Enteral High Fat-Polyunsaturated Fatty Acid Blend Alters the Pathogen Composition of the Intestinal Microbiome in Premature Infants with an Enterostomy

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Objective To determine the effect of enteral fish oil and safflower oil supplementation on the intestinal microbiome in infants with an enterostomy born premature.

Study design Infants with an enterostomy born premature were randomized to receive early enteral supplementation with a high-fat polyunsaturated fatty acid (HF-PUFA) blend of fish oil and safflower oil vs standard nutritional therapy. We used 16S rRNA gene sequencing for longitudinal profiling of the microbiome from the time of study entry until bowel reanastomosis. We used weighted gene coexpression network analysis to identify microbial community modules that differed between study groups over time. We performed imputed metagenomic analysis to determine metabolic pathways associated with the microbial genes.

Results Sixteen infants were randomized to receive enteral HF-PUFA supplementation, and 16 infants received standard care. The intestinal microbiota of infants in the treatment group differed from those in the control group, with greater bacterial diversity and lower abundance of *Streptococcus*, *Clostridium*, and many pathogenic genera within the *Enterobacteriaceae* family. We identified 4 microbial community modules with significant differences between groups over time. Imputed metagenomic analysis of the microbial genes revealed metabolic pathways that differed between groups, including metabolism of amino acids, carbohydrates, fatty acids, and secondary bile acid synthesis.

Conclusion Enteral HF-PUFA supplementation was associated with decreased abundance of pathogenic bacteria, greater bacterial diversity, and shifts in the potential metabolic functions of intestinal microbiota. *(J Pediatr 2017;181:93-101).*

Trial registration ClinicalTrials.gov: NCT01306838

Premature infants with necrotizing enterocolitis, spontaneous intestinal perforation, or intestinal atresia commonly require abdominal surgery and creation of a small bowel enterostomy.[1] Following surgery, these infants are at high risk for a number of complications, including cholestasis, liver disease, sepsis, growth failure, and death.[1-4] The etiology of these complications is multifactorial but is believed to be in part related to prolonged reliance on parenteral soybean-based lipid formulations, which are rich in proinflammatory omega-6 fatty acids and phytosterols and devoid of the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).[5-8]

Fish oil, which is rich in anti-inflammatory omega-3 fatty acids, has been associated with decreased cholestasis and liver injury in children with short bowel syndrome.[9,10] A recent randomized controlled trial of early enteral supplementation with a high-fat polyunsaturated fatty acid (HF-PUFA) blend of fish oil and safflower oil vs usual care in premature infants with an enterostomy found that infants treated with the HF-PUFA blend had lower conjugated bilirubin levels than infants in the control group.[11] Furthermore, the infants who received HF-PUFA required fewer sepsis evaluations and had improved growth after bowel reanastomosis. The mechanisms underlying the beneficial effects of enteral HF-PUFA in this setting are unclear.

The intestinal microbiome has an essential role in intestinal function, including nutrient absorption, metabolism, maintenance of barrier integrity, and protection against infection.[12,13] The microbiome is altered by diet, including fat

DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
HF-PUFA	High-fat polyunsaturated fatty acid
LC-PUFA	Long chain-polyunsaturated fatty acid
ME	Module eigengene
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
SS	Smoothing spline

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intake.[14,15] Infants who have undergone surgical enterostomy placement often have multiple predisposing factors that may perturb the intestinal microbiome, including premature birth, history of bowel injury or perforation, antibiotic exposure, prolonged withholding of enteral feeds, and intestinal surgery.[4] The objective of our study was to determine the effects of enteral supplementation with a HF-PUFA blend containing fish oil and safflower oil vs standard nutritional therapy on the intestinal microbiome in premature infants with an enterostomy. We hypothesized that treatment with enteral HF-PUFA leads to functional and community-level changes in the microbiome, which may promote the development of functional microbial communities that contribute to the clinical benefits of HF-PUFA that were observed in this cohort.

