

ARTICLE

Peppermint oil effects on the gut microbiome in children with functional abdominal pain

Santosh Thapa^{1,2} | Ruth Ann Luna^{1,2} | Bruno P. Chumpitazi^{3,4} | Numan Oezguen^{1,2} | Susan M. Abdel-Rahman⁵ | Uttam Garg⁶ | Salma Musaad^{3,4} | James Versalovic^{1,2} | Gregory L. Kearns⁷ | Robert J. Shulman^{3,4}

¹Department of Pathology, Texas Children's Microbiome Center, Texas Children's Hospital, Houston, Texas, USA

²Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, USA

³Department of Pediatrics, Baylor College of Medicine, Texas Children's Hospital, Houston, Texas, USA

⁴USDA/ARS Children's Nutrition Research Center, Texas Children's Hospital, Houston, Texas, USA

⁵University of Missouri School of Medicine, Kansas City, Missouri, USA

⁶Department of Pathology and Laboratory Medicine, Children's Mercy Hospital, University of Missouri School of Medicine, Kansas City, Missouri, USA

⁷Texas Christian University and University of North Texas Health Science Center School of Medicine, Fort Worth, Texas, USA

Correspondence

Robert J. Shulman, 1100 Bates Street, Room 8072, Houston, TX 77030, USA.
Email: rshulman@bcm.edu

Funding information

This study was supported by AT009101 (RJS) from the National Institutes of Health, the USDA/ARS under Cooperative Agreement No. 58-3092-0-001, and P30 DK56338 which funds the Texas Medical Center Digestive Disease Center. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This work is a publication of the USDA/ARS Children's Nutrition Research Center, Department of Pediatrics, Baylor College of Medicine and Texas Children's Hospital. The contents do not necessarily reflect the views or policies of the USDA, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government

Abstract

Peppermint oil (PMO) is effective in the treatment of functional abdominal pain disorders, but its mechanism of action is unclear. Evidence suggests PMO has microbicidal activity. We investigated the effect of three different doses of PMO on gut microbiome composition. Thirty children (7–12 years of age) with functional abdominal pain provided a baseline stool sample prior to randomization to 180, 360, or 540 mg of enteric coated PMO (10 participants per dose). They took their respective dose of PMO (180 mg once, 180 mg twice, or 180 mg thrice daily) for 1 week, after which the stool collection was repeated. Baseline and post-PMO stools were analyzed for microbiome composition. There was no difference in alpha diversity of the gut microbiome between the baseline and post-PMO treatment. Principal coordinate analysis revealed no significant difference in overall bacterial composition between baseline and post-PMO samples, as well as between the PMO dose groups. However, the very low abundant *Collinsella* genus and three operational taxonomic units (one belonging to *Collinsella*) were significantly different in samples before and after PMO treatment. The Firmicutes/Bacteroidetes ratio was lower in children who received 540 mg of PMO compared to the 180 mg and 360 mg dose groups ($p = 0.04$). Network analysis revealed separation between pre- and post-PMO fecal samples with the genus *Collinsella* driving the post-PMO clusters. PMO administration appeared to impact only low abundance bacteria. The 540 mg PMO dose differentially impacted the

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2021 The Authors. *Clinical and Translational Science* published by Wiley Periodicals LLC on behalf of American Society for Clinical Pharmacology and Therapeutics.

Firmicutes/Bacteroidetes ratio. A higher dose and/or longer duration of treatment might yield different results.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Peppermint oil (PMO) is used commonly to treat gut disorders. In vitro PMO can be bactericidal.

WHAT QUESTION DID THIS STUDY ADDRESS?

Does oral administration of PMO impact gut microbiome composition? Is there a dose-response impact on gut microbiome composition?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

PMO at the doses tested can impact gut microbiome composition. The highest dose of PMO (540 mg) changed the Firmicutes/Bacteroidetes ratio.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Some of the clinical benefit of PMO may be mediated through a change in gut microbiome composition. Higher doses and/or longer treatment should be tested to evaluate the impact on gut microbiome composition.

INTRODUCTION

Mint plants have been used for thousands of years as medicinals.¹ Peppermint oil (PMO) is obtained by steam distillation from peppermint leaves. PMO is commonly used to treat functional gastrointestinal conditions, such as irritable bowel syndrome and dyspepsia.²⁻⁶ The majority of evidence suggests that PMO is effective in the treatment of irritable bowel syndrome.^{2,4,5,7} How PMO exerts its beneficial effect is less clear; generally, it has been ascribed to its ability to act as an antispasmodic.⁷ However, PMO, like several other essential oils, also has been shown to have antimicrobial, antifungal, and even antiviral effects; however, these studies have been carried out primarily in vitro or in rodents.⁷⁻⁹

In a recent study, we investigated the effect of three different doses of PMO (180, 360, and 540 mg) on its pharmacokinetics in children.¹⁰ Given the use of PMO to treat functional gastrointestinal conditions, we carried out the study in children with functional abdominal pain.¹⁰ The doses studied were based on our previous pilot trial of PMO in children.¹¹ As part of that dose ranging study,¹⁰ stools were collected to investigate the effect of PMO on the gut microbiome composition in the children, with the primary outcome being the detection of a significant change in gut microbiome composition. Studying a dose range allowed us also to investigate the microbiome response to different doses of PMO. We hypothesized that PMO would impact gut microbiome composition and that there would be a dose-response impact on gut microbiome composition.

MATERIALS AND METHODS

Participants

Children 7–12 years of age with functional abdominal pain, as defined by pediatric Rome III criteria, were recruited from primary and tertiary (pediatric gastroenterology) care within the Texas Children's Hospital healthcare network based in Houston, Texas, USA, which is the largest pediatric provider in the area.¹² Children with functional abdominal pain were studied (as opposed to those with irritable bowel syndrome) based on the request from the funding agency. Informed consent was obtained from parents and assent was obtained from children. The study was approved by the Baylor College of Medicine Institutional Review Board. The study was registered at www.clinicaltrials.gov (NCT03295747).

Medical records were screened by trained research coordinators for International Classification of Disease-10th edition (ICD-10) codes for abdominal pain and functional abdominal pain. Secondary review of medical and laboratory records was performed by a pediatric gastroenterologist (author R.J.S.). Participants were screened via telephone and initially classified as having functional abdominal pain using a modified pediatric Rome III questionnaire.^{12,13}

Children were excluded if screening or review revealed a significant chronic medical condition (e.g., celiac disease and cystic fibrosis), chronic vomiting, unexplained weight loss, hematochezia, gastrointestinal (GI) tract surgery, significant developmental delay,

an organic GI disorder, or other comorbidity that could have affected study results. In addition, use of antibiotics, probiotics, PMO-containing products, or gastric acid modifying medication within the prior month was an exclusion. Women having reached menarche also were excluded in order to study a more developmentally homogeneous group.

During the baseline (prior to PMO) period, participants who passed screening maintained daily validated pain and stooling diaries for 2 weeks.^{14,15} Children self-reported the number and severity of pain episodes on a 0–10 scale with 10 being the most severe.¹⁴ The number and type of stools passed per day was recorded using the Bristol Stool Scale.¹⁵ Children also completed the Rome III Diagnostic Questionnaire for Pediatric Functional GI Disorders. Confirmation of the diagnosis of functional abdominal pain was based on evaluation of the pain and stool diaries.¹³

Study design

The decision to study 10 participants per dose was based on the results of our pilot study.¹¹ Participants were instructed to continue their usual diet during the baseline period and during administration of PMO.

Prior to administration of PMO, children collected a stool sample at home using a self-sealing container which then was kept frozen and delivered to our laboratory via courier along with the pain and stool diary. Participants subsequently came to the Children's Nutrition Research Center and were randomized (www.randomizer.org) to receive one of three doses of PMO (Pepogest; Nature's Way Products, LLC, Lehi, UT): 180, 360, or 540 mg. A pharmacist provided the PMO to the investigators based on the results of the randomization scheme. Because the investigator and participant knew how many capsules were ingested, they were not blinded to the dose received. The PMO was purchased from the manufacturer and all capsules were from the same lot number. They received their first dose of PMO and then were sent home where they continued to ingest their assigned dose of PMO daily for 1 week (180 mg once, twice, or thrice daily). The PMO was taken with the appropriate meal (breakfast; breakfast and dinner; breakfast, lunch, and dinner). They recorded the daily intake of PMO capsules and any adverse events (e.g., heartburn and gastroesophageal reflux symptoms). During this time, they again kept the pain and stooling diary and collected a stool sample during days 6 to 7 of PMO ingestion. The stool sample, PMO pill container, and pain and stool diary were returned via courier. Stool samples were stored at -80°C until further processing.

