of CD, particularly in countries where the rising disease incidence has paralleled changes in eating habits and food industrialization. Certain nutrients and food additives have been associated with CD risk. In consequence, recent studies have advocated the inclusion or exclusion of these food components in CD management.[3]

Exclusive enteral nutrition (EEN) remains the only established dietary treatment in pediatric CD, particularly in Europe. EEN induces clinical remission in approximately 80% of patients and promotes gut healing.[3–5] The mechanism of action of EEN is not yet fully understood, but work from our group and others proposed that modulation of the gut microbiome drives its therapeutic properties.[6–8] These effects reversed when patients resumed their habitual diet and gut inflammation correspondingly increased.[7]

Although successful, EEN is a very restrictive diet with limited acceptability and prolonged use, particularly in adults. Therefore, it is of critical importance that we use our understanding of CD pathogenesis and the mechanism of EEN action to develop new effective dietary therapies that are more acceptable and tolerable. This unmet need is further supported by health professionals and patient groups when asked jointly about priorities for CD research.[9]

In view of the clinical efficacy of EEN, its simple nutritional composition, and a mechanism of action involving the gut microbiome, we hypothesized that we could achieve therapeutic results similar to EEN by developing an ordinary food diet based on the composition of EEN and mimicking its effect on the gut microbiome. If this was achievable, then such a diet would be likely to induce remission in CD. Based on this concept, we devised the CD treatment-with-eating diet (CD-TREAT) and explored microbial changes after CD-TREAT and EEN with a randomized

controlled trial (RCT) in healthy subjects. We followed this RCT with experiments in animal models to explore the anti-inflammatory effect of CD-TREAT and its effect on the gut microbiome in a disease state. Then, we tested the efficacy of CD-TREAT to induce clinical remission and ameliorate blood and colonic inflammatory markers in a pilot trial of children with active CD.

Methods

CD-TREAT Diet

The CD-TREAT is a prescriptive and personalized diet. It recreates EEN by the exclusion of certain dietary components (eg, gluten, lactose, and alcohol) and matching of others (macronutrients, vitamins, minerals, and fiber) as closely as possible using ordinary food. We based the composition of CD-TREAT on Modulen IBD (Nestle, Vevey, Switzerland), the most popular formula used in Europe, and on which we have based our mechanistic studies.[6,7] Maltodextrin, an artificial glucose polymer and the commonest form of carbohydrate in EEN feeds, but not present in natural foods, was substituted by food high in starch and low in fiber. Because approximately 10% of food starch resists digestion (ie, resistant starch) and so reaches the colon,[10] this is likely to influence the gut microbiome in a different way than EEN. Hence, we purposely decreased

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Exclusive enteral nutrition is an effective treatment for patients with Crohn's disease. This study aimed to develop a nutritional therapy program that involved a less-restrictive diet for such patients.

NEW FINDINGS

CD-TREAT, an ordinary food diet, had similar effects to those of exclusive enteral nutrition on the gut microbiome and metabolome of healthy participants; reduced ileitis in a rat model of disease; and reduced disease activity and colonic inflammation in children with active Crohn's disease.

LIMITATIONS

The efficacy of CD-TREAT in patients with active Crohn's disease requires replication in large clinical trials.

IMPACT

CD-TREAT is a diet-only treatment for active Crohn's disease that might be used interchangeably with exclusive enteral nutrition; particularly in adults.

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the carbohydrate in CD-TREAT, particularly complex carbohydrates, in favor of protein. Carbohydrate and protein intake remained within the composition range of other EEN feeds with published records of efficacy in active CD (eg, Fresubin Protein, Fresenius Kabi, Dublin, Ireland; Peptamen AF, Nestle). The micronutrients from EEN were achieved with a multivitamin tablet. CD-TREAT provides the daily energy requirements and considers food preferences. A CD-TREAT daily menu is presented in Supplementary Table S1.

RCT in Healthy Volunteers

Study Design. Healthy adults (>18 years old) were recruited from the community. At study enrollment, subjects who had any acute or chronic illness (ie, illness requiring regular visits to health services) were excluded. Participants had stable weight (±2 kg) in the past month, no history of gut surgery, and had not used antibiotics or steroids during the previous 3 months.

Participants were randomly allocated to EEN and CD-TREAT for 7 days each, with a 14-day washout period in between to restore the gut microbiome to its baseline state and avoid intervention contamination bias (Figure 1A).

Modulen IBD was used for the EEN trial, as described previously.[11] During CD-TREAT, participants were provided with a food list from which to choose their preferred items. An individualized dietary plan was developed by the research dietitians providing their daily energy requirements. This plan was developed using the food group exchange methodology[12] and WinDiets 2010 (https://windiets.software.informer.com/). Energy requirements were calculated using the participant's estimated basic metabolic rate and self-reported physical activity level.[13] EEN and CD-TREAT meals were supplied to participants free of charge along with written preparation instructions.

Before initiation of the first experimental diet, participants recorded their habitual diet using 7-day estimated weight food diaries. This diet was replicated during the last week of the washout, before their second intervention. Weight changes were monitored.

Dietary Intake and Acceptability of Interventions. Appetite, gastrointestinal symptoms, and adherence to the experimental diets were assessed by self-reported questionnaire (Supplementary Files S1 and S2). Leftovers of EEN formula were returned as additional estimates of treatment adherence. Adherence to CD-TREAT was further assessed by recording intake daily using estimated weight food diaries.

Fecal Sample Collection

Fresh fecal samples were collected before and after each experimental diet, providing a total of 4 samples per participant (Supplementary File S2). The shape and texture of the samples was assessed using the Bristol Stool Form Scale.[14] Fecal water, pH, and ammonia were measured.[6]

Fecal Microbiome. Fecal genomic DNA was extracted using the chaotropic method.[6] Quantification of total bacteria (16S rRNA gene copy number per gram of stool) was carried out with quantitative polymerase chain reaction using TaqMan (ThermoFisher Scientific, Waltham, MA) chemistry.[6] The V4 region of the 16S rRNA gene was amplified and sequencing was

performed (MiSeq, Illumina, Essex, UK) using 2- × 250-bp paired-end reads.[7]

Fecal Bacterial Metabolites. Free and total fecal sulfide was measured with a solid-state ion selective electrode (HI4115; Hanna, Bedfordshire, UK). Short-chain fatty acids (SCFAs) and branched-chain fatty acids were quantified by gas chromatography.[6]

Fecal Metabolome. Untargeted fecal metabolites were measured using liquid chromatography–mass spectrometry (Supplementary File S2) in chloroform, methanol, and water (1:3:1) extracts. Metabolite profiling was performed using a SeQuant ZIC-pHILIC column (Hichrom Ltd, Reading, UK) coupled to an Orbitrap Exactive instrument (ThermoFisher Scientific).
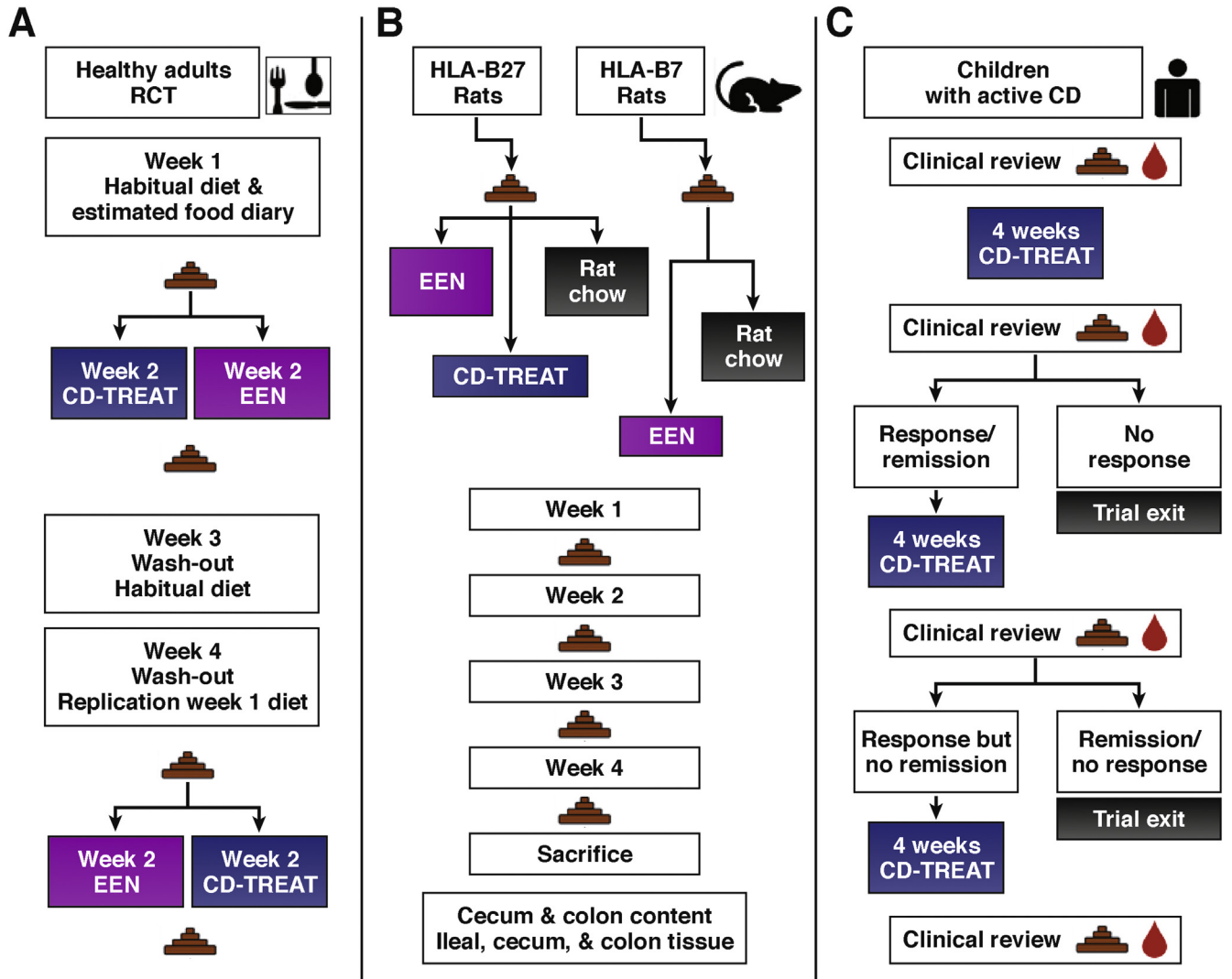
Animal Experiments

Study Design. The animal experiment explored the effect of CD-TREAT, compared with EEN and regular chow, on gut inflammation and microbiome in a disease state. We used adult (36- to 40-week-old) heterozygous HLA-B27 (B27) and HLA-B7 (B7) transgenic rats of a Piebald-Virol-Glaxo background. B27 rats express the human major histocompatibility complex class I HLA-B27 gene and the associated human β_2-microglobulin gene and develop inflammation throughout their gastrointestinal tract, including the ileum.[15] They do not present gut inflammation in a germ-free environment[16] and severity of ileitis is decreased by antibiotic treatment.[17] This model has been used extensively in inflammatory bowel disease research, including interventions with EEN.[18] The B27 gut microbiome has a similar phenotype to that seen in CD, including an increase in *Proteobacteria* and a decrease in *Firmicutes* species.[19] The HLA-B27 genotype has been associated independently with CD, and patients with ankylosing spondylitis often have subclinical gut inflammation.[20] B7 rats do not present gut inflammation and were used as controls.

