

Successful Dietary Therapy in Paediatric Crohn's Disease is Associated with Shifts in Bacterial Dysbiosis and Inflammatory Metabotype Towards Healthy Controls

Charlotte M. Verburgt,^{a,b,c*,} Katherine A. Dunn,^{d*} Mohammed Ghiboub,^{a,b} James D. Lewis,^{e,f}

Eytan Wine,⁹ Rotem Sigall Boneh,^h Konstantinos Gerasimidis,ⁱ Raanan Shamir,^j

Susanne Penny,^k Devanand M. Pinto,^k Alejandro Cohen,¹ Paul Bjorndahl,^m Vaios Svolos,ⁱ

Joseph P. Bielawski,^{e,m} Marc A. Benninga,^a Wouter J. de Jonge,^{b,m,n} Johan E. Van Limbergen^{a,b,o}

^aDepartment of Paediatric Gastroenterology and Nutrition, Amsterdam University Medical Centers, University of Amsterdam, Emma Children's Hospital, Amsterdam, The Netherlands

^bTytgat Institute for Liver and Intestinal Research, Amsterdam Gastroenterology Endocrinology Metabolism, University of Amsterdam, Amsterdam, The Netherlands

^cAmsterdam Reproduction & Development Research Institute, Emma Children's Hospital, Amsterdam, The Netherlands ^dDepartment of Biology, Dalhousie University, Halifax, NS, Canada

^eCentre for Clinical Epidemiology and Biostatistics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA Division of Gastroenterology and Hepatology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA

⁹Division of Paediatric Gastroenterology, Stollery Children's Hospital, University of Alberta, Edmonton, AB, Canada

Wolfson Medical Centre, Holon, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

Department of Human Nutrition, School of Medicine, Dentistry & Nursing, University of Glasgow, Glasgow, UK

Institute of Gastroenterology, Nutrition and Liver Diseases, Schneider Children's Medical Centre, Petach-Tikva, Sackler Faculty of Medicine, Tel Aviv University, Tel Aviv, Israel

^kHuman Health Therapeutics, National Research Council, Halifax, NS, Canada

Proteomics and Mass Spectrometry Core Facility, Faculty of Medicine, Dalhousie University, Halifax, NS, Canada

"Department of Mathematics & Statistics, Dalhousie University, Halifax, NS, Canada

ⁿDepartment of Surgery, University of Bonn, Bonn, Germany

°Amsterdam Public Health Research Institute, University of Amsterdam, Amsterdam UMC, Amsterdam, The Netherlands

Corresponding author: Dr Johan Van Limbergen, MD, PhD, Department of Paediatric Gastroenterology and Nutrition, Emma Children's Hospital, Amsterdam University Medical Centers, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Tel.: +31-20 566 3053; Email: j.e.vanlimbergen@amsterdamumc.nl *Joint first authors.

Abstract

Background and aims: Nutritional therapy with the Crohn's Disease Exclusion Diet + Partial Enteral Nutrition [CDED+PEN] or Exclusive Enteral Nutrition [EEN] induces remission and reduces inflammation in mild-to-moderate paediatric Crohn's disease [CD]. We aimed to assess if reaching remission with nutritional therapy is mediated by correcting compositional or functional dysbiosis.

Methods: We assessed metagenome sequences, short chain fatty acids [SCFA] and bile acids [BA] in 54 paediatric CD patients reaching remission after nutritional therapy [with CDED + PEN or EEN] [NCT01728870], compared to 26 paediatric healthy controls.

Results: Successful dietary therapy decreased the relative abundance of Proteobacteria and increased Firmicutes towards healthy controls. CD patients possessed a mixture of two metabotypes [M1 and M2], whereas all healthy controls had metabotype M1. M1 was characterised by high Bacteroidetes and Firmicutes, low Proteobacteria, and higher SCFA synthesis pathways, and M2 was associated with high Proteobacteria and genes involved in SCFA degradation. M1 contribution increased during diet: 48%, 63%, up to 74% [Weeks 0, 6, 12, respectively.]. By Week 12, genera from Proteobacteria reached relative abundance levels of healthy controls with the exception of *E. coli*. Despite an increase in SCFA synthesis pathways, remission was not associated with increased SCFAs. Primary BA decreased with EEN but not with CDED+PEN, and secondary BA did not change during diet.

Conclusion: Successful dietary therapy induced correction of both compositional and functional dysbiosis. However, 12 weeks of diet was not enough to achieve complete correction of dysbiosis. Our data suggests that composition and metabotype are important and change quickly during the early clinical response to dietary intervention. Correction of dysbiosis may therefore be an important future treatment goal for CD. **Key Words:** Diet; Crohn's disease; microbiome; treatment; inflammatory bowel disease

1. Introduction

The increasing incidence of Crohn's disease [CD], one of the inflammatory bowel diseases [IBD], points to an important

role for environmental determinants.¹ The pathogenesis of CD is thought to result from a complex interplay of genetic risk factors, environmental factors, and alterations of the

© The Author(s) 2022. Published by Oxford University Press on behalf of European Crohn's and Colitis Organisation.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial License (https://creativecommons.org/ licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com microbiome [dysbiosis] and subsequent perturbations of the metabolome leading to inflammation and disease.²⁻⁷ Most currently available medical therapies are directed against inflammation but not towards the microbiome or correction of its dysbiosis.^{8,9} If dysbiosis is a trigger for inflammation, correction of dysbiosis might be an unexplored future goal of therapy.

The most commonly recognised dysbiosis in treatmentnaïve patients with CD is an increase in Proteobacteria, namely Enterobacteriaceae and Pasteurellaceae, and a decrease in Firmicutes, namely Erysipelotrichales and Clostridiales.^{4,5,10} 'Functional dysbiosis', defined by changes in metagenomic pathways, and profiling of the microbiome's metabolic activity potential by 'metabotype' in the context of dietary therapy, has been less studied.^{11–13}

Exclusive enteral nutrition [EEN], a form of dietary therapy consisting of a liquid formula diet as the sole source of food for 6–8 weeks, is used as first-line therapy for induction of remission in mild to moderate paediatric CD.^{2,14,15} EEN was shown to be superior to corticosteroids in non-severe disease, avoiding medical side effects and supporting growth and more effective healing of the mucosa.^{16–20} The hypothetical mechanism whereby diet induces remission appears to be exclusion of habitual Western diet.^{2,10,21–23} Accordingly, upon re-exposure to habitual diet, there is an increase in inflammation and resurgence of Proteobacteria.¹⁰

We recently published a novel Crohn's Disease Exclusion diet + Partial Enteral Nutrition [CDED + PEN], which is characterised by a significant reduction in inflammation and improved sustained remission through Week 12 compared with EEN.^{10,24} CDED is a whole-food diet coupled with supplemental formula [partial enteral nutrition: PEN], designed to reduce exposure to dietary components which are hypothesised to negatively affect the microbiome, intestinal barrier, and intestinal immunity.^{2,4,23,25} On the microbiome side, the diet was designed to reduce the relative abundance of mucosal Proteobacteria, particularly *E.coli*, and to increase short chain fatty acid [SCFA]-producing bacteria over time by providing mandatory daily consumption of apple pectin and varying sources of resistant starch as well as other sources of dietary fibres.^{26–28}

Dysbiosis in CD may drive inflammation via an increase in pathobionts or decrease in beneficial bacteria, although causation remains to be fully elucidated. Persistence of dysbiosis might explain in part the chronicity of CD and subsequently, correction of dysbiosis might influence disease progression through decreased inflammation. Dietary modulation may induce remission via compositional change [ie correction of dysbiosis, increase in SCFA producers, or simply decrease in Proteobacteria], or via alteration of the functionality of the microbiome [metabotype].