Microbiome characterization

DNA extraction and sequencing

Microbial DNA was extracted from the stool samples using the MoBio PowerSoil DNA Isolation kit (Qiagen, Hilden, Germany) following manufacturer recommendations. The 16S rRNA amplicon sequencing was performed following the standard Illumina sequencing protocol, as previously described.¹⁶ Briefly, the V4 hypervariable region of the 16S rRNA was amplified by polymerase chain reaction (PCR) using the NEXTFlex V4 Amplicon-Seq Kit 2.0 (Bioo Scientific, Austin, TX) and sequenced on the Illumina MiSeq (Illumina, San Diego, CA) platform, yielding 2×250 bp paired-end reads (median reads per sample = 46,272). Two negative controls (no template PCR controls) also were sequenced along with the samples to monitor the potential background noise (both negative controls had fewer than 500 reads post-quality filtering and were excluded from further analysis).

Sequence processing

The Illumina-sequenced paired-end fastq files (i.e., raw sequences) were demultiplexed (by sample) using the Illumina MiSeq Reporter analysis software. Primers were removed using cutadapt.¹⁷ The sequence data then were imported into QIIME 2 (version 2019.10) for processing.¹⁸ Denoising and filtering of the sequences were completed using the DADA2 (version 1.16) pipeline.¹⁹ Any features (i.e., the products of DADA2 denoising) with less than 32 nucleotides length were filtered out of the sequence table. Chimeric sequences were removed using vsearch (version 2.7.0).²⁰ Sequences were clustered de novo into operational taxonomic units (OTUs) at 97% identity using the QIIME 2-VSEARCH plugin. Taxonomy was assigned to the sequences using VSEARCH against the SILVA (version 13.2) reference database.²¹ Any unassigned and non-bacterial sequences (e.g., archaea, chloroplast-derived, and mitochondrial, etc.) were removed from the data before proceeding with analysis.

Sequence analysis

The quality-filtered feature (sequence) table, taxonomy table, representative sequences file, and tree file generated using the QIIME2 pipeline were imported into the phyloseq R-package (version 1.32.0) for subsequent analysis.²² We performed microbiome analyses in a rarefied (i.e., subsampled without replacement) read count table where the samples were rarefied at 9643 reads (the lowest read

depth in the dataset) to account for differing read depths across samples.²³

Taxonomic profiling and diversity analysis

We calculated the relative abundance of bacterial taxa across samples at the phylum, family, genus, and OTU levels. Alpha diversity, which measures the richness and evenness of the microbial community in a sample, was calculated using the Shannon and Simpson indices. Beta diversity, which measures the similarity (or dissimilarity) of the microbial community composition between samples, was characterized using the Bray-Curtis index. Additionally, beta diversity was characterized by the Aitchison distance,²⁴ which accounts for the compositional nature of microbiome data, following the centered log-ratio transformation of the read counts.²⁵ Principal coordinate analysis (PCoA) and principal component analysis (PCA) plots were used for visualization of Bray-Curtis and Aitchison distance data, respectively.

Enterotypes classification

The classification of enterotypes to the taxonomic profiles generated from our samples was performed using the webserver <https://enterotypes.org/>. Enterotype (clusters of individuals with similar microbiome composition) assignments were based on the classifier trained on stool samples from the Human Microbiome Project and MetaHIT studies.^{26,27}

Bacterial network analysis

The genus level abundance table first was normalized within each sample to the sum of all counts to get the relative abundance. The relative abundances of each genus then were transformed into 10 quantiles across all samples. The subject-genus tuples with their respective quantiles then were imported into Cytoscape 3.8.2.²⁸ The subjects and genera were interpreted as nodes and the quantiles as weights of the connecting edges. Additional statistical information about the genera discriminatory power between the pre- and post-PMO samples (Wilcoxon *p* value) was imported into Cytoscape. In Cytoscape, the “Edge-weighted Spring Embedded Layout” algorithm with the quantiles as weights was used to generate network layouts for various slices of nodes/edges combinations. We included the network generated using only genera that were present in at least five samples, had a Wilcoxon *p* less than or equal to 0.05 between pre- and post-PMO samples, and

with abundance/edges that remained after removing the lowest 0, 0.1, and 0.2 quantiles.

Statistical analysis

Overall differences in continuous variables between groups were compared using the Kruskal-Wallis test. Differences in categorical variables between groups were compared using chi-square or Fischer’s exact test as appropriate. Average pain severity and frequency per day were calculated as the average of all pain assessments.

Differences in alpha diversity between and among groups were detected using the Wilcoxon rank sum test (two groups) or Kruskal-Wallis test followed by Dunn’s test (more than two groups). We also performed 1000 bootstraps to calculate the variance of alpha diversity metrics in baseline and post-PMO samples followed by a Wilcoxon rank sum test to identify the significance of differences in variances.²⁹ Pearson’s correlation analysis was performed to investigate the association of continuous demographic variables (age, height, and body mass index of the participants) with alpha diversity (Shannon index) of the bacterial microbiome in baseline and post-PMO samples, whereas the Wilcoxon rank sum or Kruskal-Wallis test was used to assess the alpha diversity by categorical variables (sex, race, and ethnicity of the participants).

Differences in beta diversity by treatment group (baseline and post-PMO) and PMO dose (180, 360, and 540 mg) were detected using the permutational multivariate analysis of variance (PERMANOVA)³⁰ test (Adonis function with 999 permutations in the vegan R-package³¹). Firstly, PERMANOVA was performed with an unblocked single variable design (model 1 example: `adonis (bray.dist ~ treatment group, permutations = 999)`). Second, we included a factor in the model to detect if the PERMANOVA result differs from that of the unblocked single variable design (model 2 example: `adonis (bray.dist ~ treatment group*sex, permutations = 999)`). Finally, we specified the strata to test whether or not beta diversity by treatment group differs while controlling for a factor (model 3 example: `adonis (bray.dist ~ treatment group, strata = sex, permutations = 999)`). Based on the results from the model 2, we tested whether beta diversity differs by PMO treatment while controlling for sex, ethnicity, and race.

To determine whether demographics/clinical variables influenced the microbial community composition observed in our samples, we performed PERMANOVA of the Bray-Curtis dissimilarity indices (baseline and post-PMO). We tested the effects of gender (male and female), ethnicity (Hispanic and non-Hispanic), and race (Black, White, and others) on the microbiome composition in baseline and post-PMO groups separately. We also performed a

homogeneity test of the group dispersion (betadisper function in vegan) to determine the effect of sample dispersion (variance) on a significant PERMANOVA result.

Microbiome differential abundance analysis was performed using the traditional Wilcoxon rank sum test or Kruskal-Wallis test. We also used the R package analysis of variance (ANOVA)-Like Differential Expression version 2 (ALDEx2), which takes into account sample variation and compositional nature of microbiome data for differential (relative) abundance analysis.³² Benjamini-Hochberg correction was applied to the control false-discovery rate (FDR) for multiple hypotheses testing. A *p* value less than 0.05 was considered statistically significant.

RESULTS

Participant demographics and clinical characteristics

There was a statistically, but not clinically, significant difference in mean age between the three PMO dose groups (Table 1). There were no differences among the groups in the proportion of girls, body mass index, race, or ethnicity.