The B27 animals were fed ad libitum with EEN, CD-TREAT, or standard chow (BK001E, Special Diets Services, Essex, UK) for 4 weeks (B27-EEN, B27-CD-TREAT, and B27-CONTROL, respectively). To confirm gut inflammation in the B27 animals, a healthy group of B7 rats fed on standard chow was included (B7-CONTROL). Similarly, B7 rats on EEN were included to address the net effect of EEN on their gut microbiome in the absence of inflammation (B7-EEN). A B7 group on CD-TREAT was not included because the effect of CD-TREAT against EEN on the healthy gut microbiome was addressed in the human RCT (Figure 1B). CD-TREAT food items were cooked, homogenized, and stored at −20°C. Rats were maintained in individual cages and under specific pathogen-free conditions. Weight was monitored.

Fecal Samples, Luminal Contents, and Tissue Harvesting. Fecal samples were collected at baseline and weekly until sacrifice after 28 days. Gut luminal contents and ileal, cecal, and colonic tissue were stored at −70°C. The weights of cecal luminal content and fecal water were measured.

Gut Histopathology and Cytokine Expression in Ileum. Colonic and ileal specimens were fixed in 10% formalin until the tissue was embedded in paraffin, stained with hematoxylin and eosin, and evaluated by 2 pathologists (R.K. and J.S.) in a blinded manner (Supplementary File S2).

Figure 1. (*A*) RCT in healthy volunteers. (*B*) Animal experiments. (*C*) Open-label trial in patients with active CD.

Total RNA was isolated from ileal tissues and stored immediately in RNAlater using the AllPrep DNA/RNA Mini kit (Qiagen, Manchester, UK). Reverse transcription was performed using the QuantiTect Reverse Transcription kit (Qiagen). Relative expression of genes encoding for TNF-α, interleukin (IL)-6, IL-10, IL-1b, and CXCL-1 was measured with quantitative polymerase chain reaction using TaqMan chemistry, housekeeping genes as endogenous controls, and the comparative threshold cycle method (Supplementary File S2).

Gut Microbiome. DNA from fecal, cecal, and colonic luminal contents was extracted as described earlier. DNA from cecal and colonic tissue was extracted using the AllPrep DNA/RNA Mini Kit. The 16S rRNA gene sequencing in all samples and the concentration of total bacteria and SCFAs in fecal samples and cecal luminal contents were measured as described earlier. The total quantity (micromoles) of each SCFA and branched-chain fatty acid produced in the entire cecal luminal content also was calculated.

Open-Label Trial in Patients With Active CD

Study Design. Children (6–15 years old) previously diagnosed with relapsing CD (weighted Pediatric Crohn's Disease Activity Index [wPCDAI] score $\geq$ 12.5[21]) were recruited from the Royal Hospital for Children (Glasgow, UK). Exclusion criteria included antibiotic use within 1 month; change in type or dose of CD therapy within 3 months or 1 month, respectively; enrollment in other studies; disease severity sufficient to warrant hospitalization; and food allergies incompatible with CD-TREAT.

Participants were treated exclusively with CD-TREAT for 8 weeks. All food was provided. In contrast to the healthy volunteer RCT, meals requiring cooking were prepared by a subcontracted catering company and were delivered to the participants (Supplementary Figure S1). Participants recorded their dietary intake daily (Supplementary File S3).

Clinical reviews were scheduled at weeks 2 (telephone) and 4 and 8 (visits). No alterations to CD-related medications were allowed. If disease activity deteriorated at any point or there was no improvement at 4 weeks, then patients discontinued CD-TREAT. Those patients who clinically responded but did not enter clinical remission at 8 weeks were offered the option to stay on CD-TREAT for a further 4 weeks (Figure 1*C*).

Primary and Secondary Outcomes. The outcomes of the trial were selected based on the recommendations of the

European Crohn's and Colitis Organization.[22] The primary outcome was clinical response (wPCDAI score decrease $\geq$ 17.5) or clinical remission (wPCDAI score < 12.5) at 8 weeks. Secondary outcomes included changes in fecal calprotectin (FC; CALP0170 kit, Calpro AS, Lysaker, Norway), serum albumin, and C-reactive protein.[6]

Bioinformatics

Operational taxonomic unit (OTU) assignments were generated using a modified version of the VSEARCH pipeline (https://github.com/torognes/vsearch/wiki/VSEARCH-pipeline; Supplementary File S2). For fecal metabolome, we used probabilistic quotient normalization[23] for univariate differential analysis (muma package; https://cran.r-project.org/web/packages/muma/index.html) and square-root transformation and Pareto-scaling[24] for multivariate analysis.

Statistics

Power Calculation. A confirmed microbial signal explaining the EEN therapeutic effect does not exist. Hence, to power the human RCT, we chose a microbial signal that changed significantly during EEN in children with CD, was associated with disease improvement, and showed the largest size effect with the narrowest standard deviation (SD).[6] Recruiting 25 healthy adults would produce 80% power to detect a mean fecal butyrate decrease of 19 μmol/g (SD 32) after either dietary intervention. The power calculation was revised after completion of the first 10 participants (Supplementary File S2). Animal experiments were performed at 2 independent occasions (4 rats per group).

Data Analysis. For group comparisons, we used the general linear model with Box-Cox transformation and Fisher pairwise test. For microbiome, α-diversity (Shannon and Chao1) and β-diversity (Bray-Curtis dissimilarity and weighted UniFrac distance) were performed (vegan package) in R. Discriminant taxa were identified using t tests on logarithmic proportional abundances. For metabolome data, principal component analysis and permutational multivariate analysis of variance were carried out on Manhattan distance matrices. Differentially abundant metabolites were identified using a modification of the univariate function in the muma package, adapted for paired data. All correlations were tested with the Pearson test; but for the metabolome analysis, the Spearman rank correlation test was used instead to compensate for the disproportionate influence of outliers. The significance level for the human RCT and pilot trial in patients was defined as $P <$ .05. Benjamini-Hochberg corrections for multiple testing were applied, relaxing the criteria to $P <$ .1 in the animal experiments in which samples were small (n = 4 per group).

Ethical Considerations

The RCT in healthy volunteers was approved by the ethics committee of the University of Glasgow (Glasgow, UK; reference 200130161) and the trial in patients was approved by the West of Scotland Research Ethics Committee (reference 17/WS/0119). Participants and/or caregivers provided written consent. The animal experiments were conducted under licenses by the UK Home Office.

Results

RCT in Healthy Volunteers

Participant Characteristics. Twenty-eight volunteers were recruited. Three dropped out, leaving 25 subjects who completed the trial (mean body mass index 22.4 kg/m^2, SD 2.7; mean age 24.5 years, SD 3.1; 13 women [52%]). Thirteen and 12 were randomly allocated to start with EEN and CD-TREAT, respectively. No significant changes in body weight were observed (Supplementary Table S2).

Dietary Intake and Acceptability of Interventions. Differences between the prescribed and actual intake were minimal during the 2 dietary interventions, indicating high compliance (Table 1). Nutrient intake during CD-TREAT was more similar to that of EEN than to the participants' habitual diet. During EEN and CD-TREAT, the participants consumed more total and saturated fat and less fiber and carbohydrates compared with their habitual diet. Protein intake was higher than the habitual diet during CD-TREAT.

CD-TREAT was easier to follow and more satiating than EEN. Gastrointestinal symptoms were uncommon for the 2 diets. Conversely, ratings for abdominal pain and diarrhea were higher ($P =$ .005 for the 2 comparisons) for EEN than for CD-TREAT (Supplementary Table S3).

Fecal Sample Characteristics. The 2 dietary interventions decreased Bristol Stool Chart ratings ($P <$ .001 for the 2 comparisons) and fecal output mass (EEN, $P =$ .031; CD-TREAT, $P =$ .017). Fecal water content decreased only after EEN ($P =$ .003; Supplementary Table S2).

Fecal Microbiome. The concentration of total fecal bacteria significantly decreased with the 2 diets (Supplementary Table S2). Ninety-six samples from 24 participants were analyzed after removing 4 samples with <5,000 reads. The number of unique OTUs across all samples was 1176, annotated to 224 genera. No significant changes from baseline were observed for the Chao1 index ($P =$ .143 for the 2 comparisons), a measure of microbiome richness, or the Shannon α-diversity index (EEN, $P =$ .387; CD-TREAT, $P =$ .359; Figure 2A and B). Microbiome structure clustered according to study time point ($R^2 = 0.13$, $P <$ .001; Figure 2C). Compared with baseline, EEN ($R^2 = 0.14$, $P <$ .001) and CD-TREAT ($R^2 = 0.05$, $P <$ .001) shifted the β-diversity index to the same direction, explaining 14% and 5% of the variation in microbiome structure, respectively. Weighted UniFrac distance analysis produced similar results (Supplementary Figure S2).

After filtering out OTU with abundance <0.01%, 329 OTUs remained, aggregated to 118 genera. A moderate to strong correlation between the changes after EEN and CD-TREAT was observed for OTU (r = 0.67, $P <$ .001; Figure 2D) and for genera (r = 0.65, $P <$ .001; Supplementary Figure S3).