We assessed the contribution of whole-metagenome defined microbiome features in reaching remission using standardised dietary treatments, taking into account the compositional nature of the data and metabotypes, and evaluated if correction of dysbiosis towards healthy controls occurs. We further assessed if alterations in SCFA and BA were associated with remission.

2. Methods

2.1. Trial participants and healthy controls

The CDED study was an investigator-initiated, prospective, randomised, controlled trial [RCT] with two interventional

arms comparing a CDED + PEN [Group 1] with an EEN [Group 2], diet administered orally over 12 weeks in a paediatric population with mild to moderate active luminal disease, as previously reported [NCT01728870].^{10,18} Group 1 received CDED phase 1 with recommended intake of 50% PEN for calculated energy requirements [Modulen, Nestlé Health Science, Vevey, Switzerland] for the first 6 weeks, followed by phase 2 diet with recommended intake of 25% PEN for the next 6 weeks. Group 2 received standard-of-care EEN [Modulen] for 6 weeks followed by 25% PEN during Weeks 6–12, with gradual reintroduction of table foods between Weeks 6–9 as per local preference, such that all patients were exposed to PEN + free diet by Week 12.¹⁰ Clinical remission at Week 6 was defined as PCDAI ≤10 on an intention-to-treat [ITT] analysis.²⁹

We obtained metagenomic sequence data from stool samples collected at three time points [Weeks 0, 6, 12] from 54 patients who reached remission with either CDED + PEN or EEN in the CDED-RCT. Good compliance with dietary therapies was ensured by only selecting patients who achieved remission, as dietary adherence was significantly associated with reaching remission at Week 6 in earlier analyses.^{10,24} We examined stool samples collected at three time points [Weeks 0, 6, 12] for SCFA in 53 patients and BA in 42 patients. In addition, metagenomic sequences from 26 healthy paediatric controls, publicly available from previously published data [COMBO],^{30,31} were used for comparison. Healthy controls had no history of diagnosis with IBD, coeliac disease, bowel surgery, or other chronic intestinal disorders. None of the healthy children had received antibiotics in the prior 6 months. For all in- and exclusion criteria of healthy controls, see Supplementary Table 1.

The study was approved by the local ethics board at each of the participating sites.

2.2. Metagenomic sequence data and analysis

DNA was extracted from stool samples using the MO BIO PowerFecal DNA Kit [Qiagen, Hilden, Germany] at the Integrated Microbiome Resource of Dalhousie University [imr.bio]. Shotgun metagenomic sequences were obtained using Nextera XT library preparation and 150-bp pairend Nextseq sequencing. Sequences were processed using the kneaddata pipeline, which uses Trimmomatic³² and bowtie2³³ to remove low-quality reads [<50 base pairs and quality score <Q20] and human and PhiX174 contaminants. Paired forward and reverse sequence reads were concatenated and HUMAnN2³⁴ was run in parallel³⁵ to functionally annotate reads into KEGG orthologs [KOs].³⁶ KO counts were converted to enzyme counts and substrate product pairs using BiomeNet scripts.³⁷ Only KOs present in three or more samples having summed counts >25 [>0.0001% of total counts] were included. Taxonomic assignment was determined from concatenated sequence reads using Metaphlan2³⁸ which uses a set of marker genes to assign taxonomic identity. An overview of read, KO, and EC counts can be found in Supplementary Table 2. Bray Curtis dissimilarity was calculated and we used nonmultidimensional scaling to plot the distances. Additional detailed methods regarding sequencing and sequence analysis are available as Supplementary Data.

Linear discriminant analysis of effect size [LEfSe]³⁹ was used to identify genomic features characterising the differences between healthy and active CD at various weeks of treatment. This method is useful for detecting taxa or pathways that are over- or under- represented in high-dimensional microbiome data. LEfSe couples standard statistical tests of significance [Wilcoxon test p < 0.05] and then builds a linear discriminant analysis model [threshold of two] using the relative difference between groups to rank features. To examine differential abundance associated with time, Songbird⁴⁰ and ANOVA-like differential expression [ALDEx2]⁴¹ were used. Additional analysis details are available as Supplementary Data.

Unsupervised Bayesian analysis of community metabolism [BiomeNet] was performed to identify metabotypes.³⁷ Genes that could be assigned to enzyme commission [EC] numbers were used. EC data was converted to reaction substrate product pairs to create metabolic network files. The Bayesian model was run with K = 2 metabotypes and L = 50 subsystems [see Supplementary Methods for BiomeNet hyperparameter selection]. The first 100 iterations were treated as 'burn-in' and discarded. After burn-in, an additional 10 000 iterations were performed and samples were retained for analysis every 20 iterations.

2.3. Metabolite analysis of short chain fatty acids and bile acids

The absolute dry concentration of short chain fatty acids [SCFA; C2-C8] and branched chain fatty acids [BCFA; iC4-iC6] were measured by gas chromatography coupled with flame ionisation detection in acidified ether extracts at the Gerasimidis laboratory [Dept of Human Nutrition, University of Glasgow]. Details of sample preparation, gas chromatography, and peak integration are included in Supplementary Materials. Mann–Whitney U [unpaired] and Wilcoxon signed rank tests [for paired samples] were used to compare SCFA measurements at different time points.

Primary, secondary, and total bile acid [BA] concentrations were measured at the Proteomics Core facility [Dalhousie University] using liquid chromatography coupled to mass spectrometry [MS]. Details of sample preparation, liquid chromatography-MS and peak extraction are included in Supplementary Materials. Bile acid peak heights were normalised by calculating a linear fit of the peak height of each bile acid in a pooled sample, accounting for injection number and using the fit to interpolate the peak height adjustment for samples based on injection number. Statistical analysis and graphs were performed with GraphPad Prism v8.0.2.263 [GraphPad Software Inc.]. Analyses were done according to clinical outcome groups of sustained remission, non-sustained remission, and no remission. Data were subjected to one-way analysis of variance [ANOVA] or Student's t test. The two-tailed level of significance was set at ≤ 0.05 .