No differences were noted among the dose groups in mean abdominal pain severity, number of abdominal pain episodes per day, or number of stools per day at baseline, or while taking PMO. When assessing the cohort (*n* = 30),

	Peppermint Oil Dose Group			<i>p</i> Value
	180 mg <i>n</i> = 10	360 mg <i>n</i> = 10	540 mg <i>n</i> = 10	
Age (years)	11.5 ± 0.7 ^a	11.4 ± 1.0	10.2 ± 1.3	0.03
Female	8 (80%)	7 (70%)	6 (60%)	0.62
Body mass index	24.0 ± 5.3	21.4 ± 5.0	22.7 ± 4.9	0.42
Race				0.96
White	5	4	4	
Black	5	5	5	
Asian	0	1	0	
Native American	0	0	1	
Ethnicity				1.00
Hispanic	4	4	4	
Non-Hispanic	6	6	6	
Number of pain episodes per day				
Baseline	1.3 ± 1.4	2.2 ± 2.5	1.6 ± 1.0	0.38 ^c
Post-treatment	0.4 ± 0.5	0.5 ± 0.5	1.4 ± 2.6	0.81 ^d
<i>p</i> value	0.21 ^b	0.20	0.88	0.15 ^e
Pain severity 0–10				
Baseline	1.5 ± 1.3	1.8 ± 1.2	2.8 ± 1.7	0.13 ^c
Post-treatment	0.8 ± 0.8	0.7 ± 0.7	1.2 ± 1.7	0.90 ^d
<i>p</i> value	0.18 ^b	0.16	0.04	0.004^e
Number of stools per day				
Baseline	1.0 ± 0.6	1.2 ± 0.6	1.0 ± 0.5	0.66 ^c
Post-treatment	0.8 ± 0.5	0.7 ± 0.4	0.7 ± 0.3	0.89 ^d
<i>p</i> value	0.36 ^b	0.26	0.24	0.05 ^e

TABLE 1 Participant demographics and pain/stooling data

Note: Bold = significant.

Abbreviation: PMO, peppermint oil.

^aMean ± SD.

^b*p* value for rank test baseline vs PMO.

^c*p* value for Kruskal-Wallis test for difference at baseline among groups.

^d*p* value for Kruskal-Wallis test for difference after PMO administration among groups.

^e*p* value for rank test for difference between Baseline and PMO administration for participants as a whole (*n* = 30).

mean pain severity was greater during the baseline period than during PMO treatment ($p = 0.004$) with the 540 mg dose group showing a significant decrease in pain severity ($p = 0.04$). No differences among dose groups were noted regarding the number of pain episodes. The number of stools passed per day for the cohort ($n = 30$) tended to be less during the PMO treatment period ($p = 0.05$). No adverse events were noted during the study related to administration of PMO. Percent compliance with PMO ingestion did not differ among groups ($92 \pm 16\%$, $87 \pm 14\%$, and $91 \pm 7\%$ for dosing groups 180, 360, and 540, respectively; $p = 0.30$).

Microbial alpha diversity

We found no significant difference (Wilcoxon rank sum test $p > 0.05$) in the Shannon diversity (Figure 1a) or Simpson diversity (Figure 1b) metrics between baseline and post-PMO samples. However, the variance of diversity metrics in the baseline samples was significantly greater than the post-PMO samples ($p < 0.001$) with both diversity estimators (Figure 1c,d). Alpha diversity was not associated with age, height, or body mass index (Figure S1) or with sex, race, or ethnicity of the participants (Figure S2).

There was a decreasing trend in alpha diversity in the 540 mg PMO dosing group compared with the 180 and 360 mg groups (for both Shannon and Simpson indexes; Figure 2a,b), but the values did not achieve statistical

significance after FDR correction. There was no difference in the diversity by either metric comparing baseline and PMO results (Figure 2a,b).

Microbial beta diversity

The PCoA ordinations using the Bray-Curtis dissimilarity index revealed no significant difference (Adonis $p > 0.05$) in bacterial composition between baseline and post-PMO samples as well as between the PMO dose groups (Figure 3). The overall heterogeneity in community composition across samples at baseline was relatively higher (with respect to the median profile), but not statistically significant (Wilcoxon rank sum test $p > 0.05$), from the post-PMO samples (Figure 3b). Like the Bray-Curtis result, PCA of the Aitchison distance using centered log-ratio transformed data also found no significant difference (Adonis $p > 0.05$) in the community composition between baseline and post-PMO samples (Figure S3).

Demographics variables influencing the microbial community

We found only one categorical variable (ethnicity) that seemed to be related to the microbial community composition at baseline (Adonis $p < 0.05$) via univariate analysis (Table S1). However, the homogeneity test of dispersion

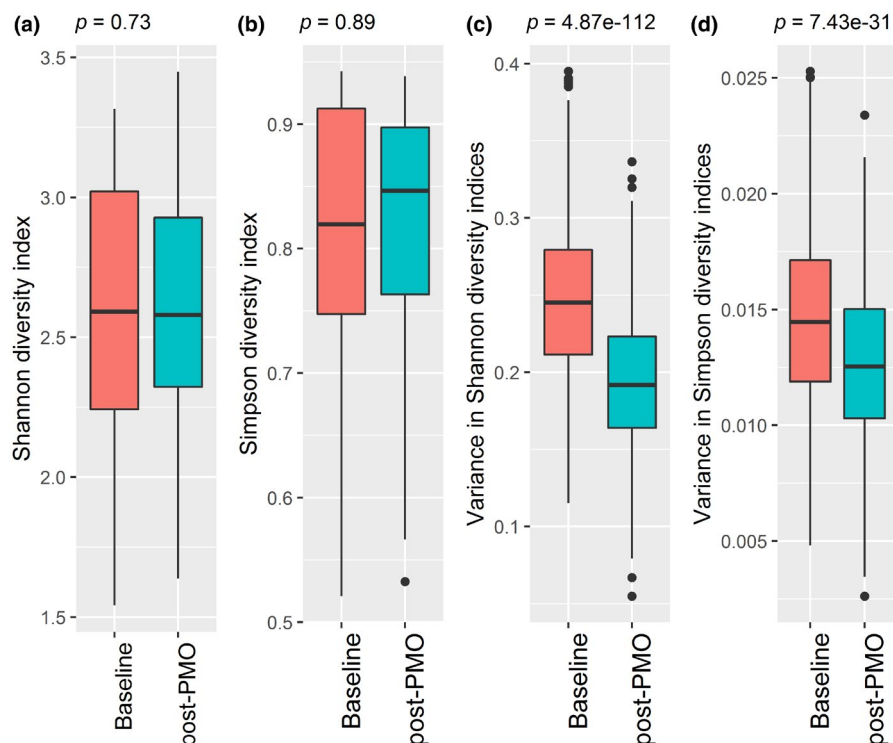


FIGURE 1 Estimators of microbial alpha diversity in baseline and PMO dose groups. Box plots showing Shannon diversity index (a), Simpson diversity index (b), variance in Shannon diversity index (c), and variance in Simpson diversity index (d) between baseline and post-PMO samples. The p values between baseline and post-PMO samples were calculated using Wilcoxon rank sum test. The variance of diversity in the baseline samples was significantly greater than that in the post-PMO samples (1000 bootstraps followed by a Wilcoxon rank sum test $p < 0.05$) for both Shannon (c) and Simpson (d) indices. PMO, peppermint oil