The relative abundance of 58 (49.3%) and 38 (32.3%) genera changed significantly after EEN and CD-TREAT, respectively. Twenty-eight of these genera changed in the same direction for the 2 dietary interventions (Table 2). Therefore, 48.3% of the EEN effect was replicated by CD-TREAT and 73.7% of the CD-TREAT effect was observed

Table 1. Dietary Intake in the RCT in Healthy Volunteers (n = 25) and Open-Label Trial in Children With Active CD (n = 5)

	RCT in healthy volunteers					Open-label trial in patients with CD	
	Habitual diet	EEN actual intake	CD-TREAT actual intake	EEN prescribed	CD-TREAT prescribed	CD-TREAT actual intake	CD-TREAT prescribed
Energy (kcal)	2093 (497)	2234 (428)	2407 (344)[a,e]	2183 (363)	2261 (282)	2298 (222)	2451 (519)
Energy (% TEE)	96.8 (21.44)	103.4 (16.7)	111.9 (14.8)[a,e]	100.4 (3.3)	104.9 (9.1)	98.7 (12.6)	103.1 (8.0)
Fat (%)	33.9 (4.6)	40.3 (0.8)[a,f]	42.6 (3.3)[a,b,f]	41.4 (0.0)[a,f]	43.9 (0.9)[a,b,f]	39.8 (3.9)	41.4 (2.5)
Saturated fat (%)	12.2 (2.7)	22.8 (0.5)[a,c,f]	21.4 (2.1)[a,b,f]	23.4 (0.0)[a,f]	21.2 (0.6)[a,b,f]	22.7 (2.8)	23.0 (1.9)
Protein (%)	13.6 (1.9)	14.2 (0.2)	19.0 (1.8)[a,b,f]	14.4 (0.0)[a,d]	18.3 (0.7)[a,b,f]	17.5 (2.4)	18.2 (0.8)
Carbohydrates (%)	49.9 (4.4)	45.4 (1.0)[a,c,e,f]	38.2 (3.2)[a,b,f]	43.2 (0.0)[a,f]	37.9 (0.4)[a,b,f]	41.1 (3.4)	39.0 (3.1)
Fiber (g/1000 kcal)	7.8 (2.2)	0.0 (0.0)[a,f]	4.7 (1.0)[a,b,f]	0.0 (0.0)[a,f]	4.2 (0.3)[a,b,f]	4.5 (0.4)	5.1 (0.8)
Gluten free	No	Yes	Yes	Yes	Yes	Yes	Yes
Lactose free	No	Yes	Yes	Yes	Yes	Yes	Yes
Alcohol free	No	Yes	Yes	Yes	Yes	Yes	Yes

NOTE. Data are displayed as mean (standard deviation).
TEE, total energy expenditure.
[a]Significantly different than "habitual diet."
[b]Significantly different than "EEN."
[c]Significantly different than "prescribed."
[d]$P < .05$.
[e]$P < .01$.
[f]$P < .001$ for Fisher pairwise comparisons after general linear modeling with Box-Cox transformation.

in EEN. Thirty genera changed significantly only during EEN and 10 changed only during CD-TREAT (Supplementary Tables S4–S6).

Similar findings were observed from OTU assignments. In total, 109 OTUs (33.1%) for EEN and 64 OTUs (19.5%) for CD-TREAT changed significantly compared with baseline. The relative abundance of 47 OTUs was similarly altered after EEN and CD-TREAT, indicating that 43.1% of the EEN-induced OTU changes were replicated by CD-TREAT and 73.4% of CD-TREAT-induced OTU changes were observed in EEN. The OTUs that changed significantly during only EEN or CD-TREAT were 62 or 17, respectively
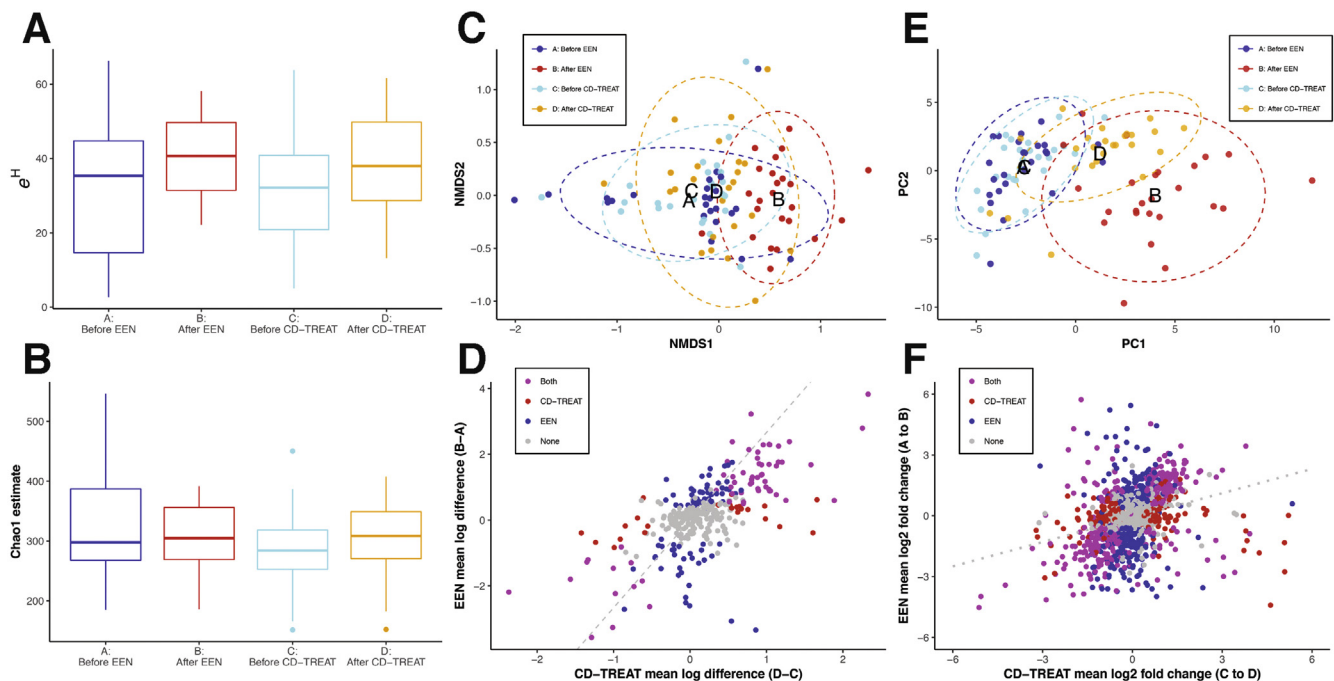
Figure 2. Effect of experimental diets on the gut microbiome of healthy volunteers (n = 24). (A) Exponential of Shannon index (e^H). (B) Chao1 index. (C) NMDS using Bray-Curtis distances of OTU community structure. (D) Correlation of OTU abundance changes. (E) Principal component analysis of annotated metabolites. (F) Correlation of annotated metabolite changes. NMDS, nonmetric multidimensional scaling; PC, principal component.

Table 2. Mean Difference in Relative Abundance ($\log_{10}$) of Genera and Fold Change ($\log_2$) in Metabolites That Similarly ($P < .05$) Changed After EEN and CD-TREAT

	EEN	Adjusted P value	CDT	Adjusted P value
Genera increased during the 2 diets				
Actinobacteria				
Actinomyces	1.67	<.001	0.85	.003
Eggerthella	2.06	<.001	0.90	.001
Senegalimassilia	1.02	.008	0.63	.015
Bacteroidetes				
Alistipes	1.01	.012	0.67	.024
Butyricimonas	1.16	.020	0.76	.014
Prevotella	1.83	<.001	0.57	.030
Firmicutes				
Anaerofilum	1.65	<.001	0.93	.003
Anaerotruncus	1.21	<.001	1.32	<.001
Candidatus Soleaferrea	1.09	<.001	0.71	.014
Eisenbergiella	2.61	<.001	2.17	<.001
Family XIII AD3011 group	0.99	<.001	0.80	<.001
Flavonifractor	2.15	<.001	0.95	.003
Hungatella	1.72	<.001	1.04	.002
Lachnoclostridium	2.20	<.001	0.63	.002
Oscillibacter	1.97	.001	1.02	.006
Ruminiclostridium 5	0.96	.002	0.70	.014
Tyzzerella 4	1.68	<.001	1.14	.015
Proteobacteria				
Bilophila	0.80	.024	1.14	.002
Escherichia, Shigella	1.71	<.001	1.59	.001
Genera decreased during the 2 diets				
Actinobacteria				
Bifidobacterium	−1.66	<.001	−1.51	<.001
Firmicutes				
Dialister	−2.39	<.001	−1.00	.014
Faecalibacterium	−1.82	<.001	−0.63	.011
Lachnospiraceae FCS020 group	−0.85	.029	−0.62	.001
Lachnospiraceae UCG-004	−2.18	<.001	−0.95	.011
Pseudobutyrivibrio	−3.35	<.001	−1.27	.002
Ruminiclostridium 6	−1.25	.004	−1.42	.018
Ruminococcaceae UCG-013	−1.94	<.001	−0.83	.011
Ruminococcus 1	−3.00	<.001	−3.78	<.001
Top 25 metabolites changed during the 2 diets (ranked by EEN change in *P*)				
Metabolism of lipids				
3a,7a,12a-Trihydroxy-5b-cholestanoate	1.76	<.001	1.21	<.001
3a,7a-Dihydroxy-5b-cholestanate	1.48	<.001	1.26	<.001
FA hydroxy(20:4)	1.70	<.001	0.64	.023
DG(15:0/20:4[5Z,8Z,11Z,14Z]/0:0)	2.73	<.001	1.75	<.001
DG(15:0/18:2[9Z,12Z]/0:0)	−2.95	<.001	−1.41	.042
ST hydroxy(3:2/3:0/3:0)	2.01	<.001	1.06	.001
11-Acetoxy-3b,6a-dihydroxy-9,11-seco-5a-cholest-7-en-9-one	1.56	<.001	1.21	<.001
ST hydroxy(4:0)	1.45	<.001	1.23	<.001
26-Hydroxycholesterol 3-sulfate	2.07	<.001	1.47	<.001
Metabolism of proteins				
Ala-Leu-Gln-Gln	3.43	<.001	3.79	<.001
Lys-Ala-Gln	−4.52	<.001	−5.12	<.001
N2-acetyl-L-aminoadipate	−3.85	<.001	−1.63	.006
N-succinyl-LL-2,6-diaminoheptanedioate	−3.49	<.001	−1.04	.010
Metabolism of cofactors or vitamins				
(1R,6R)-6-hydroxy-2-succinylcyclohexa-2,4-diene-1-carboxylate	−2.75	<.001	−1.24	<.001
Biosynthesis of secondary metabolites				
Antheraxanthin	−2.88	<.001	−1.73	.002

Table 2. Continued

	EEN	Adjusted P value	CDT	Adjusted P value
Unknown				
Crotanecine	−2.42	<.001	−1.05	<.001
Glutamylalanine	−2.14	<.001	−0.90	.002
N-(3-hydroxybutanoyl)-L-homoserine	−2.34	<.001	−0.84	.001
Formylpyruvate	−2.44	<.001	−0.50	.018
6-hydroxy-indole-3-acetyl-valine	1.53	<.001	0.68	.001
Evodone	1.83	<.001	0.73	<.001
Androstane-3,17-diol dipropionate	2.68	<.001	1.18	<.001
13′-hydroxy-&a;-tocopherol	2.03	<.001	1.30	<.001
(24R,24′R)-fucosterol epoxide	2.10	<.001	1.17	<.001
N-acetyl-L-histidine	−2.87	<.001	−1.45	<.001

NOTE. For the genera analysis, 96 samples from 24 healthy humans were used; for metabolite analysis, 100 samples from 25 healthy volunteers were used. P values were analyzed by paired t test or Wilcoxon signed-rank test.