3. Results

Whole metagenome sequences were obtained from 54 of 74 patients who entered ITT, diet-induced, steroid-free remission [PCDAI ≤ 10] at Week 6 [146 samples over three time points; Weeks 0, 6, 12]. All three time points from the same patient were available for 41 participants [CDED + PEN *n* = 23, EEN *n* = 18]. We analysed SCFA data from 53 patients [151 samples] and BAs from 42 patients [93 samples], due to sample availability. There were no clinically significant differences in baseline characteristics with regards to age, gender, disease location, duration, activity, C-reactive protein [CRP], or use of medication between the two study groups [Supplementary Table 3].¹⁰

3.1. Microbiome shifts with diet-induced remission

3.1.1. Weeks 0–6: decrease in Proteobacteria

Examination of the pairwise community dissimilarity [Brav-Curtis], based on taxonomic assignment from metagenomic sequence data, shows changes between baseline and Week 12 towards the healthy samples in both diets [Permanova p = 0.001; Figure 1]. We examined these community changes in more detail using LEfSe to identify taxonomic differences that were over- or under-represented between the weeks of treatment and healthy controls. Comparison between all baseline samples and healthy controls identified taxa over-represented in baseline samples to include Proteobacteria, specifically Escherichia, Burkholderiales, and Klebsiella, as well as Fusobacteria Akkermansia and Bifidobacterium, and within Firmicutes, Streptococcus, Veillonella, Holdemania, Peptostreptococcaceae, and Blautia were also over-represented. In addition Saccharomyces, Bacteroidales, Alistipes, and Parabacteroides along with the Firmicutes, Eubacterium, Anaerostipes, Lachnospiraceae, Roseburia, Oscillibacter, Anaerotruncus, Clostridaceae, and *Ruminoccocus* were under-represented [Figure 2; Supplementary Figure 1 and Supplementary Table 4].

Once samples are split for the two diets achieving clinical remission at Week 6, we see that CDED + PEN changes, as compared with baseline, were associated with a significant increase in Firmicutes, particularly *Clostridiales*, driven by *Roseburia*, *Oscillibacter*, *Anaerotruncus*, and *Ruminococcus*; and a significant [p < 0.05] decrease in Proteobacteria, particularly Gammaproteobacteria [Supplementary Figure 2a and Supplementary Table 5]. In comparison with healthy samples, we still see differences: increased relative abundance of Proteobacteria [*Escherichia*, *Klebsiella*, *Citrobacter*, *Burkholderiales*, and *Agrobacterium*], whereas Firmicutes-like bacilli [*Lactococcus* and *Streptococcus*] remained more predominant in healthy samples [Figure 3a; Supplementary Figure 3b and Supplementary Table 7].

Changes in EEN patients showed a significant [p <0.05] increase in Firmicutes taxa in Clostridiales, Erysipelotrichaceae, and Veillonellaceae, as well as a decrease in Proteobacteria [Supplementary Figure 2b and Supplementary Table 6]. In comparison with healthy samples, EEN-diet samples at Week 6 differed: Firmicutes overall decreased [notably Lachnospiraceae, Peptostreptococcus, Blautia, Subdoligranulum, Veillonella, and Gemella] but specific taxa showed increases [Dialister, Ruminococcus, Roseburia, Coprococcus, Eubacterium, Lactococcus, and Streptococcus]. Relative abundance of Proteobacteria [Klebsiella, Escherichia, Enterobacter, Citrobacter, and Parasuterella] was also still increased after 6 weeks of EEN compared with healthy controls [Figure 3b; Supplementary Figure 3b and Supplementary Table 8].

Overall, from Week 0 to Week 6 both therapies significantly reduced Proteobacteria and increased Firmicutes [Supplementary Figure 2]. Although Proteobacteria abundance had decreased when clinical remission was achieved with dietary therapy at Week 6, it was still increased compared with healthy controls [Figures 3 and 4]. The relative abundance of *E. coli* in the baseline samples compared with healthy controls is particularly striking [Wilcoxon BH corrected p = 0.0005; Figure 4].



Figure 1. Beta-diversity plots of Bray–Curtis distances showing a gradual correction at Weeks 0, 6, 12 remission and Week 12 non-remission for patients achieving remission in Week 6 with EEN [n = 22, 20, 14, 6, respectively] and CDED + PEN [n = 30, 28, 25, 1, respectively] compared with healthy paediatric controls [n = 26] [Permanova p = 0.001]. EEN, exclusive enteral nutrition; CDED + PEN, Crohn's disease exclusion diet + partial enteral nutrition.



Figure 2. LEfSe cladogram of taxa showing significant [p < 0.05] differences in abundance of baseline treatment-naïve [Week 0] CD children [CDED + PEN and EEN groups, n = 52] compared with healthy controls [HC] [n = 26]. Analyses include only samples from patients reaching remission at Week 6. Each dot represents identified taxa in this data. Taxa highlighted in red are increased and highlighted in green are decreased in treatment-naïve [Week 0] CD samples compared with HC. Detailed marked figure can be found in Supplementary Figure 1. CD, Crohn's disease; EEN, exclusive enteral nutrition; CDED + PEN, Crohn's disease exclusion diet + partial enteral nutrition; LEfSe, linear discriminant analysis of effect size.

As analyses were based on compositional data, which can be sensitive to other taxa increasing and/or decreasing, we used log ratios to examine differential abundance associated with covariates [time in this case] as implemented in Songbird⁴⁰ and visualised using Qurro.⁴² Bacteroidetes was used as the reference group for comparisons as it was most consistent across samples and time points and not the taxon of interest. Participants following CDED + PEN showed an overall nonsignificant increase in Firmicutes at all levels of taxonomy in Week 6 [all p > 0.05]. For Proteobacteria however, there was a significant decrease in abundance during the first 6 weeks [p = 0.02], mostly driven by γ -proteobacteria [w0 vs w6, p = 0.073], namely from *Escherichia* genera [Supplementary Figure 4]. Interestingly for EEN, changes in both Firmicutes



Figure 3. LEfSe cladogram of taxa showing significant [p < 0.05] differences in abundance of Week 6 remission samples split by diet versus healthy controls [HC] [n = 26]. Each dot represents identified taxa in these data. Taxa highlighted in red are increased and in green are decreased in CD [Week 6] samples compared with HC. Detailed marked Figure can be found in Supplementary Figure 3. A] CDED + PEN Week 6 [n = 28] vs HC [n = 26]. B] EEN Week 6 [n = 20] vs HC [n = 26]. CD, Crohn's disease; EEN, exclusive enteral nutrition; CDED + PEN, Crohn's disease exclusion diet + partial enteral nutrition; LEfSe, linear discriminant analysis of effect size.

and Proteobacteria were not significant in patients during the first 6 weeks of treatment [all p > 0.05, Supplementary Figure 5]. ALDEx2 compositional analysis at the phylum level identified significant differences in Proteobacteria at Weeks 0 and 6 compared with healthy controls in both diets [Supplementary Tables 11–20]. For additional ALDEx2 compositional data analysis, see Supplementary Results.⁴¹

3.1.2. Weeks 6–12: further decrease in Proteobacteria

Continued dietary treatment with CDED + PEN between Weeks 6 and 12 resulted in a further decrease in Proteobacteria towards healthy controls. However, *Escherichia* and *Sutterella* among Proteobacteria genera remained more abundant in CD patients and remained significantly different from healthy samples [Wilcoxon *p* <0.05 and LDA >2] [Figure 4; Supplementary Figure 6]. Of note, there was a much higher relative abundance of *Escherichia* at baseline during the inflamed state compared with other Proteobacteria [Figure 4]. Among the expansion in Firmicutes, *Faecalibacterium* and *Blautia* became more abundant in CD patients but were still significantly different from healthy controls. *Anaerostipes, Ruminococcus*, bacilli, Porphyromonadaceae, Bifidobacteriales, Ascomycota, and Caudovirales were more abundant in healthy samples, compared with CDED + PEN at Week 12 [Supplementary Figure 6].