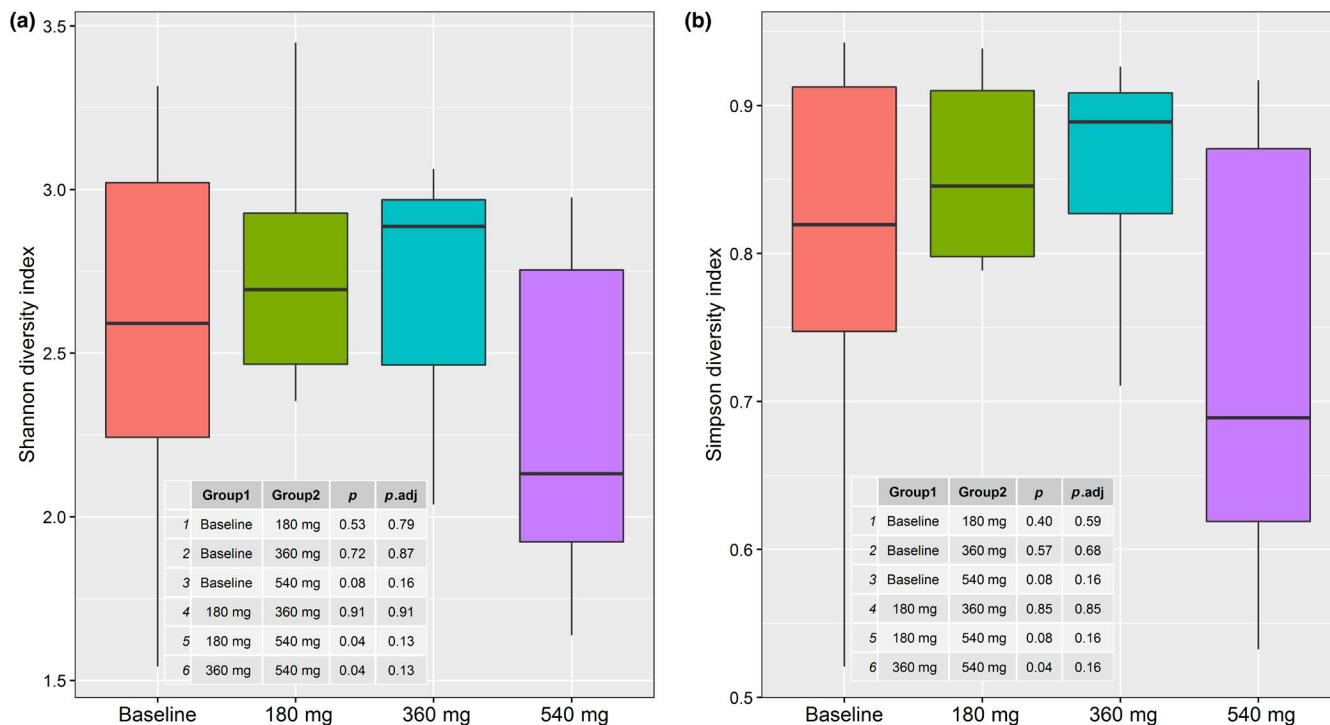


FIGURE 2 Alpha diversity among PMO dose groups. Box plots of Shannon (a) and Simpson (b) diversity indices plotted among the PMO dose groups. The *p* values were calculated using pairwise Wilcoxon test. *p*.adj = adjusted *p* value after false discovery rate (FDR) correction. PMO, peppermint oil

suggested that the difference could potentially be because of sample dispersion (Betadisper, $p < 0.05$). Additionally, the effect of ethnicity on the community composition was not significant via multivariate analysis (see Table S1 for details).

Because we did not detect any significant difference in the microbial community composition (beta diversity) of our samples stratified by treatment groups with the reported race, ethnicity, and sex of the children, we did not expect demographics to be a confounder when identifying taxa specific to baseline and post-PMO samples. Thus, demographics were not used in identifying taxa.

Microbial taxon abundance

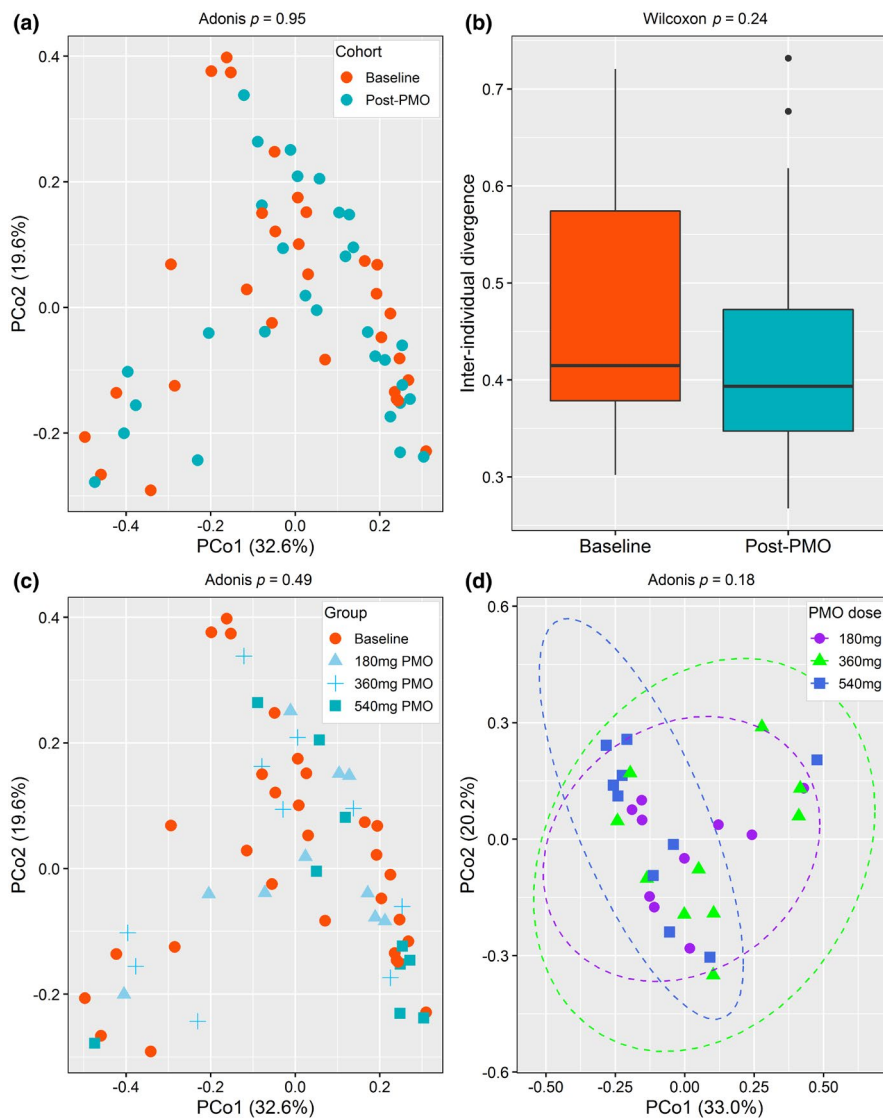
Both baseline and post-PMO gut microbiome were dominated by two bacterial phyla, Firmicutes (mean relative abundance across baseline samples = 47%, post-PMO = 46%) and Bacteroidetes (baseline = 43%, post-PMO = 46%; Figure 4a). The major bacterial families reported were Bacteroidaceae (baseline = 27%, post-PMO = 30%), Lachnospiraceae (baseline = 21%, post-PMO = 18%), Ruminococcaceae (baseline = 19%, post-PMO = 18%), Prevotellaceae (baseline = 10%, post-PMO = 8%), and Rikenellaceae (baseline = 4%, post-PMO = 5%; Figure 4b). The genera *Bacteroides* (baseline = 27%, post-PMO = 30%), *Prevotella* 9 (baseline = 8%,

post-PMO = 7%), *Bifidobacterium* (baseline = 5%, post-PMO = 4%), *Faecalibacterium* (baseline = 5%, post-PMO = 5%), *Alistipes* (baseline = 4%, post-PMO = 5%), and *Blautia* (baseline = 3%, post-PMO = 3%) were dominant in the participants before and after PMO treatment (Figure 4c).

We found no differentially abundant major taxa (at the phylum, family, or genus levels) between baseline and post-PMO samples using the traditional Wilcoxon rank sum test (all FDR-corrected *p* values > 0.05), except a very low abundant family, Coriobacteriaceae (baseline = 0.08%, post-PMO = 0.22%; $p = 0.002$; Figure 4d) and the genus *Collinsella* (baseline = 0.08%, post-PMO = 0.22%; $p = 0.007$; Figure 4e) belonging to the family Coriobacteriaceae. Three OTUs were differently abundant before and after PMO treatment. They were otu65, belonging to the genus *Collinsella* (baseline = 0.1%, post-PMO = 0.2%; $p = 0.004$); otu92, belonging to the genus *Adlercreutzia* (baseline = 0.04%, post-PMO = 0.01%; $p = 0.023$), and otu128, belonging to an unclassified member of the Prevotellaceae family (baseline = 0.01%, post-PMO = 0.002%; $p = 0.023$). ALDEx2 also identified the OTU belonging to the genus *Collinsella* (family: Coriobacteriaceae) as the only differentially abundant taxon between baseline and post-PMO samples.