(Supplementary Tables S7–S9). No OTU or genera demonstrated a significant change in the opposite direction when comparing EEN and CD-TREAT.

Fecal Bacterial Metabolites. Fecal pH increased to alkaline levels, with a mean increase of 1.3 units during EEN ($P < .001$) and 0.9 unit during CD-TREAT ($P < .001$). Similarly, the fecal concentration of total sulfide increased after the 2 dietary interventions (EEN, 213%; CD-TREAT, 109%; $P < .001$ for the 2 comparisons); free sulfide decreased only after EEN. The mean concentrations of acetate, propionate, and butyrate significantly decreased after EEN and CD-TREAT, with a similar effect size observed between the 2 diets. Branched-chain fatty acids increased only after EEN and valerate decreased only after CD-TREAT (Supplementary Table S2).

Fecal Metabolome. The number of annotated metabolites across all 100 samples was 886. The metabolome significantly changed toward the same direction after EEN ($R^2 = 0.21$, $P < .001$) and CD-TREAT ($R^2 = 0.13$, $P < .001$), with 21% of the variation in the metabolome profile explained by the 4 study time points ($P < .001$; Figure 2E). The $\log_2$-fold baseline changes of metabolite abundance were correlated between EEN and CD-TREAT (Spearman $\rho = 0.38$, $P < .001$; Figure 2F).

Of the 886 annotated metabolites, 675 (76.2%) and 573 (64.7%) changed significantly after EEN and CD-TREAT, respectively (Supplementary Table S10). Two-hundred ninety-four metabolites changed in the same direction after the 2 dietary interventions. Therefore, 43.6% of the EEN effect was replicated by CD-TREAT and 51.3% of the CD-TREAT effect was observed in EEN. Most of these metabolites were associated to metabolism of lipids (n = 90, 30.6%), proteins (n = 47, 16.0%), carbohydrates (n = 8, 2.7%), nucleotides (n = 5, 1.7%), cofactors or vitamins (n = 7, 2.4%), biosynthesis of polyketides and nonribosomal peptides (n = 4, 1.4%), and secondary metabolites (n = 9, 3.1%; Supplementary File S4). The 25 most statistically significant metabolites ranked by P value for EEN change are presented in Table 2. Metabolites that changed significantly in only one of the diets are presented in

Supplementary File S4. Sixty-eight (7.7%) had changes in the opposite direction for the 2 diets.

For all analyses performed, there were no significant differences in the microbiome of the 2 pretreatment diets (ie, pre-EEN and pre–CD-TREAT), indicating that the washout period and replication of the pre-intervention diet reverted the microbiome to the same baseline characteristics (metabolome: $R^2 = 0.01$, $P = .391$; microbiome: $R^2 = 0.01$, $P = .187$; Figure 2C and E).

Animal Experiments

Animal Characteristics. Twelve B27 and 8 B7 rats were included. Each group included 2 male and 2 female rats with no group differences in weight (mean 285.4 g, SD 80.3). Only animals on the experimental diets, including B27-EEN, B27-CD-TREAT, and B7-EEN, gained weight (Supplementary Table S11).

The B27-CONTROL group had higher colonic and ileal inflammation and fecal water content than the B7-CONTROL group (Figure 3, Supplementary Figure S4, Supplementary Tables S11 and S12). The B27-CONTROL microbiome was characterized by compositional and functional dysbiosis, including decreased fecal, cecal bacterial diversity, and butyrate production (Figure 4, Supplementary Tables S11 and S13–S17, Supplementary Figure S5).

Gut Histopathology and Cytokine Expression in Ileum. The histopathology scores were highly correlated between the 2 assessors (ileum: Spearman $\rho = 0.76$, $P < .001$; colon: Spearman $\rho = 0.84$, $P < .001$). Individual components of the histopathology scoring are presented in Supplementary Table S12 and representative sections are presented in Figure 3B, Supplementary Figure S4, and Supplementary File S5. EEN and CD-TREAT ameliorated ileitis compared with the B27-CONTROL group (Figure 3; $P = .015$, $P = .044$). No such effect was observed for colonic inflammation. There was no effect of EEN on B7 gut inflammation.

Expression of genes encoding for TNF-α, IL-6, IL-10, and CXCL-1 in the ileum did not differ significantly between the

A

B
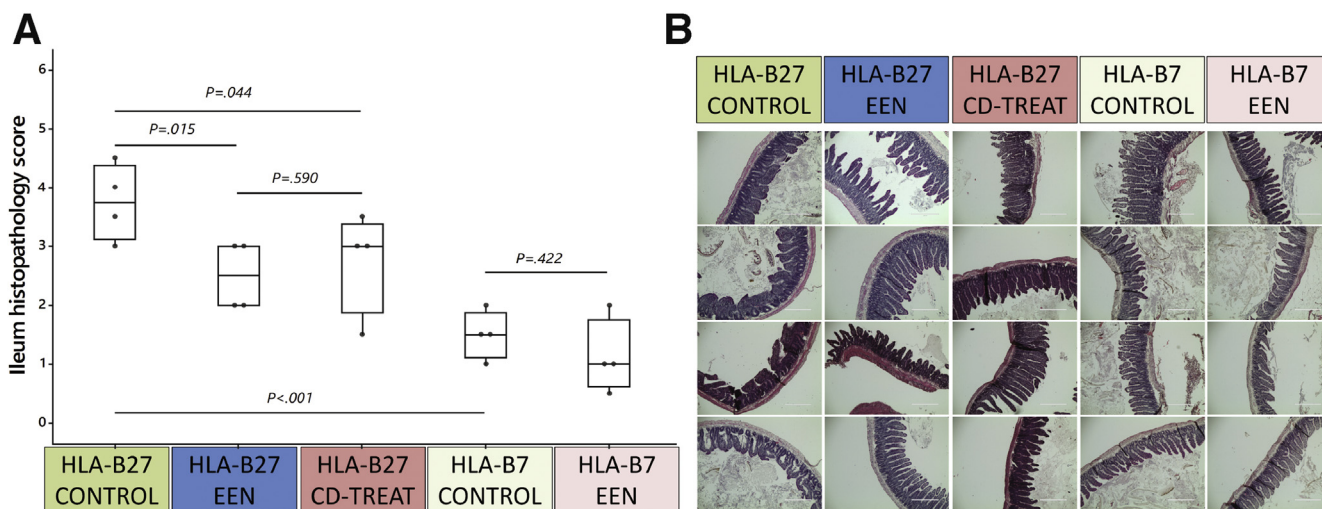
Figure 3. (*A*) Rat ileal histopathology scores. (*B*) Representative sections of all (n = 20) animal groups (*P* values for Fisher pairwise comparisons by general linear model).

B7-CONTROL and B27-CONTROL groups despite the higher average values observed in the latter group. When we looked at the effect of the experimental diets, the B27-CD-TREAT group had lower mean expression of IL-6 than the B27-CONTROL group (*P* = .036), and although CXCL-1 also was lower, this did not reach significance (*P* = .089). Regardless of intervention, fecal water content did not change in the B27 rats. A significant increase was observed in the B7-EEN group (Supplementary Tables S11 and S12).

Gut Luminal Content Characteristics. The 2 experimental diets decreased the weight of cecal luminal contents in the B27 animals (EEN, *P* < .001; CD-TREAT, *P* = .017 compared with B27-CONTROL). This effect also was observed in the colon of the B27-EEN group (*P* = .040) and in the cecum and colon of the B7-EEN group (Supplementary Table S13).

Gut Microbiome. The B27-EEN and B27-CD-TREAT groups had a higher concentration of total bacteria in cecal luminal contents and in fecal samples than the B27-CONTROL group (Supplementary Table S13). Rat microbiome was characterized in 100 fecal, 20 cecal, and 17 colonic luminal contents and 19 cecal and 10 colonic tissue samples after removing samples with low reads (<5000). There were 1072 unique OTUs and 218 unique genera.

The 2 experimental diets shifted the B27 fecal and cecal luminal microbiome structures in the same direction. Microbiome signatures were significantly different than the B27-CONTROL group in fecal samples (EEN: R^2 = 0.45; CD-TREAT: R^2 = 0.43; *P* = .034 for the 2 comparisons) and cecal luminal contents (EEN: R^2 = 0.47; CD-TREAT: R^2 = 0.54; *P* = .029 for the 2 comparisons; Figure 4*C* and *D*). The effect of EEN and CD-TREAT on the B27 fecal microbiome was already evident from the first week of intervention (EEN: R^2 = 0.57; CD-TREAT: R^2 = 0.58; *P* = .030 for the 2 comparisons). A similar effect was observed for the B7-EEN group compared with the B7-CONTROL group (fecal: R^2 = 0.43, *P* = .034; cecal: R^2 = 0.54, *P* = .029). Nonmetric multidimensional scaling plots for the microbiome of cecal

tissue, colon tissue, and colon luminal contents are presented in Supplementary Figure S5. Analysis using weighted UniFrac distances produced similar results (Supplementary Figures S6 and S7).

After the 4-week intervention, the B27-CD-TREAT group had a higher Shannon α-diversity index in feces (*P* = .020) and cecal luminal contents (*P* = .006) than the B27-CONTROL group. A similar pattern of effect was observed for the B27-EEN group, but did not reach significance (Figure 4*A* and *B*).