Figure 4. Changes in relative abundance [incl. standard deviation] of selected Proteobacteria across time points for children on CDED + PEN and EEN compared to healthy controls. EEN, exclusive enteral nutrition; CDED + PEN, Crohn's disease exclusion diet + partial enteral nutrition.

Gradually resuming normal diet between Weeks 6 and 12 after EEN resulted in an increase in Proteobacteria, namely *Enterobacter*, away from healthy controls. Among Firmicutes, *Streptococcus*, *Blautia*, and *Faecalibacterium* increased with resuming normal diet, whereas Peptostreptococacceae, *Eubacterium*, *Anaerostipes*, *Coprococcus*, and *Ruminococcus* remained more abundant in healthy controls [Supplementary Figure 6 and Supplementary Table 10].

Compositional analysis with Songbird in CDED + PEN samples again revealed no significant changes in Firmicutes [for w6 vs w12, nor w0 vs w12], except for Oscillospiraceae [w0 vs w12, p = 0.03]. At a phylum level, Proteobacteria continued to decrease between Weeks 6 and 12, sustaining the significant decrease compared with baseline [w6 vs w12, p = 0.16; w0 vs w12, p < 0.01]. Again this observation was mostly explained by a decline in γ -Proteobacteria [p < 0.01] mainly through *Escherichia* genera [p < 0.01, Supplementary Figure 4]. Changes in EEN were not significant across all time points in this analysis [all p > 0.05; Supplementary Figure 5]. ALDEx2 analysis at the phylum level identified a significant difference in Proteobacteria at Week 12 for EEN samples compared with healthy controls. However, CDED + PEN samples at Week 12 did not differ significantly from healthy controls [Supplementary Tables 11–20]. For additional ALDEx2 analysis see Supplementary Results.

3.2. Microbiome metabotypes

In order to move beyond association studies with separate enzymes/metabolites, we applied unsupervised Bayesian analysis of community metabolism [BiomeNet] to identify microbial metabotypes associated with health, disease, and achieving remission. In comparing healthy controls with all CD patients in unsupervised analyses, we found that patients' samples grouped into two metabotypes [M1 and M2; Supplementary Figure 7]. All healthy controls were grouped within one metabotype [M1] at a posterior probability [PP] >0.9. CD patients possessed a mixture of the two metabotypes, with mixtures related to stage of treatment. CD patients achieving remission showed a steady increase in the M1 contribution as nutritional therapy progressed. Patients achieving remission increased M1 over treatment from 48% prior to treatment to 63% at Week 6 and 74% at Week 12, and changes in the mixture weights were significant (paired Wilcoxon sign rank test; baseline vs Week 6 [p = 0.03]; baseline vs Week 12 [p < 0.01]; Week 6 vs 12 [p < 0.01]).

Nineteen major subnetworks of reactions were identified with differing contributions to M1 and M2. Among the pathways identified within metabotypes, one subnetwork of reactions differed substantially between the two metabotypes [subsystem 29—Supplementary Figure 7 and SupplementaryTable 21]. Within this subnetwork were a number of reactions involving the metabolism of various sugars, notably their modification by phosphotransferases [Supplementary Table 21].

The identified metabotypes were also significantly different when comparing metagenomic diversity of samples across different time points using Bray-Curtis distances [Permanova p < 0.01, Supplementary Figure 8]. Main taxa that contributed to M1 [based on coefficient, see Supplementary Figure 8] were Prevotella copri, Bacteroides cacceae, Alistipes putredinis, Faecalibacterium praustnitzii, Bacteroides stercoris, and Bacteroides uniformis. Taxa contributing mainly to M2 [based on coefficient, see Supplementary Figure 8] were Bacteroides ovatus, Ruminococcus torques, Akkermansia muciniphila, Escherichia coli, Clostridium bolteae, Veillonella parvla, and Enterococcus faecalis. To get more insight into the microbiome community structure within the metabotypes, we analysed 16S rRNA gene data using BioMiCo [for the same CD patients as were used in whole-metagenome analysis above]. We identified four



Figure 5. Metabotype 1 community structure. Cytoscape visualisation of the community structure identified from BioMiCo analysis of Metabotype 1 samples using taxa identified in 16S rRNA gene data. Nodes represent predominate taxa identified in the analysis, with size showing the summed posterior probability of the contribution of those taxa across assemblages. Edges indicate taxa that co-occurred in an assemblage.

major microbial assemblages [PP >0.1], two defining M1 and two defining M2, and eight minor microbial assemblages [0.1 >PP >0.05], five defining M1 and three defining M2. M2 metabotype was associated with high Proteobacteria with similar contributing taxa as described above (M1 [blue] Figure 5, M2 [red] Figure 6]). Main contributing taxa to metabotype communities identified with the BioMiCo analysis were similar to the taxa with the largest coefficients from the Permanova test based on metabotype labels shown in the non-metric multi-dimensional scaling plot of Bray– Curtis distances [Supplementary Figure 8]. In addition, community structure identified using topic models [NMF and STM] yielded nearly identical results to BioMiCo [see Supplementary Data: Metabotypes -NMF and STM].

3.3. SCFA

There were no significant differences between SCFAs at Week 0 between CDED + PEN and EEN. Faecal SCFA concentrations did not change significantly across the three time points in CDED + PEN [p > 0.05]. Total SCFA concentration as well as acetate, propionate, and butyrate concentrations, declined significantly between baseline and Week 6 for samples reaching remission with EEN [Welch's t test p < 0.01, p < 0.01, p = 0.02, and p < 0.01, respectively] and increased between Week 6 and 12 with exposure to other food [p = 0.01, p = 0.02, p = 0.11, and p < 0.01, respectively] [Supplementary Figure 9 and Supplementary Tables 22–25]. Total SCFA, acetate, proprionate, and butyrate differed significantly between CDED + PEN and EEN samples reaching remission at Week 6 [p < 0.01, p < 0.01 p = 0.02, p = 0.01, p < 0.01, respectively], but these differences were lost at Week 12.

SCFA concentrations were also associated with M1 and M2 mixtures in patients [90% PP cut-off]. Metabotype M1 was associated with higher concentrations in butyrate [p = 0.02] and valerate [p < 0.001] [Supplementary Figure 10]. Genes involved in butyrate formation were also associated with M1 and M2 [Supplementary Figures 12 and 13]. Using 90% PP [the most informative cut-off



Figure 6. Metabotype 2 community structure. Cytoscape visualisation of the community structure identified from BioMiCo analysis of metabotype 2 samples using taxa identified in 16S rRNA gene data. Nodes represent predominate taxa identified in the analysis, with size showing the summed posterior probability of the contribution of those taxa across assemblages. Edges indicate taxa that co-occurred in an assemblage.

for comparison with healthy controls], genes significantly increased in M1 were butyrate kinase [K00929], phosphate butyryltransferase [K00634], 4-hydroxybutyryl-CoA dehydratase [K14534], and β -lysine-5,6-aminomutase [K01844],and genes significantly decreased in M1 included genes in L-glutamate degradation to butyrate and enoyl-CoA hydratase [K00134, K01035, K08318, K01029, K01615, K01039, K01040, K01825, K01782, K01692, K01715] [Supplementary Figures 13–16].