Network analysis of bacterial genera using Cytoscape showed a clear separation between the pre- and post-PMO samples, with few overlaps (Figure 5). The genus *Collinsella* was driving the post-PMO clusters, whereas

FIGURE 3 Bray-Curtis dissimilarity-based microbial beta diversity and inter-individual divergence in baseline and PMO dose groups. (a) Principal coordinate analysis (PCoA) plot of the Bray-Curtis index between the baseline and post-PMO cohorts. (b) Inter-individual divergence in the bacterial community composition across samples in the baseline and post-PMO cohorts. (c) Bray-Curtis index-based PCoA plot of the samples grouped by baseline and PMO dose groups. (d) Bray-Curtis index-based PCoA plot of the samples grouped by PMO dose groups. The percentage of variance accounted for by the first two principal coordinates (PCo) that explained the largest fractions of variability in our data are shown in the axis labels in the PCoA plots. Each point represents a sample and closeness of the points indicates high similarity in the microbial community. The ellipses were drawn at 95% confidence interval. PMO, peppermint oil



Adlercreutzia and Prevotellaceae unclassified, among others, were the major drivers in pre-PMO (baseline) samples.

No significant differences among the PMO dose groups (180, 360, and 540 mg) were found in mean relative abundance of bacteria at the phylum, family, or genus levels (FDR-corrected Kruskal–Wallis p values > 0.05). Similar results were obtained with ALDEx2. Firmicutes and Bacteroidetes represented more than 90% of the total bacterial community in the dose groups (Table S2). The Firmicutes/Bacteroidetes ratio was lower in children who received 540 mg of PMO compared to the 180 and 360 mg dose groups (chi-square test $p = 0.04$; Figure 6a). Family level bacterial relative abundance is summarized in Table S3. Although none of the genera were differently abundant among the dosing groups, *Bacteroides* was the most dominant genus across the groups (mean relative abundance in 180 mg group = 28%, 360 mg = 22% and 540 mg = 41%), followed by *Prevotella* and *Bifidobacterium* (for details see Table S4).

Enterotypes of the gut microbiome

Based on the variation in relative abundance of the bacterial taxa, we identified two enterotypes: *Bacteroides*-enriched (80%) and Firmicutes-enriched (20%) in our samples ($n = 60$). Bray-Curtis dissimilarity-based PCoA analysis of the enterotypes revealed two separate clusters of samples in both baseline (Figure S4A) and post-PMO (Figure S4B) samples. We further found a transition of the enterotypes (*Bacteroides* to Firmicutes and vice-versa) from pre- (baseline) to post-PMO treatment in six subjects. Seventeen percent of the participants whose gut microbiome were *Bacteroides*-enriched at baseline transitioned to Firmicutes-enriched enterotype after PMO treatment, whereas only one participant with Firmicutes-dominant gut microbiome at baseline transitioned to *Bacteroides*-dominant type upon PMO treatment. A subgroup analysis of the *Bacteroides*-dominant samples, after excluding those which transitioned from one enterotype to another in post-PMO treatment,

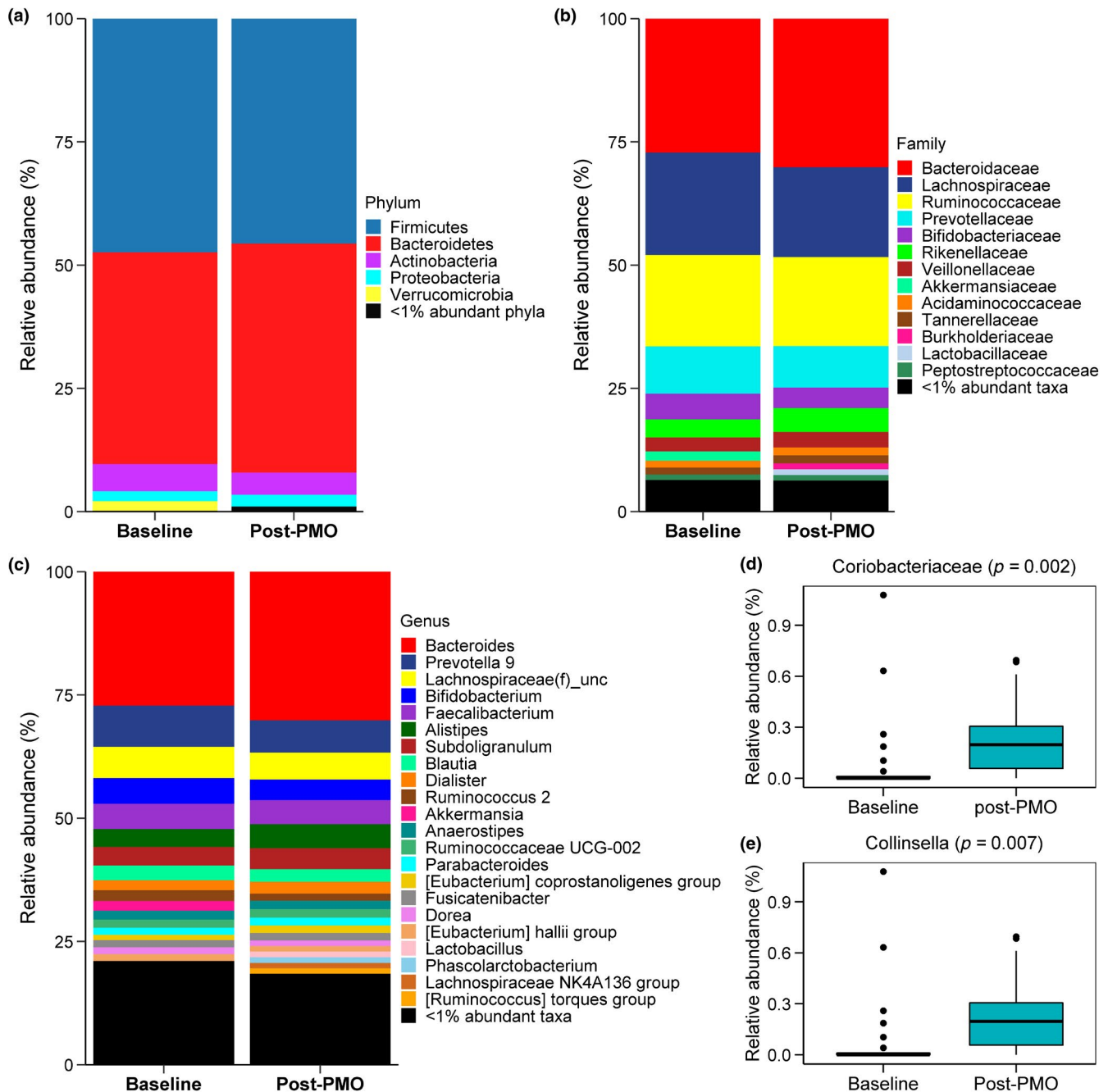


FIGURE 4 Gut microbiome composition of baseline and post-PMO samples. Stacked bar plots showing the average relative abundance (%) of 16S V4 rRNA gene sequences assigned to each bacterial phylum (a), family (b), and genus (c). Taxa with less than 1% relative abundance were grouped together for visualization. A higher taxonomic level is reported if the genus level classification could not be assigned. Unc = unclassified. Box plots showing differently abundant (FDR-corrected $p < 0.05$) family (d) and genus (e) levels taxa in baseline and post-PMO groups. FDR, false discovery rate; PMO, peppermint oil

found no significant differences in diversity and composition by PMO treatment (Figure S5) and dose (Figure S6).