After filtering out OTUs with abundance <0.01%, 92 genera remained at baseline, 109 at 7 days and 105 at 28 days of intervention in fecal samples, and 104 genera were assigned to cecal luminal contents. To assess the effect of the experimental diets on B27 microbiome, we compared the genus relative abundance of fecal samples and cecal luminal contents for the B27-EEN and B27-CD-TREAT groups against the B27-CONTROL group. Of the 105 genera tested in cecal luminal contents, the relative abundance of 27 (25.7%) for the B27-EEN group and of 25 (23.8%) for the B27-CD-TREAT group significantly differed compared with the B27-CONTROL group. The relative abundance of 13 genera responded similarly for EEN and CD-TREAT, indicating that 48% of EEN-induced changes were replicated by CD-TREAT and 52% of CD-TREAT–induced changes were observed in EEN. Twenty-six genera changed in the opposite direction between CD-TREAT and EEN (Supplementary Tables S18–S20). Similar effects were observed in fecal samples (Supplementary Tables S21–S26) and for OTU assignments (Supplementary Tables S27–S35), although the small sample precluded robust statistical analysis.

Gut Bacterial Metabolites. The B27-EEN and B27-CD-TREAT groups had higher cecal concentrations of valerate, isobutyrate, and isovalerate (*P* < .001 for all comparisons) compared with the B27-CONTROL group. In addition, the B27-CD-TREAT group had more butyrate (*P* = .004) and propionate (*P* = .021) in the cecum, whereas the B27-EEN group had lower acetate (*P* < .001) and
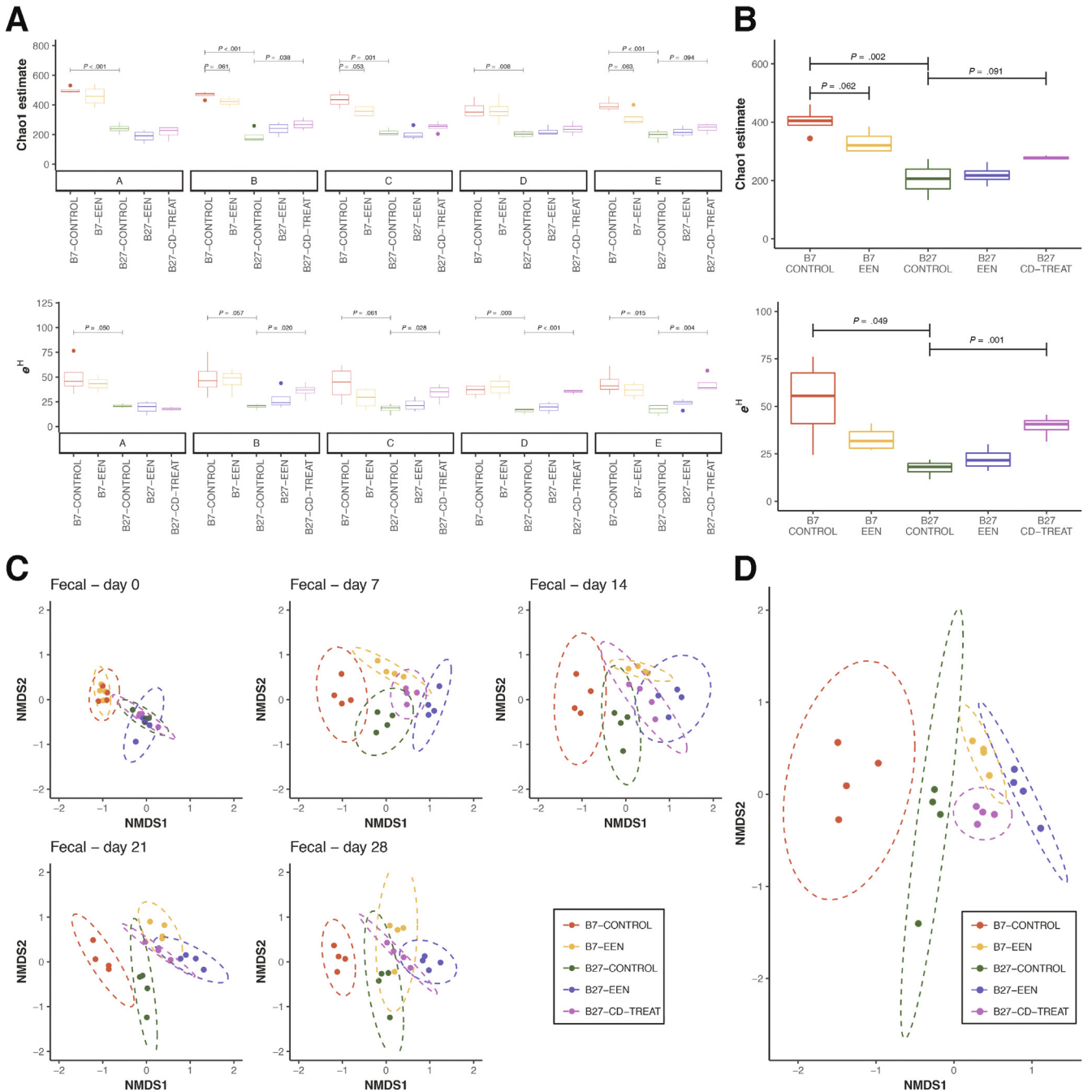
Figure 4. Chao1 and exponential of Shannon index (e^H) of (*A*) rat fecal microbiome during experimental diets and (*B*) rat cecal luminal content microbiome. NMDS using Bray-Curtis distances of OTU community structure of (*C*) rat fecal microbiome during experimental diets and (*D*) rat cecal luminal content microbiome in all animals (n = 20). NMDS, nonmetric multidimensional scaling.

propionate ($P = .003$). For total amount of SCFA in the entire cecum, a more reliable proxy of net production, in the B27 groups, EEN decreased acetate, propionate, and butyrate ($P < .001$ for all comparisons) and increased isobutyrate ($P = .028$) and, to a lesser extent, isovalerate ($P = .057$). CD-TREAT increased isobutyrate ($P < .001$) and isovalerate ($P = .001$) and tended to decrease ($P = .096$) total cecal amount of acetate. In the B7 groups, the effect of EEN on cecal concentration and total SCFA production was similar to that of the B27 groups (Supplementary Table S13).

In support of these data, sequential data from the weekly fecal sample collection showed that EEN and CD-TREAT significantly decreased the concentrations of acetate and propionate and increased those of isobutyrate and isovalerate in B27 groups. Similar effects were observed for the B7-EEN group (Supplementary Table S11).
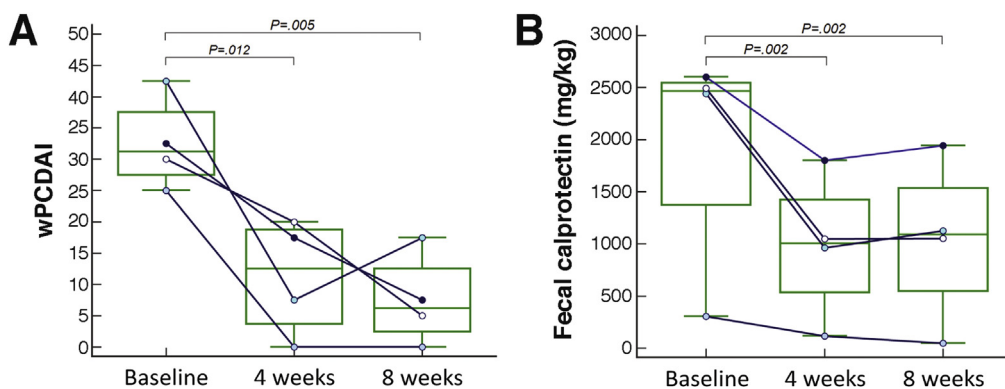
Figure 5. (A) wPCDAI score and (B) FC in children with CD at study enrollment and at 4 and 8 weeks on CD-TREAT (P values for Fisher pairwise comparisons by general linear model).

CLINICAL AT

Open-Label Trial in Patients With Active CD

Five children with mild to moderate active luminal disease (wPCDAI score 22.5–42.5) were enrolled from October 2017 to April 2018 and followed up until June 2018. All had been treated previously with EEN. Three patients were on monotherapy with thiopurine, 1 was on combination therapy with thiopurine and anti-TNF, and 1 had no background treatment (Supplementary Table S36). Compliance to CD-TREAT was high (Table 1). Four children (80%) completed 8 weeks of treatment and 1 child discontinued CD-TREAT after 9 days because of symptom exacerbation.

After 4 weeks on CD-TREAT, 60% (3 of 5) clinically responded (wPCDAI score change > 17.5) and 40% (2 of 5) were in clinical remission (wPCDAI score < 12.5). At the end of 8 weeks of treatment, 80% (4 of 5) clinically responded and 60% (3 of 5) entered clinical remission. In the 4 children who completed 8 weeks of CD-TREAT, the wPCDAI score decreased from a baseline mean of 32.5 (SD 7.5) to 11.3 (SD 9.2) and 7.5 (SD 7.4) at 4 (P = .012) and 8 (P = .005) weeks, respectively (Figure 5A and Supplementary Table S37). One patient had increased C-reactive protein and decreased albumin at CD-TREAT initiation; these inflammatory markers normalized by treatment completion. All 4 patients who completed 8 weeks on CD-TREAT had increased FC (range 307–2602 mg/kg) at treatment initiation (Figure 5B and Supplementary Table S36). By weeks 4 and 8 on CD-TREAT, the baseline concentration of FC significantly decreased by 53% and 55%, respectively (baseline: mean 1960 mg/kg, SD 1104; 4 weeks: mean 981 mg/kg, SD 690; 8 weeks: mean 1042 mg/kg, SD 776). In 1 patient (25%), FC decreased to normal (<50 mg/kg) levels (Figure 5B). Interestingly, FC also decreased in the patient who discontinued CD-TREAT after 9 days (2026 mg/kg at baseline vs 1072 mg/kg at 9 days).

Discussion

This series of studies aimed to develop an ordinary food-based therapy for active CD using EEN as an exemplar of effective nutritional therapy. EEN is likely to work by exclusion or inclusion of dietary components that modify an inflammatory microbiome.[8] The specific dietary components and how these interact with the gut microbiome are not yet fully elucidated. In the absence of detailed knowledge, we developed CD-TREAT, a personalized and tolerable diet with comparable composition to EEN. We anticipated that CD-TREAT would induce similar broad-spectrum alterations in the gut microbiome and provide similar efficacy in treating gut inflammation and improving clinical activity.