3.4. Bile acids

There were no significant differences in conjugated or unconjugated primary BA [priBA], secondary BA [secBA], or total BA concentrations between CDED + PEN and EEN samples at baseline. Total BA levels were stable throughout all time points in both EEN and CDED + PEN [Supplementary Figure 17]. EEN samples showed a decrease in priBA concentrations at Week 6 [p = 0.04], which was not sustained through Week 12 [p = 0.095]. CDED + PEN did not induce changes in the concentrations of priBA across all time points. When looking at the predominant secBA only [LCA and DCA] across the time points, there was a non-significant increase in EEN samples at Week 6 [p = 0.06] [Supplementary Figure 18], whereas total secBA levels remained stable over all different time points with both dietary strategies.

4. Discussion

We have demonstrated that diet-induced remission is associated with a correction of dysbiosis and change in both the composition of the microbial community as well as its metabolic pathways towards that of the microbial community in healthy controls, which was particularly striking with CDED + PEN. However, 6 weeks of diet was not enough to fully correct the microbiome to a community comparable to healthy controls.

We have previously shown, using 16S rRNA amplicon data limited to phylum level, that CDED + PEN continued to change the microbiome between Week 6 and Week 12. Patients on EEN generally rebounded to pre-treatment dysbiosis at Week 12, with exposure to habitual diet accompanied by a rebound in inflammation, despite ongoing treatment with similar amounts of PEN.^{10,43} In the present study, we show that dietary therapy decreased the relative abundance of Proteobacteria, increased Firmicutes and Bacteroidetes down to a genus level with metagenomic analysis, and induced a gradual change in metabotype towards that of healthy controls. Diet-induced remission on the other hand was not associated with any significant sustained changes in SCFAs or BAs. Our results provide more insight into the roles of microbiota composition, functionality, and metabolism in dietinduced remission.

Dietary Therapy in Paediatric Crohn's Disease

Looking for commonalities between EENand CDED + PEN-induced remission at the genus level may provide insight as to which change in taxa is driving response and correction of dysbiosis, i.e. 're-biosis'. Concordance for entering remission between diets was found only among genera from Proteobacteria, which demonstrated a decline. Escherichia, Haemophilus, and Citrobacter demonstrated the highest relative abundance during the pre-treatment inflammatory state and declined towards that of healthy controls with diet. This could be due to a direct effect of the diet [low fat, low haeme, low simple carbohydrates in EEN and CDED + PEN] as well as indirectly due to the reduction in inflammation. The increase in Firmicutes was not concordant across the different treatment strategies, as different genera increased or decreased depending more on which diet the patient was following rather than the general response. Whereas improvements in clinical condition [eg. gastrointestinal transit time, reduced intestinal inflammation, and oxygen availability] can also lead to microbiome modification, there was a repeated association of diet-induced clinical remission with a reduction in Proteobacteria, using various methodologies including compositional analysis, although results differed according to the technique used. Correcting for the compositional nature of the microbiome is especially challenging in a dysbiotic environment with a large sparse dataset. Our results suggest that the drop in Proteobacteria is likely to play an important role in achieving clinical remission with dietary therapy, particularly with CDED + PEN. The observed increase in Firmicutes may be in part due to secondary niche expansion, and in part to different substrates in the two diets. The possibility of secondary niche expansion is also supported by the observation that Firmicutes expansions occurred with EEN, which is devoid of fibre which normally drives Firmicutes expansion.

A previous study compared compositional dysbiosis between patients and healthy controls with EEN, high caloric intake PEN, and anti-tumour necrosis factor [TNF], and demonstrated that PEN [ranging from 50% to 90% of the volume of formula used in EEN] with exposure to free diet steered composition away from that of healthy controls.³⁰ Here we show that CDED + PEN is associated with a gradual correction of dysbiosis and the associated metabolite pathway profile towards healthy controls, which can contribute to a more sustained remission in children with mild to moderate CD. The metabotype of patients receiving CDED + PEN for active disease entering diet-induced remission shifted gradually towards the M1 metabotype that characterises healthy children. This metabotype represents a compositional profile of primarily Bacteroidetes and Firmicutes. It is interesting to note that the CDED is a low-fat diet with mandatory exposure to fruits and resistant starch. Low-fat diets will increase Bacteroidetes at the expense of Firmicutes, but during a high Proteobacteria state, the combination of low fat/high fibre exposure will likely favour correction of dysbiosis by decreasing Proteobacteria and increasing both Bacteroidetes and Firmicutes.44 This observation shows that the dysbiosis present in CD is also functional. However, these community and functional changes towards a healthy microbiome 'state' likely take longer than the period it takes to achieve clinical remission, as most patients achieving clinical remission by Week 6 were already in remission or clinical response by Week 3, whereas metabotype continued to correct over 12 weeks

and still did not reach 100% M1 metabotype.⁴⁵ Reaching 'normobiosis'—ie. microbiome patterns as found in healthy hosts—and sustaining a healthy metabotype for a longer period of time with dietary therapy could therefore prolong sustained clinical remission, once it has been reached by avoiding rebound changes to baseline signatures.

The gradual metabotype improvements from baseline to Weeks 6 and 12 indicate that also at the functional level, microbiome-focused therapy might require a more sustained intervention. We found that M2 [predominant in active disease] is associated with lower SCFA concentrations and pathways associated with phosphotransferases, which have been described to have a wide range of regulatory roles including biofilm formation and virulence and are involved in a lot of different complex sugar metabolisms.⁴⁶⁻⁴⁹ Using our metagenome data, combining various Bayesian analytical frameworks, we show that lower SCFAs in the higher M2 state may be due to both reduced production [less SCFA producers from pyruvate] as well as increased usage [more SCFA consumption by members of the disease-associated community and use of alternative butyrate production pathways] [Supplementary Data].50-55 Increased metabolic capacity of SCFA synthesis and higher butyrate levels at baseline have been shown to play a role as markers of a more stable, health-associated metabotype, as was shown recently to be important for maintaining anti-TNF and azathioprine remission.56,57 However, measured SCFA concentrations [which only reflect a small amount of total intestinal SCFA metabolism] were not associated with achieving clinical remission with EEN or CDED + PEN.