DISCUSSION

In the present study, we characterized the gut bacterial microbiome of children with functional abdominal pain

before and after treatment with different doses of PMO. Although the composition of the gut microbiome overall did not differ significantly between baseline (untreated) and after treatment with PMO, abundance of some bacteria (e.g., *Collinsella*) differed significantly before and after PMO treatment. In addition, the Firmicutes/Bacteroidetes ratio was lower in children who received 540 mg of PMO compared to the 180 and 360 mg dose groups. The human

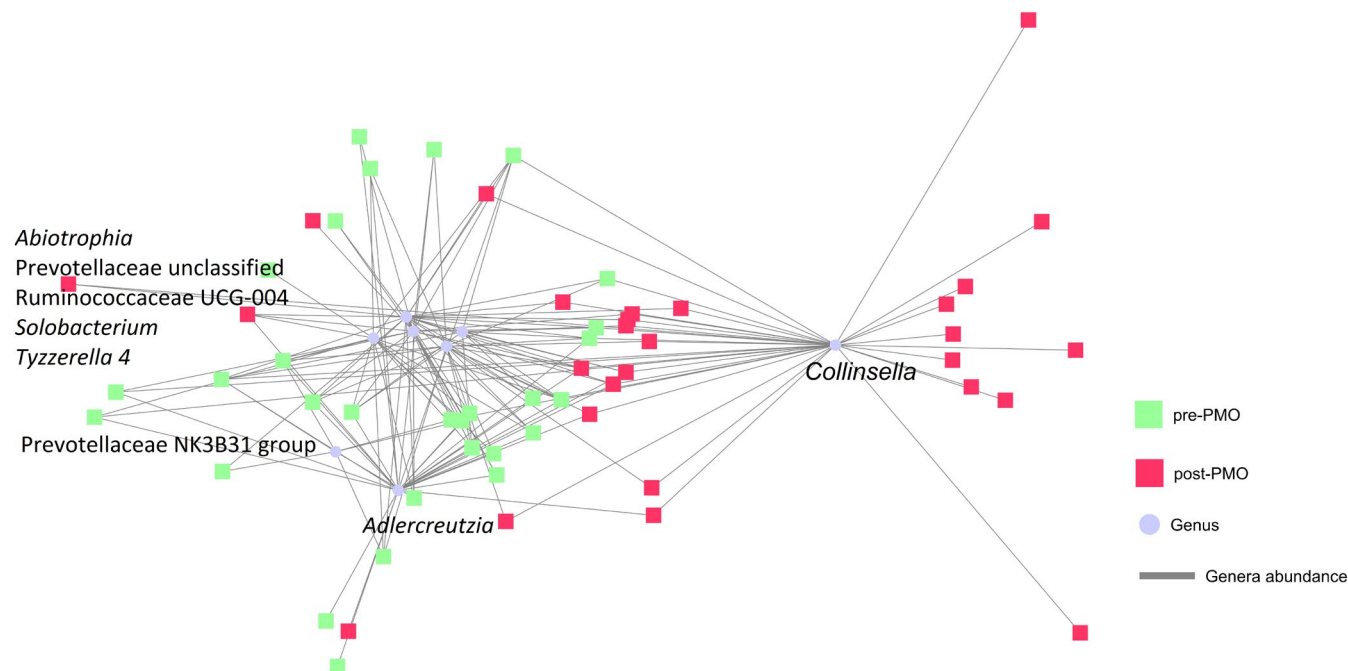


FIGURE 5 Genus level bacterial network in pre-PMO (baseline) and post-PMO samples. Cytoscape was used to generate the network. The subjects and genera were interpreted as nodes and the quantiles as weights of the connecting edges. The network included only genera that were present in at least five samples, had a Wilcoxon p less than or equal to 0.05, and abundance/edges that remained after removing the lowest 0, 0.1, and 0.2 quantiles. PMO, peppermint oil

gut microbiota is mostly composed of these two phyla that represent greater than 90% of the total community.²⁷ In addition, network analysis revealed separation between pre- and post-PMO fecal samples with the genus *Collinsella* driving the post-PMO clusters.

We found a lower abundance of *Collinsella* at baseline than after PMO treatment. Lower gut levels of *Collinsella* have been reported in adult patients with irritable bowel syndrome (another common functional abdominal pain disorder) compared to sex- and age-matched control individuals.³³ Somewhat in contrast, *Collinsella* has been implicated in increasing gut permeability (known to occur in irritable bowel syndrome³⁴) by reducing the expression of tight junction protein in epithelial cells and inducing expression of IL-17.³⁵ Thus, the role(s) of *Collinsella* in symptom generation in functional abdominal pain disorders, such as functional abdominal pain (FAP) in children requires further investigation as does whether the potential beneficial effect of PMO in treatment of these pain disorders relates to the impact of PMO on gut microbiome composition and/or function.

The majority of evidence suggests that PMO is effective in the treatment functional disorders, particularly irritable bowel syndrome, although two recent studies question these findings.^{2-6,36,37} Generally, its beneficial effect has been ascribed to its ability to act as an antispasmodic.⁷ However, PMO contains essential oils which are defined as volatile secondary metabolites of plants that give the

plant a distinctive smell, taste, or both.⁸ The main constituent and active ingredient of PMO appears to be menthol, although it contains a large number (~ 300) of other components.^{8,38} A number of in vitro studies have described the ability of PMO to exhibit bactericidal activity.³⁹⁻⁴³ That said, comparison among studies is difficult because of, among other issues, differences in the assay used and the units used to describe the results.^{9,41} Hence, there is some discrepancy as to the extent of the antimicrobial nature of PMO.^{8,41}

Dysbiosis of the gut microbiome may contribute to the symptom manifestations of functional GI disorders.^{44,45} Given the potential bactericidal effect of PMO, we speculated that PMO might also exert its beneficial effects on functional disorders like irritable bowel syndrome through alteration of gut microbiome composition. To our knowledge no other studies have examined the effect of PMO on the gut microbiome in humans. One study in adults with dyspeptic symptoms, constipation, and/or diarrhea carried out profiling of commensal bacteria using polymerase chain reaction DNA analysis; however, PMO was one of multiple ingredients in the treatment, complicating interpretation.⁴⁶ Similarly, interpretation of a study by Giannenas et al. is unclear; they examined the effect in chickens of a feed additive with high concentrations of menthol (the primary ingredient in PMO) on fecal *Escherichia coli* and *Lactobacillus acidophilus* concentrations as subsequently grown in culture; the additive