Our RCT in healthy volunteers confirmed the a priori hypothesis that CD-TREAT would induce similar effects on the human microbiome as EEN. Applying multifaceted, targeted, and untargeted multi-omics methodology, we interrogated changes in microbiome profiling and metabolic signatures before and after the 2 diets. The microbiome composition, fecal pH, SCFA, total sulfide, fecal bacterial load, and fecal metabolome significantly changed in the same direction for the 2 diets with many parallel changes in specific metabolites and species. Some effects seen in the present study have been previously associated with gut dysbiosis or an "unhealthy" microbiome, but paradoxically associated with decreased disease activity and amelioration of colonic inflammation in children with active CD on EEN.[6,7] This paradox remains one of the fundamental, yet unresolved, questions regarding the mechanism of EEN action and what this in turn indicates about the pathophysiology of CD.[25] EEN could deplete the nutrients necessary for bacterial growth, such as fiber, or create a luminal micro-environment where the functionality of inflammatory microbes is suppressed or altered, blunting activation of the gut-associated immune system in patients with CD. This theoretical mechanism of EEN action also is supported by the clinical effectiveness of antibiotics[26] and the relative ineffectiveness of probiotics, prebiotics, and fiber interventions aiming to reconstitute a "healthy" microbiome.[27]

Several effects observed in the healthy volunteer RCT were replicated in our animal model of gut inflammation, which features a microbial dysbiosis similar to human CD.[19] Encouragingly, the changes in metabolites and microbiome composition in fecal samples, cecal luminal contents, and mucosa-associated microbiome were similar between the 2 experimental diets and significantly different than the B27-CONTROL group.

The effects of the 2 experimental diets on secondary outcomes, such as abundance of species and metabolites, were very similar, but not always the same. This was

anticipated because CD-TREAT is a far more diverse diet than EEN, the number of microbial signals assessed at one time was large, and inter- and intraindividual variations in microbiome characteristics were considerable, effects we also observed even after the end of intervention solely with EEN (Figure 2C). Importantly, unlike EEN, CD-TREAT is not an identical diet in all individuals. This means interindividual food differences can be significant within the CD-TREAT group and so the potent effect of ingredients in this food (eg, phenols, type of amino acids, and fiber) on the microbiome also will naturally differ. In truth, one advantage of the natural variability inherent in CD-TREAT is the possibility of identifying food-based signals in larger studies of efficacy. Transgressions from prescribed CD-TREAT are less likely to explain microbial differences because prescribed and reported intake did not differ. Moreover, a longer intervention might have mitigated the deviation in microbiome effects between the 2 diets. Indeed, this was observed in the animal experiments in which β-diversity was more similar after 28 days than after 7 days of intervention (Figure 4C).

However, it is noteworthy that several changes in the taxon abundance of certain species and metabolites were similar to those described before in children with CD on EEN.[6,7] Specifically, the abundance of genera belonging to *Actinobacteria*, *Bacteroidetes*, and *Firmicutes*, such as *Bifidobacterium*, *Alistipes*, *Dialister*, *Faecalibacterium*, *Pseudobutyrivibrio*, and *Ruminococcus*, fecal pH, metabolites such as SCFA, sulfide, and adenine substructures, which significantly changed in children with CD after EEN,[6-8,24] also changed in the present RCT. Likewise, differences in fecal amino acid and lipid concentrations and metabolism have been described between patients with CD and healthy controls,[28] inferring their role in disease etiology, several of which also changed during CD-TREAT and EEN. Although important microbial messages emerge from these studies, the main purpose of this study was to demonstrate comparability of microbial changes and, by proxy, putative microbial mechanism of action and not to look specifically at the mechanism of CD-TREAT or EEN action. Such a study is important but would be better specifically addressed by exploring CD-TREAT against EEN in patients with active CD.

The attenuation of ileitis histopathology scores and the lower expression of IL-6 and CXCL-1 in our B27-CD-TREAT rat model also indicate that CD-TREAT can deliver therapeutic benefit in a disease state strongly associated with microbial dysbiosis, much like EEN in CD. In particular, the effect of EEN on ileal inflammation in our animals aligns with evidence of its efficacy in ileal mucosal healing in pediatric CD.[4]

Because preclinical findings require replication in clinical trials, we next tested CD-TREAT in children with active CD. CD-TREAT produced the same clinical efficacy signal and the same decrease in FC as in children with newly diagnosed CD on EEN.[4,5] Adherence to CD-TREAT was high, suggesting it was relatively easy to follow, although meals were cooked and provided free to explore the efficacy signal under maximum treatment adherence conditions. In this small number of patients, it is not possible to test whether

clinical efficacy is associated with an EEN-like modification of the microbiome.

We could not directly relate our findings with those from clinical research of other exclusion diets.[3] Although such previous studies have shown encouraging efficacy signals in uncontrolled trials in patients with active CD, they lack mechanistic evidence that would allow a direct comparison with the present data. However, we argue that these diets work by decreasing exposure to free sugars, dietary fat, or food additives and emulsifiers or by promoting gut "normobiosis." Indeed, several EEN feeds used in the management of active CD also are rich in fat and sucrose, contain maltodextrins and carrageenan, and lack fiber, and as discussed earlier, EEN-induced dysbiosis is paradoxically associated with mucosal healing.[6] The clinical efficacy signals of these diets could be attributed to other excluded components yet undescribed.

EEN acceptability and long-term use, particularly in adult patients, might be limited by taste fatigue and poor palatability. In this study, CD-TREAT ranked as more palatable, easier to follow, more satiating, and causing fewer gastrointestinal side effects than EEN. These observations are in agreement with the opinions of families of children with CD, previously treated with EEN, who reported that a solid food-based alternative would be preferable.[29] We did not observe differences in satisfaction, fullness, and desire to eat ratings or in gastrointestinal symptoms, such as abdominal discomfort, passing wind, and constipation, between the 2 diets. This is likely to be because the diets were isocaloric, had very similar composition, including low fiber content, and perhaps most importantly, the duration of intervention was not long enough to observe a significant effect.

The strengths of the study are the well-designed experiments in humans, for whom the provision of the CD-TREAT was freely available to maximize adherence; the replication of our findings in animals with gut inflammation and microbiome dysbiosis similar to human CD; the extension of these experiments to demonstrate an early pilot signal of efficacy in our target clinical population; and the comprehensive multi-omics methodology applied.

However, the healthy human microbiome analysis was solely performed on fecal samples and thus might not reflect the mucosal microbiome. Undertaking repeat colonoscopy assessments in healthy volunteers would be ethically unacceptable. However, the microbial signals of the 2 experimental diets on the fecal and cecal luminal contents and tissue specimens were similar in the animal experiments. The duration of the intervention lasted for 7 days in the human RCT and for 4 weeks in the animal experiment, which is shorter than the 6–8 weeks of EEN that patients with CD typically receive. However, the immediate response of the fecal rat microbiome within the first week of treatment, with fewer changes observed thereafter, suggests the observation was long enough, at least for the main study outcomes. We also believe that the effect of animal overfeeding and weight gain on the gut microbiome was insignificant because comparable microbial signals were observed in the healthy volunteer RCT

and previously in children with CD gaining weight during EEN.[6,7] To ensure the most conservative approach to animal experiments, and for animal welfare, we kept the group sizes modest; hence, this experiment might be underpowered for additional and exploratory outcomes, with substantial inter-animal variation, such as the effects on specific taxon abundance. This also might have been the issue in the expression of some ileal tissue cytokines, in which, despite apparent mean differences between groups, the small number of animals and substantial inter-animal variation, as observed previously,[19] weakened statistical power. However, for a preliminary study developing and supporting our core hypothesis, the rat model, as reported, provided sufficient support to subsequently interrogate CD-TREAT in our pilot trial in patients with active CD.

In addition, for some outcome measures for which we infer equivalence between the EEN and CD-TREAT interventions, we might have needed a larger sample. Such outcome measures could include the absence of differences in the expression of cytokines in the ileum of the animal experiments and some microbiome characteristics in the RCT of healthy volunteers. However, the primary objective of these preclinical studies was to explore the effects of each experimental diet against the control diet, with the equivalence between experimental diets studied as a secondary, yet important, objective. Likewise, the efficacy signals of CD-TREAT in children with CD need replication in larger studies and against EEN.

Conclusion

We have produced robust proof-of-concept data to support a novel dietary treatment trialed in healthy volunteers, subsequently in rats with gut inflammation and microbiome dysbiosis similar to human CD, and in a pilot trial in children with active CD. With the evidence produced within this scientific body of work, the efficacy of CD-TREAT on human CD clinical outcomes needs to be ascertained in large well-controlled clinical trials and with inclusion of mechanistic studies to explore microbial signals of response. If successful, CD-TREAT has the potential to be used interchangeably with EEN, particularly in adults in whom EEN uptake is low, and raises the prospect of long-term dietary maintenance therapy.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at https://doi.org/10.1053/j.gastro.2018.12.002.

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CLINICAL AT

Supplementary Methods

RCT in Healthy Volunteers

Acceptability of Interventions. Appetite, gastrointestinal symptoms, and adherence to the experimental diets were assessed by self-reported questionnaire. This was compiled and checked for content validity by the research team and for readability by lay people. The questionnaire collected information from each participant on the participant's appetite on days 1 and 6 of each intervention using visual analog scales extracted from an existing questionnaire.[1] Gastrointestinal symptoms and level of adherence to the diet also were assessed at the end of each intervention using Likert scales from a validated questionnaire.[2] Additional meals outside the experimental diets were recorded.

Fecal Sample Collection. The entire bowel movement was collected, stored immediately under anaerobic (Oxoid AnaeroGen Sachet; ThermoFisher Scientific) cold conditions, and processed within 60 minutes of defecation. The entire bowel movement was weighed, homogenized, and stored appropriately for downstream methods.

Untargeted Fecal Metabolomics. *Extraction.* Freeze-dried fecal samples were extracted by chloroform, methanol, and water at a volume ratio of 1:3:1. The extraction was carried out by mixing extraction mixture 800 μL with a 20-mg freeze-dried fecal sample. The supernatant was recovered to another vial and extracts were stored at -80°C until liquid chromatography–mass spectrometry (LC-MS) analysis.

Solvents and Chemicals. High-performance LC (HPLC)-grade acetonitrile, chloroform, and water were obtained from ThermoFisher Scientific. Ammonium carbonate and methanol were purchased from Sigma-Aldrich (Poole, Dorset, UK). HPLC-grade water was produced in house by a Direct-Q 3 Ultrapure water purification system (Millipore, Watford, UK). The metabolite standards were obtained from Sigma-Aldrich and were prepared as previously described.[3]

HPLC Conditions. <u>Mobile Phase and Column.</u> The mobile phase solutions were freshly prepared and stored at room temperature for up to 48 hours. Mobile phase A (ammonium carbonate buffer 20 mmol/L, pH 9.2) was prepared by dissolving ammonium carbonate 1.92 g in HPLC-grade water 800 mL followed by adjustment to pH 9.2 with ammonia solution and then made up to 1 L. Mobile phase B was prepared using HPLC-grade acetonitrile. The column used was a ZIC-pHILIC (L150 $\times$ 4.6-mm inner diameter, 5 μm, polymeric bead support) column from Hichrom Ltd (Reading, UK). The column was attached to a ZIC-pHILIC guard column.