Methods

A randomized, controlled trial of enteral fish oil and safflower oil supplementation vs usual care was conducted in the Neonatal Intensive Care Unit of Brenner Children's Hospital at Wake Forest Baptist Medical Center (ClinicalTrials.gov: NCT01306838). Details of the study design have been described previously by Yang et al.[11] In brief, inclusion in the study required the presence of a jejunostomy or ileostomy, birth gestational age of less than 37 weeks, and postnatal age less than 2 months at the time of study enrollment. Infants were excluded if they had a colostomy, major congenital anomaly, or metabolic disease. Infants in the control group received usual nutritional care. Once tolerating minimal enteral feedings of 20 mL/kg/d, infants in the treatment group received enteral fat supplementation with fish oil (Major Fish Oil 500, Major Pharmaceuticals, Livonia, Michigan, or Rugby Sea Omega 50, Rugby Laboratories, Inc, Corona, California) and safflower oil (Microlipid, Nestle Nutrition, Florham Park, New Jersey). The fish oil supplements contained EPA, DHA, and vitamin E (tocopherol). Fish oil was initiated at a dose of 0.2 g every 12 hours for infants weighing less than 1000 g or 0.25 g every 12 hours for infants weighing more than 1000 g and increased to a maximum dose of 0.5 g every 6 hours. Safflower oil, which is enriched in the omega-6 fatty acid linoleic acid, was started at 1 g/kg/d and increased by 0.5 g/kg/d to 2.5 g/kg/d for a goal omega-6 to omega-3 fatty acid ratio of 3.75 to 5.1. Parenteral Intralipid (Baxter Healthcare, Deerfield, Illinois) was decreased by 0.5 g/kg/d as enteral fat supplementation advanced and discontinued when the dose reached <1 g/kg/d.

Enteral feedings were initiated on recovery of bowel function after ostomy placement and advanced gradually. Infants received their mother's milk when available or infant formula. As enteral nutrition advanced, parenteral nutrition and intravenous lipid were decreased to achieve a growth rate of 15 g/kg/d. Goal caloric intake was generally 120-130 kcal/kg/d. Stool ostomy output was collected weekly from the time of study enrollment to the time of bowel reanastomosis, up to a maximum of 10 weeks. Samples were stored at −80°C until further processing. Four subjects included in the original randomized trial were excluded from this analysis because of missing samples (2 subjects in treatment group and 2 subjects in control group). The trial was approved by the Wake Forest University Health Science institutional review board, and written informed consent was obtained from parents. The microbiome analysis was a secondary study of the existing, deidentified fecal specimens and was deemed exempt research by the Duke institutional review board.

DNA Extraction, Polymerase Chain Reaction (PCR) Amplification, and DNA Sequencing

Total genomic DNA was extracted from fecal samples with the use of a commercial bead-beating method (Zymo Research Soil Microbe DNA Kit, Irvine, California). PCR amplification of the V4 region of the 16S rRNA gene was performed with the use of 12 nucleotide barcode-indexed primers and previously described standardized PCR conditions for the Earth Microbiome Project.[16] PCR amplicons were pooled in equimolar concentrations and purified by gel extraction. Sequencing was performed on the Illumina HiSeq Platform (Illumina, Inc, San Diego, California) with the use of 150 nucleotide single-end reads.

Sequences were split, quality-trimmed, demultiplexed, and chimera-reduced with the use of QIIME tools.[17] High-quality sequences sharing ≥99% nucleotide sequence identity were clustered into operational taxonomic units (OTUs) with USEARCH (version 6; http://drive5.com/usearch/), aligned to the Greengenes database (version 13.8.99), and representative sequences were given a taxonomic assignment using BLAST against the SILVA bacterial database (Release 111).[18,19] Sparse OTUs with less than 3 occurrences in at least 20% of samples were excluded from subsequent analyses because they were unlikely to significantly contribute to the overall composition and inferred metabolic capacity in any one sample. In the case of calculating alpha-diversity, however, only singleton OTUs were excluded. Counts were normalized with the cumulative sums scaling approach (percentile $P = .5$, determined using the 'cumNormStatFast' function) in the metagenomeSeq package (version 1.12.0).[20]

Microbiome Composition and Diversity

Microbial composition and diversity were determined by the use of tools within the phyloseq (version 1.14.0) and metagenomeSeq packages (version 1.12.0).[20,21] Smoothing spline (SS)-ANOVA was used from the metagenomeSeq and gss (version 2.1-5) packages in R to identify time intervals in which alpha-diversity and bacterial taxonomic groups differed between treatment groups, with controlling for repeat sampling of individual infants as a random effect.[20,22] The area between the observed distributions was measured for each time interval of difference. P values were calculated with a permutation-based method in which the groups were permutated 10 000 times and the observed areas were compared with the empiric null distributions. All analyses were performed with R statistical software (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was considered $P < .05$, with Benjamini-Hochberg correction for multiple testing.