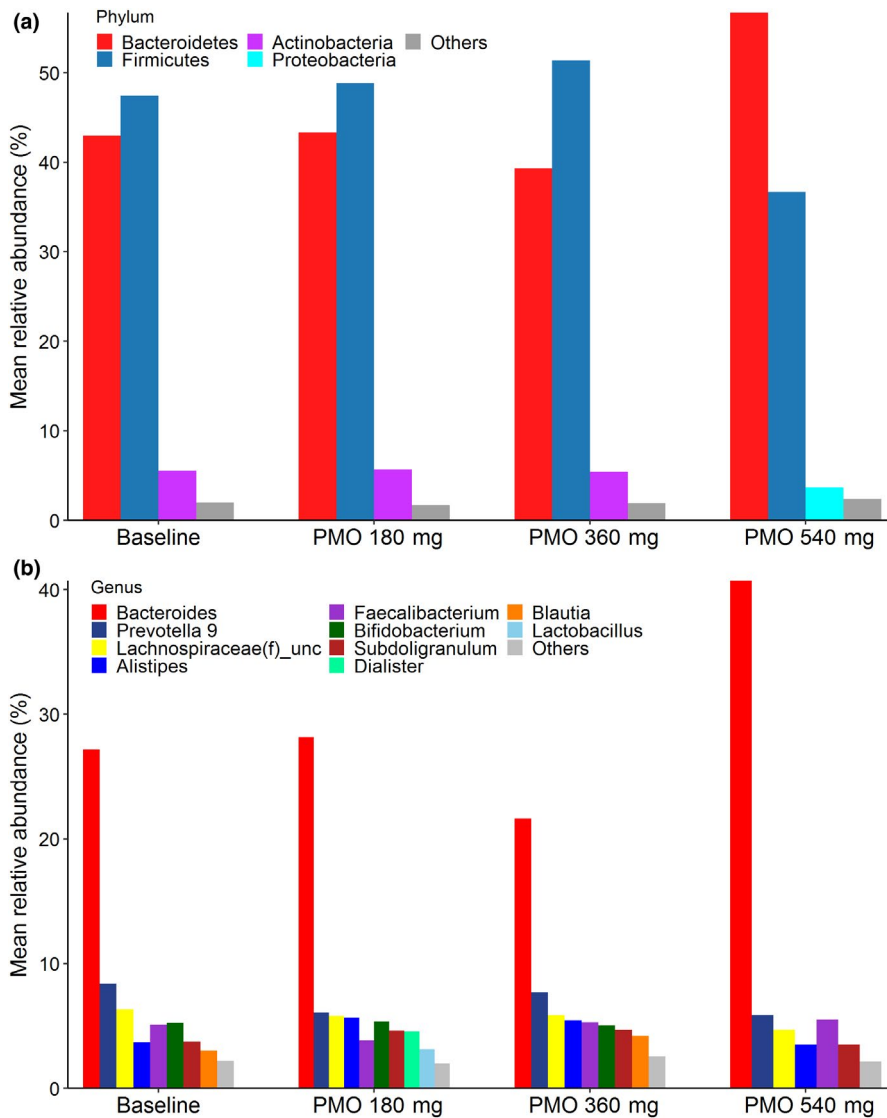


FIGURE 6 Gut microbiome composition at baseline and in PMO dose groups. Phylum (a) and genus (b) levels bar graphs by PMO dosing groups. Taxa with less than 3% relative abundance were grouped together as “Others” for visualization. Baseline samples are also included in the plots for comparisons. PMO, peppermint oil

resulted in higher *Lactobacillus* counts in the jejunum and *E. coli* in the colon.⁴⁷

Contrary to our hypothesis, we found no dramatic effect of PMO on the gut microbiome. Our findings of no significant differences in alpha diversity of the bacterial microbiome between the baseline and post-PMO samples may be attributed, in part, to the significantly greater variance in alpha diversity scores in the baseline samples than in the post-PMO samples. At baseline, the overall pattern of the microbiome, particularly, a higher relative abundance of *Bacteroides* and lower abundance of *Faecalibacterium* spp., and *Akkermansia* spp. suggest an inflammatory, dysbiotic gut community that fits with previous descriptions of the gut microbiome in functional disorders, such as irritable bowel syndrome.^{48,49} Following treatment with PMO there were no changes in the abundance of *Bacteroides* or potentially beneficial bacteria, such as *Bifidobacterium* spp., *Faecalibacterium* spp., and *Akkermansia* spp.

It is not clear why we saw only a small impact of PMO on the gut microbiome even at the highest dose in contrast to what is reported in in vitro studies. It is possible that, in humans, PMO does not impact gut microbiome composition significantly. The formulation of PMO that we used is enteric coated to reduce the risk of exacerbating gastroesophageal reflux and is not released solely in the colon.¹¹ In a study using a similar formulation to ours that presumably was absorbed primarily in the small intestine, Weerts et al. showed in adults with irritable bowel syndrome that it improved symptoms.³⁶ In contrast, in the same study, a PMO formulation released in the colon was not efficacious.³⁶ These results call into question the long-standing presumption that PMO exerts its beneficial effect in the colon. Future studies evaluating the effect of PMO on the gut microbiome may seek to collect samples at more proximal sites such as the duodenum or jejunum, as changes in small bowel (or gastric) microbiome composition may be playing a larger role.

It also is possible that there are other mechanisms by which PMO exhibits its protective effects that do not necessarily involve changes in the bacterial composition of the gut. For example, Botschuijver et al. using a rat maternal separation model for irritable bowel syndrome reported that the gut mycobiome of maternally separated and non-separated rats differed.⁵⁰ Treatment of both groups with a combination of PMO and caraway shifted the gut mycobiome composition so that they were similar after treatment.⁵⁰ The change in mycobiome composition was associated with a reversal of visceral hypersensitivity in the maternally separated rats.⁵⁰ In contrast, treatment with PMO and caraway did not lead to a coherent shift in microbiome composition; the change in visceral hypersensitivity was related to the change in yeast but not bacteria.⁵⁰ PMO has been described as being capable of inhibiting growth of yeast.^{9,50} Given these reports, future human studies potentially should interrogate the gut mycobiome after PMO administration.

Limitations of this study are primarily those of small sample size. Although our sample size was reasonably robust (30 samples each in the baseline and post-PMO groups), given the trends in microbial shifts that we identified, a larger sample size will be necessary to capture these shifts in their full complexity. Use of whole genome shotgun sequencing, although significantly more expensive than the 16S sequencing used in this study, would provide greater sequencing depth and precision.

Strengths of the study include the use of a well-characterized clinical cohort, a trial in which each participant acted as their own control (providing pre- and post-PMO treatment stool samples), expertise in microbiome studies, and following well-established protocols derived from the Human Microbiome Project. As noted above, the impact of PMO on the human microbiome has yet to be studied in adults, much less in children. Our findings provide the first benchmark information about the effect of PMO (and its dosing) on gut bacterial community and suggest that, at the doses studied, the clinical benefit of PMO may not be mediated through changes in gut microbiome composition. The study duration should have been long enough to detect potential changes in microbiome composition based on previous (in vitro and rodent) studies of the effect of PMO on gut bacteria.⁷⁻⁹ Further studies using a multi-omics approach (microbiome, metabolomics, and metaproteomics) may provide new information about how PMO exerts its beneficial effects in functional disorders and potentially reveal targets for manipulation for prevention or management of the associated gastrointestinal symptoms.

ACKNOWLEDGEMENTS

The authors thank the participants and their parents who were kind enough to take part in the study.

CONFLICT OF INTEREST

The authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

R.J.S., S.T., B.P.C., R.-A.L., N.O., S.M.A.-R., U.G., S.M., J.V., and G.L.K. wrote the manuscript. R.J.S., B.P.C., U.G., and G.L.K., designed the research. R.J.S. and B.P.C. performed the research. R.J.S., S.T., R.-A.L., N.O., S.M.A.-R., U.G., S.M., J.V., and G.L.K. analyzed the data.

REFERENCES

1. Ulbricht C, Costa D, M Grimes Serrano J, et al. An evidence-based systematic review of spearmint by the natural standard research collaboration. *J Dietary Suppl.* 2010;7:179-215.
2. Black CJ, Yuan Y, Selinger CP, et al. Efficacy of soluble fibre, antispasmodic drugs, and gut-brain neuromodulators in irritable bowel syndrome: a systematic review and network meta-analysis. *Lancet Gastroenterol Hepatol.* 2020;5:117-131.
3. Hawrelak JA, Wohlmuth H, Pattinson M, et al. Western herbal medicines in the treatment of irritable bowel syndrome: A systematic review and meta-analysis. *Complement Ther Med.* 2020;48:102233.
4. Alammari N, Wang L, Saberi B, et al. The impact of peppermint oil on the irritable bowel syndrome: a meta-analysis of the pooled clinical data. *BMC Complement Altern Med.* 2019;19:21-30.
5. Khanna R, MacDonald JK, Levesque BG. Peppermint oil for the treatment of irritable bowel syndrome: a systematic review and meta-analysis. *J Clin Gastroenterol.* 2014;48:505-512.
6. Ruepert L, Quartero AO, de Wit NJ, van der Heijden GJ, Rubin G, Muris JWM. Bulking agents, antispasmodics and antidepressants for the treatment of irritable bowel syndrome. *Cochrane Database Syst Rev* 2011;8:CD003460.
7. Chumpitazi BP, Kearns GL, Shulman RJ. Review article: the physiological effects and safety of peppermint oil and its efficacy in irritable bowel syndrome and other functional disorders. *Aliment Pharmacol Ther.* 2018;47:738-752.
8. Wińska K, Mączka W, Łyczko J, Grabarczyk M, Czubaszek A, Szumny A. Essential oils as antimicrobial agents-myth or real alternative? *Molecules.* 2019;24:2130.
9. Stringaro A, Colone M, Angiolella L. Antioxidant antifungal, antibiofilm, and cytotoxic activities of *Mentha* spp. Essential oils. *Medicines.* 2018;5(4):112.
10. Shulman RJ, Chumpitazi BP, Abdel-Rahman SM, Garg U, Musaad S, Kearns GL. Randomised trial: Peppermint oil (menthol) pharmacokinetics in children and effects on gut motility in children with functional abdominal pain [published online ahead of print September 16, 2021]. *Br J Clin Pharmacol.* <https://doi.org/10.1111/bcp.15076>.
11. Kearns GL, Chumpitazi BP, Abdel-Rahman SM, Garg U, Shulman RJ. Systemic exposure to menthol following administration of peppermint oil to paediatric patients. *BMJ Open.* 2015;5:e008375.