<u>HPLC Setup.</u> A gradient elution method (Supplementary Table 1) was used as described previously.[3] The flow rate was set to 0.3 mL/min.

<u>Orbitrap Exactive Mass-Spectrometer Setup.</u> Samples were analyzed using an Accela HPLC system connected to an Orbitrap Exactive mass spectrometer (ThermoFisher Scientific, Bremen, Germany). The quality of the data and identified metabolites were confirmed by standard mixtures introduced within the running sequence.[4] During the analysis, peak characteristics such as width and height were determined using relative SD values. The acceptable relative SD value was set to not exceed 20% for each standard and the retention time shift should not be more than 0.3 minute between the initial and final sample in a sequence.

Data Extraction. The raw files for the samples were extracted and processed using mzMatch/Peak ML metabolomics data analysis.[5] IDEOM provided a free data extraction application that can identify spectrum peaks while considering noise sources in the LC-MS analysis. This macro-based spreadsheet was obtained from http://mzmatch.sourceforge.net/ideom.htm and the analysis depends on the LC-MS data extraction method.[6] The R package from http://www.r-project.org was preinstalled to create a readable environment for LC-MS processing.[7] The identified metabolites and quality of peaks were examined manually using different databases, including HMDB (http://www.hmdb.ca/), KEGG (http://www.genome.jp/kegg/), and LIPID MAPS (http://www.lipidmaps.org/).

Animal Experiments

Gut Histopathology. Colonic and ileal specimens were fixed in 10% formalin until the tissue was embedded in paraffin, stained with hematoxylin and eosin, and evaluated by 2 pathologists (R.K. and J.S.) in a blinded manner. Tissue sections (5 μm) were stained with hematoxylin and eosin, imaged (EVOS FL Auto Cell Imaging System, Life Technologies, Carlsbad, CA), and evaluated by 2 pathologists (R.K. and J.S.) in a blinded manner.

The colon was assessed for leukocyte infiltration, mucosal damage, neutrophil infiltration, crypt abscesses or hyperplasia, and goblet cell depletion. The ileum was assessed for leukocyte infiltration, villous fusion, shortening or hyperplasia, and mucosal necrosis, erosion, and ulceration. Each parameter was scored from 0 to 3 (0 = no; 1 = mild; 2 = moderate; 3 = severe presence). The sum of the individual parameters generated an ileal (minimum–maximum score 0–12) and a colonic (minimum–maximum score 0–15) inflammatory score.[8]

Expression of Cytokines in Ileum. The expression of the following genes was quantified: TNF-α (GenBank accession number Rn99999017_m1), IL-6 (GenBank accession number Rn01410330_m1), IL-10 (GenBank accession number Rn01483988_g1), IL-1b (GenBank accession number Rn00580432_m1), and CXCL-1 (GenBank accession number Rn00578225_m1). Amplification reactions were analyzed by relative quantification using the comparative threshold cycle method and using the housekeeping genes GAPDH (GenBank accession number Rn01775763_g1) and RPLNO (GenBank accession number Rn03302271_gH) as endogenous controls. Data are displayed as fold change of gene expression against the average of B7-CONTROL samples.

Bioinformatics. We retained the paired-end reads between 225 bp and 275 bp in length, overlapped the reads, and performed OTU clustering on de-replicated reads after removing singletons and using 98% similarity. Afterward, we removed chimera based on the de novo approach (using the most abundant de-replicated reads) and the

reference-based approach (as mentioned in the link) and generated OTU at 97% similarity with taxonomy assigned using functions given in the Dada2 pipeline after filtering out OTUs with mean proportional abundance $<0.01\%$.

Statistics

Power Calculation. The power calculation for the RCT of health volunteers was revised after completion of the first 10 participants, at which point we were already powered to detect significant changes for SCFA and bacterial load during EEN and CD-TREAT (Supplementary Table 2). However, we continued recruitment to our original target number of 25 participants to maximize statistical power for secondary outcomes of the study in which higher interindividual variation and potentially smaller effect size (ie, 16S rRNA sequencing, metabolome) were expected.

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Supplementary Figure S1. Example CD-TREAT meals prepared by the catering company.
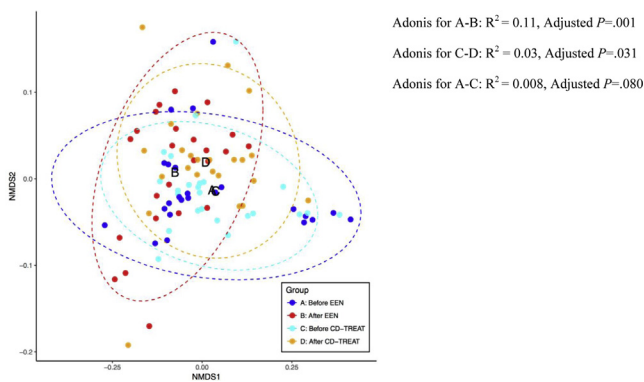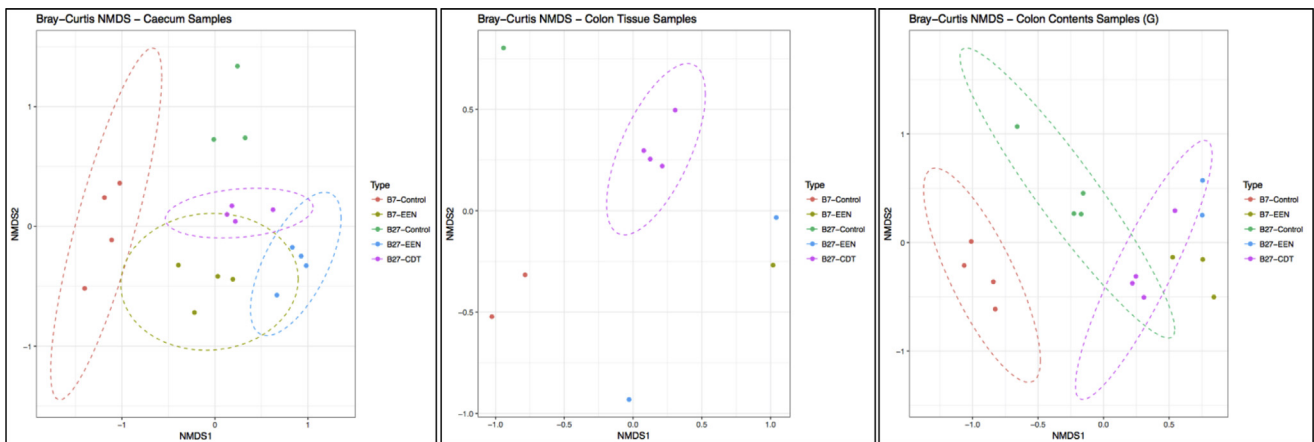

Supplementary Figure S2. NMDS using weighted UniFrac distances of the 3% OTU community structures during dietary interventions on healthy human fecal microbiome (n = 24). Adonis for *A* and *B*: $R^2 = 0.11$, adjusted $P = .001$; Adonis for *C* and *D*: $R^2 = 0.03$, adjusted $P = .031$; Adonis for *A–C*: $R^2 = 0.008$, adjusted $P = .080$. NMDS, nonmetric multidimensional scaling.
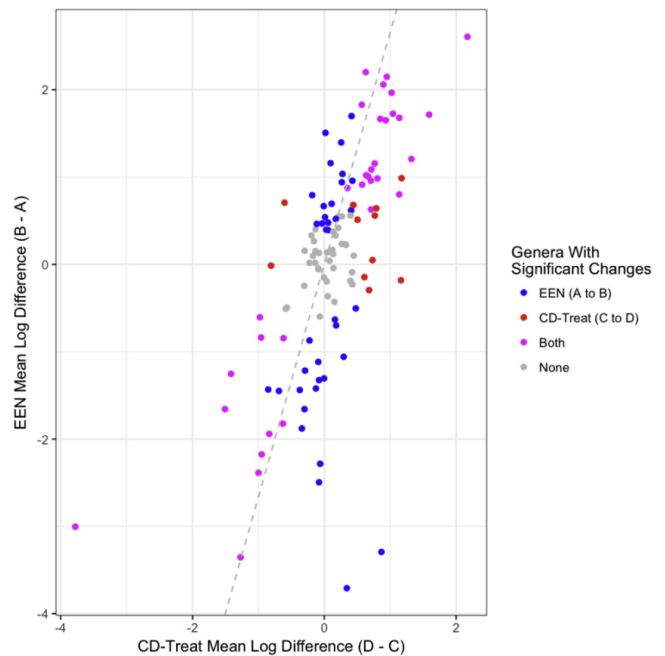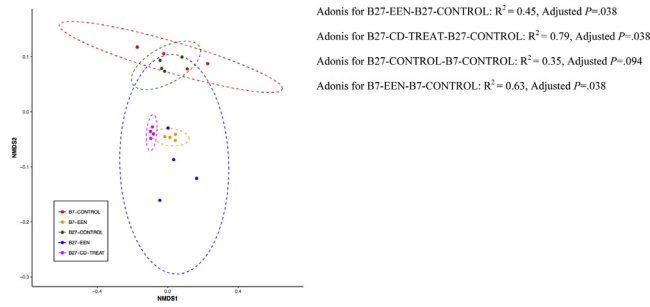