Bile acids [BA] can modulate the intestinal immune response and it is known that their composition can be altered by changes in the gut bacterial community.^{58,59} We have previously shown that deconjugation [via bile-salt hydrolase] remains intact and that reduced dehydroxylation capacity leads to impaired conversion of priBA into secBA, resulting in a high priBA/low secBA profile in CD dysbiosis.⁶⁰ Some secBA have been described to have anti-inflammatory functions.^{61,62} Here, we show that EEN induced a decrease in priBA and, when focusing only on LCA + DCA, there was a corresponding increase of secBA. This was not seen in CDED + PEN. The change in bile acid pool produced by EEN seems to be influenced more by a radical change in diet exposure, rather than being the driver behind diet-induced remission in general. Taken together, our data for SCFA and BA suggest that these metabolites are not the main drivers for the early clinical response to dietary intervention as a whole.

Our study raises the interesting notion that the microbiome could—and perhaps should—be a treatment target in CD. We show re-biosis as a response to therapy and a concomitant change in the microbiome metabolic signature over the course of dietary treatment, consistent with healthy controls. Functional and taxonomic capacity of re-biosis might therefore become a more expansive biomarker of remission, showing a holistic perspective of gut 'health' during disease.

There are several limitations to our study. We did not study mucosal bacteria, as this would have required a full colonoscopy after 12 weeks, which is currently not indicated in paediatric CD. Our analysis was metagenomic in nature, implying metagenomic functional capacity, and only SCFA and BA were measured. However, this is one of the few studies in which metagenomic pathways and measurement of an end product of the metabolome were performed simultaneously in rigorously defined patients with uniform therapies and outcomes. Our data were not cross-sectional and most of the patients were not on any medication at baseline, as medications also induce changes to the microbiome. Samples were not available for BA analysis for all time points and for all patients for whom metagenome sequencing data was obtained, due to limited stool sample volume. A common limitation of metagenomics sequencing studies is tackling the relative abundance issue of microbiome changes, which we addressed through a combination of various analytical approaches. We used healthy controls from previously published data as a comparator in this study, which were sequenced at a different facility. However, in comparing CD patients with these healthy controls, we believe our findings clearly show that we are looking at biological changes over time rather than a finding induced by differences in sequencing methods, as healthy controls clearly possessed M1 metabotypes and CD patients had a mixture of M1 + M2, with M1 contribution increasing in time with reaching remission at Week 6 across all samples. Last, although we have provided data to suggest that a decline in Proteobacteria is a major pathway associated with dietary therapy-induced remission, it is impossible to know from 6-week intervals which taxa change first with clinical remission, as suppression of one group tends to lead to niche expansion of another.

The data presented here confirm our previously reported 16S analysis that sustained dietary therapy beyond 6 weeks with CDED + PEN avoids the 'rebound' in disease-associated species, notably Proteobacteria and without an early drop in Firmicutes, when oral diet is reintroduced. In the previous study,¹⁰ we demonstrated an increase in faecal calprotectin associated with reintroduction of free oral diet following EEN, and this was recently also reported in an independent Scottish cohort.²² These translational studies suggest that longer sustained dietary therapy >12 weeks is necessary to consolidate the early microbiome changes associated with dietary change, even if clinical remission is already achieved.^{15,63} As CDED + PEN is more tolerable than the monotonous EEN, this could be feasible in daily practice.

5. Conclusion

In conclusion, correction of dysbiosis with dietary therapy appears to occur both from a compositional and functional perspective. Twelve weeks of diet brings the microbiome to a state more similar to healthy controls, but this time frame appears insufficient to achieve complete re-biosis. Although M1 ['healthy'] metabotype increases with time and is associated with increased SCFA levels, measured faecal SCFA do not increase by 12 weeks and do not correlate with achieving clinical remission. The contribution of such 'beneficial' SCFAproducing species to achieving clinical remission does not appear to be as important as the reduction of Proteobacteria. This suggests that reduction in 'pathobionts' is either an earlier stage on the path to recovery or that it is more important for reduction in inflammation than the recovery of beneficial taxa to prevent inflammation. Indeed, reduction in Proteobacteria may enable niche expansion of beneficial taxa due to a change in the microenvironment. Other metabolic processes [notably of Proteobacteria] may therefore contribute more important features of the microbiome community functional shift associated with achieving clinical remission and healing. Restoration of the microbiome and

metabolome with beneficial taxa might be required to maintain this state to prevent recurrence of inflammation or to achieve a 'cure' in the future. As the role of the microbiota and dysbiosis as instigators of gut inflammation becomes clearer, correction of dysbiosis might become an important future clinical goal of therapy, leading to incorporation of strategies targeting both the microbiome and inflammation to alter progression or recurrence of disease.

The whole shotgun metagenomic sequencing data of CDED-RCT participants used in this study are available under accession number PRJEB25264 at the European Nucleotide Archive. Whole metagenomics sequencing data from healthy controls are available under accession number SRP057027 at the Sequence Read Archive.

Funding

JVL was supported by a Canadian Institutes of Health Research [CIHR]-Canadian Association of Gastroenterology-Crohn's Colitis Canada New Investigator Award [2015-2019], a Canada Research Chair Tier 2 in Translational Microbiomics [2018-2019] and a Canadian Foundation of Innovation John R. Evans Leadership fund [awards #35235 and #36764], a Nova Scotia Health Research Foundation [NSHRF] establishment award [2015-2019], an IWK Health Centre Research Associateship and a CIHR-SPOR-Chronic Diseases grant [Inflammation, Microbiome, and Alimentation: Gastro-Intestinal and Neuropsychiatric Effects: the IMAGINE-SPOR chronic disease network], by the Wetenschappelijke Adviesraad of Stichting Steun Emma kinderziekenhuis, and by a Clinical Network Research Award from the Crohn's and Colitis Foundation [#585718]. EW was supported by grants from the Crohn's and Colitis Foundation and Crohn's Colitis Canada as well as CIHR. Nestlé Health Science kindly provided Modulen to all participating sites to ensure uniformity of the formula used among participants, and provided the formula to enrolled patients for the duration of the study. The conduct of the study in Canada [Halifax, Edmonton] was supported by local divisional funds, a Women and Children's Health Research Institute [WCHRI] Research Capacity Building Award [EW], a Canadian Institutes of Health Research [CIHR] New Investigator award [JVL], and Canada Research Chair Tier 2 in Translational Microbiomics [VL]. The funders of the study had no role in the design of the study, data collection or analysis, interpretation of data, writing of the report, or in the decision to submit the paper for publication. None of the funders had access to the data.

Conflict of Interest

JVL reports consulting, travel, and/or speaker fees and research support from AbbVie, Janssen, Nestlé Health Science, Novalac, Pfizer, Merck, P&G, GSK, Illumina, Otsuka; EW reports personal fees from Janssen, AbbVie, Nestlé Health Science, and Mead Johnson Nutrition outside the submitted work; RSB reports personal fees from consulting to Nestlé Health Science, during the conduct of the study, and personal fees as invited speaker by Nestlé Health Science and Takeda, outside the submitted work. RS: reports personal fees from consulting to Nestlé Health Science. KG received research grants and speaker's fees and had conference attendance paid by Nutricia, Nestlé, Mylan, and Dr Falk. WJdJ is funded by the Dutch Ministry of Economic Affairs, LSH-TKI, Health Holland. MAB is consultant for Norgine, Coloplast, Danone, Takeda, Allergan, Shire, FrieslandCampina, United Pharmaceuticals. CMV, MG, KAD, JPB, VS, JDL, SP, AC, and DMP have nothing to disclose.