12. Rasquin A, Di Lorenzo C, Forbes D, et al. Childhood functional gastrointestinal disorders: child/adolescent. *Gastroenterology*. 2006;130:1527-1537.
13. Czyzewski DI, Lane MM, Weidler EM, Williams AE, Swank PR, Shulman RJ. The interpretation of Rome III criteria and method of assessment affect the irritable bowel syndrome classification of children. *Aliment Pharmacol Ther*. 2011;33:403-411.
14. von Baeyer CL, Spagrud LJ, McCormick JC, Choo E, Neville K, Connelly MA. Three new datasets supporting use of the Numerical Rating Scale (NRS-11) for children's self-reports of pain intensity. *Pain*. 2009;143:223-227.
15. Lewis SJ, Heaton KW. Stool form scale as a useful guide to intestinal transit time. *Scand J Gastroenterol*. 1997;32:920-924.
16. Luna RA, Oezguen N, Balderas M, et al. Distinct microbiome-neuroimmune signatures correlate with functional abdominal pain in children with autism spectrum disorder. *Cell Mol Gastroenterol Hepatol*. 2017;3:218-230.
17. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal*. 2011;17:10-12.
18. Estaki M, Jiang L, Bokulich NA, et al. QIIME 2 enables comprehensive end-to-end analysis of diverse microbiome data and comparative studies with publicly available data. *Curr Protoc Bioinform*. 2020;70:e100.
19. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes S. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. 2016;13:581-583.
20. Rognes T, Flouri T, Nichols B, Quince C, Mahe F. VSEARCH: a versatile open source tool for metagenomics. *PeerJ*. 2016;4:e2584.
21. Quast C, Pruesse E, Yilmaz P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res*. 2013;41:D590-D596.
22. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One*. 2013;8:e61217.
23. Weiss S, Xu ZZ, Peddada S, et al. Normalization and microbial differential abundance strategies depend upon data characteristics. *Microbiome*. 2017;5:27.
24. Aitchison J. *The statistical analysis of compositional data*. Blackburn Press; 2003.
25. Martino C, Morton JT, Marotz CA, et al. A novel sparse compositional technique reveals microbial perturbations. *mSystems*. 2019;4(1):e00016-19.
26. Le Chatelier E, Nielsen T, Qin J, et al. Richness of human gut microbiome correlates with metabolic markers. *Nature*. 2013;500:541-546.
27. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. *Nature*. 2012;486:207-214.
28. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003;13:2498-2504.
29. David MM, Tataru C, Daniels J, et al. Children with autism and their typically developing siblings differ in amplicon sequence variants and predicted functions of stool-associated microbes. *mSystems*. 2021;6(2):e00193-20.
30. Anderson MJ. A new method for non-parametric multivariate analysis of variance. *Austral Ecol*. 2001;26:32-46.
31. Oksanen JBF, Friendly M, Kindt R, et al. Ordination methods, diversity analysis and other functions for community and vegetation ecologists 2012. Available at: https://www.researchgate.net/publication/323265386_Ordination_methods_diversity_analysis_and_other_functions_for_community_and_vegetation_ecologists.
32. Fernandes AD, Macklaim JM, Linn TG, Reid G, Gloor GB. ANOVA-like differential expression (ALDEx) analysis for mixed population RNA-Seq. *PLoS One*. 2013;8:e67019.
33. Kassinen A, Krogius-Kurikka L, Mäkituokko H, et al. The fecal microbiota of irritable bowel syndrome patients differs significantly from that of healthy subjects. *Gastroenterology*. 2007;133:24-33.
34. Camilleri M, Lasch K, Zhou W. Irritable bowel syndrome: methods, mechanisms, and pathophysiology. The confluence of increased permeability, inflammation, and pain in irritable bowel syndrome. *Am J Physiol Gastrointest Liver Physiol*. 2012;303:G775-G785.
35. Chen J, Wright K, Davis JM, et al. An expansion of rare lineage intestinal microbes characterizes rheumatoid arthritis. *Genome Med*. 2016;8:43.
36. Weerts Z, Masclee AM, Witteman JM, et al. Efficacy and safety of peppermint oil in a randomized, double-blind trial of patients with irritable bowel syndrome. *Gastroenterology*. 2020;158:123-136.
37. Nee J, Ballou S, Kelley JM, et al. Peppermint oil treatment for irritable bowel syndrome: a randomized placebo-controlled trial. *Am J Gastroenterol*. 2021;116(11):2279-2285.
38. Grigoleit HG, Grigoleit P. Pharmacology and preclinical pharmacokinetics of peppermint oil. *Phytomedicine*. 2005;12:612-616.
39. Muntean D, Licker M, Alexa E, et al. Evaluation of essential oil obtained from *Mentha piperita* L. against multidrug-resistant strains. *Infect Drug Resist*. 2019;12:2905-2914.
40. Husain FM, Ahmad I, Khan MS, et al. Sub-MICs of *Mentha piperita* essential oil and menthol inhibits AHL mediated quorum sensing and biofilm of Gram-negative bacteria. *Front Microbiol*. 2015;6:420.
41. Akdemir Evrendilek G. Empirical prediction and validation of antibacterial inhibitory effects of various plant essential oils on common pathogenic bacteria. *Int J Food Microbiol*. 2015;202:35-41.
42. Silva N, Alves S, Goncalves A, Amaral JS, Poeta P. Antimicrobial activity of essential oils from Mediterranean aromatic plants against several foodborne and spoilage bacteria. *Food Sci Technol Int*. 2013;19:503-510.
43. Yap PS, Lim SH, Hu CP, Yip BC. Combination of essential oils and antibiotics reduce antibiotic resistance in plasmid-conferred multidrug resistant bacteria. *Phytomedicine*. 2013;20:710-713.
44. Hollister EB, Oezguen N, Chumpitazi BP, et al. Leveraging human microbiome features to diagnose and stratify children with irritable bowel syndrome. *J Mol Diagn*. 2019;21:449-461.
45. Shin A, Preidis GA, Shulman R, Kashyap PC. The gut microbiome in adult and pediatric functional gastrointestinal disorders. *Clin Gastroenterol Hepatol*. 2019;17:256-274.
46. Ried K, Travica N, Dorairaj R, Sali A. Herbal formula improves upper and lower gastrointestinal symptoms and gut health in Australian adults with digestive disorders. *Nutr Res*. 2020;76:37-51.
47. Giannenas I, Bonos E, Skoufos I, et al. Effect of herbal feed additives on performance parameters, intestinal microbiota, intestinal morphology and meat lipid oxidation of broiler chickens. *Br Poult Sci*. 2018;59:545-553.

48. Sokol H, Pigneur B, Watterlot L, et al. Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. *Proc Natl Acad Sci U S A*. 2008;105:16731-16736.
49. Zhuang X, Xiong L, Li L, Li M, Chen M. Alterations of gut microbiota in patients with irritable bowel syndrome: A systematic review and meta-analysis. *J Gastroenterol Hepatol*. 2017;32:28-38.
50. Botschuijver S, Welting O, Levin E, et al. Reversal of visceral hypersensitivity in rat by Menthacarin®, a proprietary combination of essential oils from peppermint and caraway, coincides with mycobiome modulation. *Neurogastroenterol Motil*. 2018;30:e13299.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Thapa S, Luna RA, Chumpitazi BP, et al. Peppermint oil effects on the gut microbiome in children with functional abdominal pain. *Clin Transl Sci*. 2022;15:1036-1049. doi:[10.1111/cts.13224](https://doi.org/10.1111/cts.13224)