Supplementary Figure S3. Scatterplot with genera changes before and after EEN and CD-TREAT in the crossover RCT of healthy volunteers (n = 24).
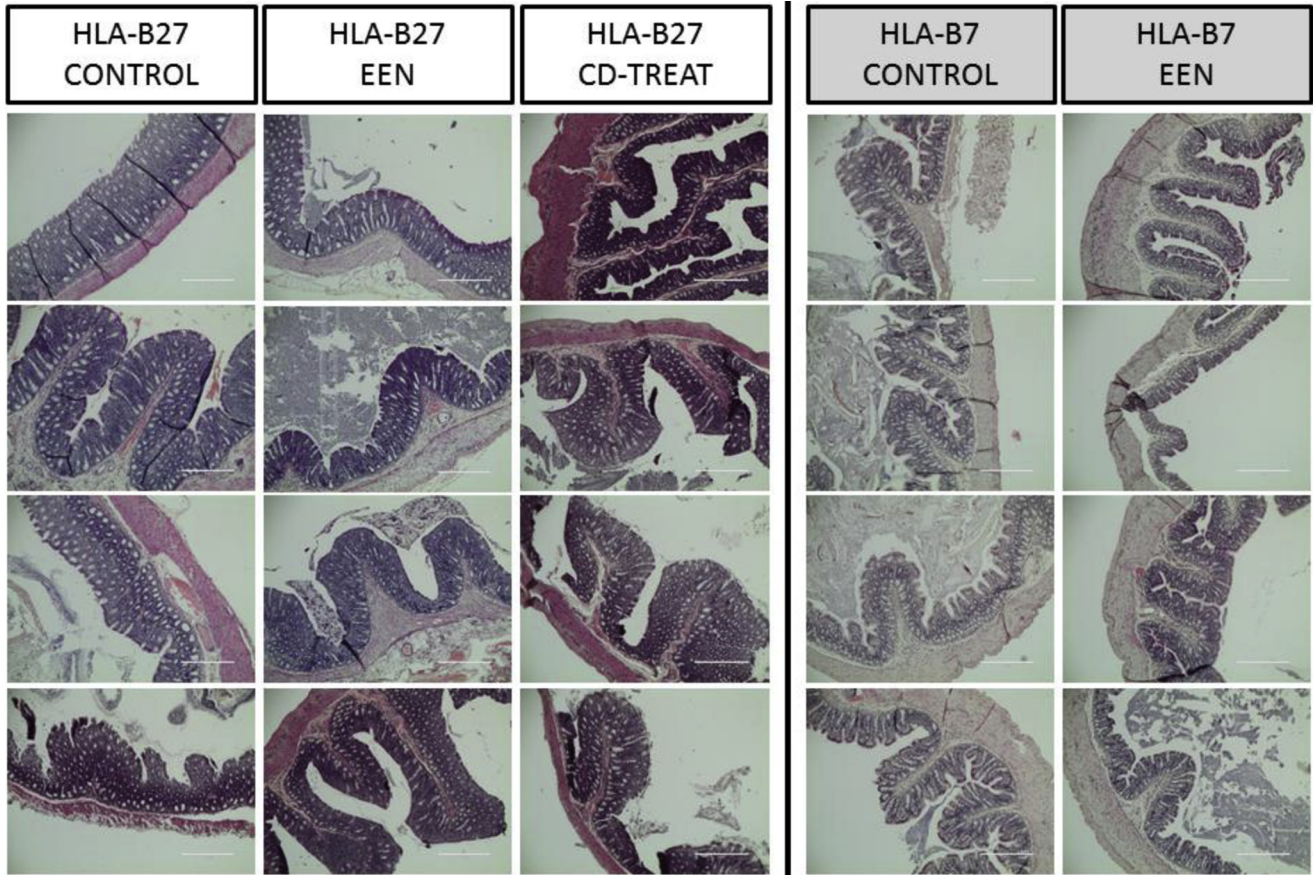
Supplementary Figure S4. Representative hematoxylin and eosin stained sections of the colon of EEN, CD-TREAT, and control HLA-B27 rats and EEN and control HLA-B7 rats. Colon tissue sections were visualized and digital images were captured using an EVOS FL Auto Cell Imaging System (Life Technologies) with a 10× objective (in all cases, data of all studied animals were included; n = 20).

Supplementary Figure S5. NMDS using Bray-Curtis distances of the 3% OTU community structures of B27-CONTROL, B27-EEN, B27-CD-TREAT, B7-CONTROL, and B7-EEN animals' (*A*) cecum tissue microbiome (n = 19), (*B*) colon tissue microbiome (n = 10), and (*C*) colon content microbiome at sacrifice (n = 17). NMDS, nonmetric multidimensional scaling.

Adonis for B27-EEN-B27-CONTROL: $R^2 = 0.45$, Adjusted P=.038

Adonis for B27-CD-TREAT-B27-CONTROL: $R^2 = 0.79$, Adjusted P=.038

Adonis for B27-CONTROL-B7-CONTROL: $R^2 = 0.35$, Adjusted P=.094

Adonis for B7-EEN-B7-CONTROL: $R^2 = 0.63$, Adjusted P=.038

Supplementary Figure S6. NMDS using weighted UniFrac distances of the 3% OTU community structures of the rat cecal luminal content microbiome at sacrifice (n = 20). Adonis for B27-EEN and B27-CONTROL: $R^2 = 0.45$, adjusted $P = .038$; Adonis for B27-CD-TREAT and B27-CONTROL: $R^2 = 0.79$, adjusted $P = .038$; Adonis for B27-CONTROL and B7-CONTROL: $R^2 = 0.35$, adjusted $P = .094$; Adonis for B7-EEN and B7-CONTROL: $R^2 = 0.63$, adjusted $P = .038$. NMDS, nonmetric multidimensional scaling.
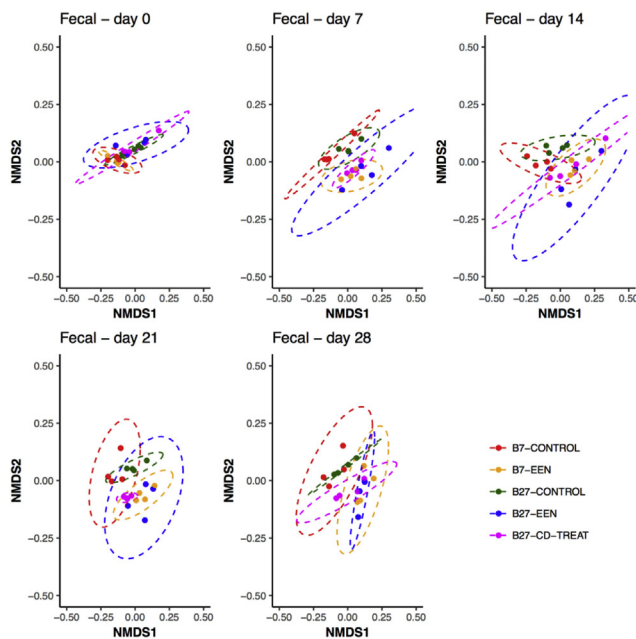
Time	Group 1	Group 2	R^2	Adjusted P
Day 0	B27-CONTROL	B7-CONTROL	0.515736	.315
Day 7	B27-EEN	B27-CONTROL	0.389642	.066
Day 7	B27-CD-TREAT	B27-CONTROL	0.434009	.066
Day 7	B27-CONTROL	B7-CONTROL	0.445806	.066
Day 7	B7-EEN	B7-CONTROL	0.540451	.066
Day 14	B27-EEN	B27-CONTROL	0.416568	.080
Day 14	B27-CD-TREAT	B27-CONTROL	0.238897	.148
Day 14	B27-CONTROL	B7-CONTROL	0.330802	.148
Day 14	B7-EEN	B7-CONTROL	0.650334	.080
Day 21	B27-EEN	B27-CONTROL	0.440758	.067
Day 21	B27-CD-TREAT	B27-CONTROL	0.604733	.039
Day 21	B27-CONTROL	B7-CONTROL	0.562680	.039
Day 21	B7-EEN	B7-CONTROL	0.704100	.039
Day 28	B27-EEN	B27-CONTROL	0.584165	.070
Day 28	B27-CD-TREAT	B27-CONTROL	0.291496	.188
Day 28	B27-CONTROL	B7-CONTROL	0.230795	.201
Day 28	B7-EEN	B7-CONTROL	0.596205	.070

Supplementary Figure S7. NMDS using weighted UniFrac distances of the 3% OTU community structures of the rat fecal microbiome during the course of dietary interventions (n = 20). NMDS, nonmetric multidimensional scaling.

Supplementary Table 1. Gradient Elution Program Applied for ZIC-pHILIC Column in LC-MS Analysis

Time (*min*)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (*mL/min*)
0	20	80	0.3
30	80	20	0.3
31	92	8	0.3
36	92	8	0.3
37	20	80	0.3
46	20	80	0.3

Supplementary Table 2. Fecal Sample Characteristics, Metabolites, and Bacterial Load Before and After Dietary EEN and CD-TREAT in the First 10 Participants Who Completed the Healthy Volunteers RCT (n = 40; Fecal Samples From 10 Participants)

	Before EEN	After EEN	Before CD-TREAT	After CD-TREAT
Bristol Stool Form Scale	3.7 (1.6)	1.6 (1.0)[a,e]	3.0 (1.3)	1.9 (0.9)[a,c]
Fecal pH	7.0 (0.5)	8.2 (0.2[a,f]	7.0 (0.6	7.7 (0.4)[a,b,e]
Acetate (*μmol/g*)	67.6 (11.9)	48.6 (14.4)[a,d]	76.9 (17.6)	65.5 (19.8)[a,b,c,e]
Propionate (*μmol/g*)	15.6 (5.4)	9.9 (4.9)[a,e]	15.8 (5.8)	13.3 (9.2)[a,d]
Butyrate (*μmol/g*)	11.9 (5.0)	6.3 (2.5)[a,e]	14.1 (10.3)	7.9 (5.4[a,e]
Valerate (*μmol/g*)	2.2 (0.6)	2.0 (0.8)	2.4 (1.5)	1.9 (1.1)
Isobutyrate (*μmol/g*)	1.7 (0.8)	2.4 (1.0)[a,d]	2.0 (1.1)	2.1 (1.0)
Isovalerate (*μmol/g*)	1.8 (1.0)	2.7 (1.1)[a,e]	2.1 (1.4)	2.3 (1.1)
Free sulfide (*nmol/g*)	5.1 (2.6)	1.5 (1.6)[a,f]	4.2 (1.0)	6.0 (4.7)[c,f]
Total sulfide (*nmol/g*)	82.2 (62.9)	250.4 (83.7)[a,f]	66.5 (30.6)	141.4 (69.8)[a,b,e]
Total bacteria	11.3 (0.2)	11.1 (0.2)[a,e]	11.2 (0.2)	11.0 (0.3)[a,d]

NOTE. Data are displayed as mean (standard deviation). Total bacteria are displayed as $\log_{10}$ 16S rRNA gene copy number per gram of stool.
[a]Significant difference between before EEN and after EEN or before CD-TREAT and after CD-TREAT.
[b]Significant difference between after EEN and after CD-TREAT.
[c]$P < .1$.
[d]$P < .05$.
[e]$P < .01$.
[f]$P < .001$ for Fisher pairwise comparisons after general linear modeling with Box-Cox transformation.