Acknowledgements

The authors would like to thank all participating children and their families. The authors also thank Arie Levine for design of the diet and his contribution to the study, as well as the many colleagues in paediatric gastroenterology and nutrition at all participating sites, who made this study possible: in Tel Aviv [Tamar Pfeffer-Gik who initially coordinated the study and Chen Sarbagili Shabat], Halifax [Anthony Otley, Mohsin Rashid, Angela Noble, Jessica Connors, Jennifer Haskett, Lisa Parkinson-McGraw, Brad MacIntyre], Edmonton [Hien Huynh, Matthew Carroll, Alexandra Petrova, Min Chen, Jessica Wu].

Antonia Harvey, registered dietitian, and M. Whebby, research assistant, modified the CDED recipes for use in Canada and maintained the Canadian CDED study website, supervised by Shannan Grant, Department of Applied Human Nutrition, Mount Saint Vincent University.

Author Contributions

JDL: control enrolment, manuscript review. EW: patient enrolment, data analysis, writing of manuscript. RS: patient enrolment and review of manuscript. RSB: design of support system, design of case report form, coordination of study, data management and analysis, article figures and tables. JVL, principal investigator for the Canadian arm: design of patient support system, funding of study, patient enrolment, data analysis and writing of manuscript, perform ance of the microbiome translational aspects of the study along with PB, KAD, and JPB. SV, KG: performed the SCFA analysis. MAB, WJdJ: manuscript writing and funding of translational analysis. CMV: manuscript writing, BA analysis. MG, AC, SP, DMP: performed the BA analysis.

Supplementary Data

Supplementary data are available at ECCO-JCC online.

References

- 1. Coward S, Clement F, Benchimol EI, *et al.* Past and future burden of inflammatory bowel diseases based on modeling of population-based data. *Gastroenterology* 2019;**156**:1345–53.e1344.
- Levine A, Sigall Boneh R, Wine E. Evolving role of diet in the pathogenesis and treatment of inflammatory bowel diseases. *Gut* 2018;67:1726–38.
- Lloyd-Price J, Arze C, Ananthakrishnan AN, et al. Multi-omics of the gut microbial ecosystem in inflammatory bowel diseases. *Nature* 2019;569:655–62.
- Gevers D, Kugathasan S, Denson LA, et al. The treatment-naive microbiome in new-onset Crohn s disease. Cell Host Microbe 2014;15:382–92.
- Duvallet C, Gibbons SM, Gurry T, et al. Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. Nat Commun 2017;8:1784.
- Douglas GM, Hansen R, Jones CMA, *et al.* Multi-omics differentially classify disease state and treatment outcome in pediatric Crohn's disease. *Microbiome* 2018;6:13.

- Ding NS, McDonald JAK, Perdones-Montero A, et al. Metabonomics and the gut microbiome associated with primary response to anti-TNF therapy in Crohn's disease. J Crohns Colitis 2020;14:1090–102. doi: 10.1093/ecco-jcc/jjaa039.
- Kennedy NA, Heap GA, Green HD, et al. Predictors of anti-TNF treatment failure in anti-TNF-naive patients with active luminal Crohn's disease: a prospective, multicentre, cohort study. Lancet Gastroenterol Hepatol 2019;4:341–53.
- Levine A, Wine E, Assa A, *et al.* Crohn's disease exclusion diet plus partial enteral nutrition induces sustained remission in a randomized controlled trial. *Gastroenterology* 2019;157:440–50. e448.
- 11. Turnbaugh PJ. Diet should be a tool for researchers, not a treatment. *Nature* 2020;577:S23.
- Sanna S, van Zuydam NR, Mahajan A, et al. Causal relationships among the gut microbiome, short-chain fatty acids and metabolic diseases. Nat Genet 2019;51:600–5.
- Quince C, Ijaz UZ, Loman N, *et al.* Extensive modulation of the fecal metagenome in children with Crohn's disease during exclusive enteral nutrition. *Am J Gastroenterol* 2015;110:1718–29; quiz 1730.
- 14. Connors J, Basseri S, Grant A, *et al.* Exclusive enteral nutrition therapy in paediatric Crohn's disease results in long-term avoidance of corticosteroids: results of a propensity-score matched cohort analysis. *J Crohns Colitis* 2017;11:1063–70.
- 15. Cohen-Dolev N, Sladek M, Hussey S, et al. Differences in outcomes over time with exclusive enteral nutrition compared with steroids in children with mild to moderate Crohn's disease: results from the GROWTH CD study. J Crohns Colitis 2018;12:306–12.
- Grover Z, Lewindon P. Two-year outcomes after exclusive enteral nutrition induction are superior to corticosteroids in pediatric Crohn's disease treated early with thiopurines. *Dig Dis Sci* 2015;60:3069–74.
- 17. Levine A, Turner D, Pfeffer Gik T, et al. Comparison of outcomes parameters for induction of remission in new onset pediatric Crohn's disease: evaluation of the porto IBD group 'growth relapse and outcomes with therapy' [GROWTH CD] study. Inflamm Bowel Dis 2014;20:278–85.
- Ruemmele FM, Veres G, Kolho KL, *et al.* Consensus guidelines of ECCO/ESPGHAN on the medical management of pediatric Crohn's disease. *J Crohns Colitis* 2014;8:1179–207.
- Van Limbergen J, Haskett J, Griffiths AM, *et al.* Toward enteral nutrition for the treatment of pediatric Crohn disease in Canada: a workshop to identify barriers and enablers. *Can J Gastroenterol. Hepatol* 2015;29:351–6.
- Lawley M, Wu JW, Navas-Lopez VM, et al. Global variation in use of enteral nutrition for pediatric Crohn disease. J Pediatr Gastroenterol Nutr 2018;67:e22–9.
- Svolos V, Hansen R, Nichols B, *et al.* Treatment of active Crohn's disease with an ordinary food-based diet that replicates exclusive enteral nutrition. *Gastroenterology* 2019;156:1354–67.e1356.
- Logan M, Clark CM, Ijaz UZ, *et al.* The reduction of faecal calprotectin during exclusive enteral nutrition is lost rapidly after food re-introduction. *Aliment Pharmacol Ther* 2019. doi: 10.1111/ apt.15425.
- 23. Gerasimidis K, Bryden K, Chen X, *et al.* The impact of food additives, artificial sweeteners and domestic hygiene products on the human gut microbiome and its fibre fermentation capacity. *Eur J Nutr* 2019. doi:10.1007/s00394-019-02161-8.
- 24. Sigall Boneh R, Van Limbergen J, Wine E, et al. Dietary therapies induce rapid response and remission in active paediatric Crohn's disease. Clin Gastroenterol Hepatol 2021;19(4):752–59. doi:10.1016/j.cgh.2020.04.006.
- 25. ChassaingB,CompherC,BonhommeB,etal.Randomized controlledfeeding study of dietary emulsifier carboxymethylcellulose reveals

detrimental impacts on the gut microbiota and metabolome. *Gastroenterology* 2022;**162**:743–56.

- Maslowski KM, Mackay CR. Diet, gut microbiota and immune responses. Nat Immunol 2011;12:5–9.
- Houron C, Ciocan D, Trainel N, *et al*. Gut microbiota reshaped by pectin treatment improves liver steatosis in obese mice. *Nutrients* 2021;13. doi:10.3390/nu13113725.
- Shinohara K, Ohashi Y, Kawasumi K, Terada A, Fujisawa T. Effect of apple intake on fecal microbiota and metabolites in humans. *Anaerobe* 2010;16:510–5.
- Turner D, Griffiths AM, Walters TD, et al. Mathematical weighting of the pediatric Crohn's disease activity index [PCDAI] and comparison with its other short versions. *Inflamm Bowel Dis* 2012;18:55– 62.
- Lewis JD, Chen EZ, Baldassano RN, et al. Inflammation, antibiotics, and diet as environmental stressors of the gut microbiome in pediatric Crohn's disease. Cell Host Microbe 2015;18:489–500.
- Wu GD, Chen J, Hoffmann C, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science 2011;334:105–8.
- Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 2014;30:2114–20.
- Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods 2012;9:357–9.
- Franzosa EA, McIver LJ, Rahnavard G, et al. Species-level functional profiling of metagenomes and metatranscriptomes. Nat Methods 2018;15:962–8.
- Tange O. GNU Parallel 2018. Geneva: CERN, Zenodo, 2018, doi: 10.5281/zenodo.1146014.
- Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 2000;28:27–30.
- 37. Shafiei M, Dunn KA, Chipman H, Gu H, Bielawski JP. BiomeNet: a Bayesian model for inference of metabolic divergence among microbial communities. *PLoS Comput Biol* 2014;10:e1003918.
- Segata N, Waldron L, Ballarini A, et al. Metagenomic microbial community profiling using unique clade-specific marker genes. Nat Methods 2012;9:811–4.
- Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60.
- Morton JT, Marotz C, Washburne A, *et al.* Establishing microbial composition measurement standards with reference frames. *Nat Commun* 2019;10:2719.
- 41. Fernandes AD, Reid J, Macklaim JM, McMurrough TA, et al. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome* 2014;5:15.
- Fedarko MW, Martino C, Morton JT, et al. Visualizing 'omic feature rankings and log-ratios using Qurro. NAR Genom Bioinform 2020;2:lqaa023.
- Verburgt CM, Dunn KA, Van Limbergen JE. Dietary therapy reduces pro-inflammatory microbiome features in pediatric Crohn's disease. *J Crohns Colitis* 2022;16:682–4. doi:10.1093/ecco-jcc/jjab197.
- 44. Abulizi N, Quin C, Brown K, *et al.* Gut mucosal proteins and bacteriome are shaped by the saturation index of dietary lipids. *Nutrients* 2019;11. doi:10.3390/nu11020418.
- 45. Sigall Boneh R, Van Limbergen J, Assa A, et al. DOP42 Dietary therapies induce rapid response and remission in active paediatric Crohn's disease. J Crohns Colitis 2019;13:S050.
- 46. Horng YT, Wang CJ, Chung WT, *et al.* Phosphoenolpyruvate phosphotransferase system components positively regulate

Klebsiella biofilm formation. J Microbiol Immunol Infect 2018;51:174-83.

- 47. Lin D, Fan J, Wang J, *et al*. The fructose-specific phosphotransferase system of Klebsiella pneumoniae is regulated by global regulator CRP and linked to virulence and growth. *Infect Immun* 2018;86. doi:10.1128/iai.00340-18.
- 48. Lim S, Seo HS, Jeong J, Yoon H. Understanding the multifaceted roles of the phosphoenolpyruvate: phosphotransferase system in regulation of Salmonella virulence using a mutant defective in ptsI and crr expression. *Microbiol Res* 2019;223–225:63–71.
- 49. Deutscher J, Aké FMD, Derkaoui M, et al. The bacterial phosphoen olpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent proteinprotein interactions. *Microbiol Mol Biol Rev* 2014;78, 231–56.
- 50. Jenkins LS, Nunn WD. Regulation of the ato operon by the atoC gene in Escherichia coli. *J Bacteriol* 1987;**169**:2096–102.
- Llewellyn SR, Britton GJ, Contijoch EJ, et al. Interactions between diet and the intestinal microbiota alter intestinal permeability and colitis severity in mice. *Gastroenterology* 2018;154:1037–46. e1032.
- 52. Singh V, Yeoh BS, Walker RE, et al. Microbiota fermentation-NLRP3 axis shapes the impact of dietary fibres on intestinal inflammation. Gut 2019. doi:10.1136/gutjnl-2018-316250.
- Makki K, Deehan EC, Walter J, Backhed F. The impact of dietary fiber on gut microbiota in host health and disease. *Cell Host Microbe* 2018;23:705–15.
- 54. Koh A, De Vadder F, Kovatcheva-Datchary P, Backhed F. From dietary fiber to host physiology: short-chain fatty acids as key bacterial metabolites. *Cell* 2016;165:1332–45.
- 55. Ijaz UZ, Quince C, Hanske L, *et al.* The distinct features of microbial 'dysbiosis' of Crohn's disease do not occur to the same extent in their unaffected, genetically-linked kindred. *PLoS One* 2017;12:e0172605.
- 56. Aden K, Rehman A, Waschina S, et al. Metabolic functions of gut microbes associate with efficacy of tumor necrosis factor antagonists in patients with inflammatory bowel diseases. *Gastroenter*ology 2019;157:1279–92.e1211.
- Effenberger M, Reider S, Waschina S, et al. Microbial butyrate synthesis indicates therapeutic efficacy of azathioprine in IBD patients. J Crohns Colitis 2021;15:88–98. doi:10.1093/ecco-jcc/jjaa152.
- Chen ML, Takeda K, Sundrud MS. Emerging roles of bile acids in mucosal immunity and inflammation. *Mucosal Immunol* 2019;12:851–61.
- Wahlström A, Sayin SI, Marschall HU, Bäckhed F. Intestinal crosstalk between bile acids and microbiota and its impact on host metabolism. *Cell Metab* 2016;24:41–50.
- 60. Connors J, Dunn KA, Allott J, *et al.* The relationship between fecal bile acids and microbiome community structure in pediatric Crohn's disease. *Isme J* 2020;14:702–13.
- 61. Duboc H, Rajca S, Rainteau D, *et al.* Connecting dysbiosis, bileacid dysmetabolism and gut inflammation in inflammatory bowel diseases. *Gut* 2013;62:531–9.
- Sinha SR, Haileselassie Y, Nguyen LP, *et al.* Dysbiosis-induced secondary bile acid deficiency promotes intestinal inflammation. *Cell Host Microbe* 2020;27:659–70.e655.
- 63. Lee D, Baldassano RN, Otley AR, et al. Comparative effectiveness of nutritional and biological therapy in North American children with active Crohn's disease. *Inflamm Bowel Dis* 2015;21:1786–93.