

Increases in Colonic Bacterial Diversity after ω -3 Fatty Acid Supplementation Predict Decreased Colonic Prostaglandin E₂ Concentrations in Healthy Adults

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ABSTRACT

Background: The intestinal microbiome is an important determinant of inflammatory balance in the colon that may affect response to dietary agents.

Objective: This is a secondary analysis of a clinical trial, the Fish Oil Study, to determine whether interindividual differences in colonic bacteria are associated with variability in the reduction of colonic prostaglandin E₂ (PGE₂) concentrations after personalized supplementation with ω -3 (n-3) fatty acids.

Methods: Forty-seven healthy adults (17 men, 30 women, ages 26–75 y) provided biopsy samples of colonic mucosa and luminal stool brushings before and after personalized ω -3 fatty acid supplementation that was based on blood fatty acid responses. Samples were analyzed using 16S ribosomal RNA sequencing. The data analyses focused on changes in bacterial community diversity. Linear regression was used to evaluate factors that predict a reduction in colonic PGE₂.

Results: At baseline, increased bacterial diversity, as measured by the Shannon and Inverse Simpson indexes in both biopsy and luminal brushing samples, was positively correlated with dietary fiber intakes and negatively correlated with fat intakes. Dietary supplementation with ω -3 fatty acids increased the Yue and Clayton community dis-similarity index between the microbiome in luminal brushings and colon biopsy samples post-supplementation ($P = 0.015$). In addition, there was a small group of individuals with relatively high *Prevotella* abundance who were resistant to the anti-inflammatory effects of ω -3 fatty acid supplementation. In linear regression analyses, increases in diversity of the bacteria in the luminal brushing samples, but not in the biopsy samples, were significant predictors of lower colonic PGE₂ concentrations post-supplementation in models that included baseline PGE₂, baseline body mass index, and changes in colonic eicosapentaenoic acid-to-arachidonic acid ratios. The changes in bacterial diversity contributed to 6–8% of the interindividual variance in change in colonic PGE₂ ($P = 0.001$).

Conclusions: Dietary supplementation with ω -3 fatty acids had little effect on intestinal bacteria in healthy humans; however, an increase in diversity in the luminal brushings significantly predicted reductions in colonic PGE₂. This trial was registered at www.clinicaltrials.gov as NCT 01860352. *J Nutr* 2019;149:1170–1179.

Keywords: gut microbiome, colon cancer prevention, fish oils, prostaglandin E₂, fatty acids

Introduction

The composition of the intestinal microbial community is thought to play an important role in the host's intestinal proinflammatory state, which, in turn, modulates the risk of colon cancer. Alterations in the abundance of specific members of the intestinal microbiota are useful in stool-based screening for uncovering the presence of colon cancer and precancerous lesions (1). The mechanisms by which the

microbiota can contribute to the proinflammatory state and the risk of colon cancer include a bidirectional interaction between immunologic signaling in the host intestinal mucosa with the microbiota and/or the products of microbial metabolism (2–5). A heightened proinflammatory state in the colon that can result from these interactions, as characterized by increased production of prostaglandin E₂ (PGE₂), is a critical event in stimulating the process of colonic carcinogenesis (6).

We conducted a clinical trial with a primary goal of reducing colonic PGE₂ concentrations using personalized ω -3 FA supplementation. Both animal and human data support the role of increased dietary intakes of ω -3 FAs in reducing proinflammatory processes and preventing colon cancer (7). The preponderance of rodent data using a variety of preventive agents, including ω -3 FAs, suggests that a reduction in colonic neoplasia is associated with a reduction of approximately $\geq 50\%$ in colonic mucosal PGE₂ (8–15). The trial goal was a 50% reduction, the minimal reduction associated with prevention, because PGE₂ does have an important role in maintaining homeostasis in normal colon tissue. In immune cells, PGE₂ functions to dampen inflammatory responses and PGE₂ has an important role in repair of intestinal injury via its proliferative effects on the epithelium (16, 17). This may be one reason why clinical trials using fish oils for treatment of inflammatory bowel diseases have not proven to be beneficial, and proinflammatory effects of fish oils have been reported in colitis animal models (18, 19).

Our clinical trial utilized an ω -3 FA formulation that is highly enriched in EPA. There are important distinctions in the mechanisms of action of different ω -3 FAs that likely contribute to variation in results between studies using varying types of fish-oil supplements (20, 21). EPA is an ω -3 FA that effectively binds to the active site of cyclooxygenase-1 to inhibit the oxygenation of arachidonic acid (AA; ω -6) and hence formation of PGE₂ (22, 23). Other long-chain ω -3 FAs, including DHA, are very modest inhibitors of cyclooxygenase-1, the major cyclooxygenase isoform found in normal colon tissue (22, 24, 25). For cyclooxygenase-2, EPA and other ω -3 FAs are poor inhibitors and ω -3 FAs function mainly to supplant AA in membranes, thereby decreasing available substrate for PGE₂ production via cyclooxygenase-2 action (26, 27). In vivo, we established that ω -3 FA feeding can reduce PGE₂ formation by 50% in normal colonic tissue, and we utilized the relation between serum EPA-to-AA ratios and colonic PGE₂ for constructing the personalized dosing model in the clinical trial (15, 23, 28).

Supplementation with ω -3 FAs could also exert effects via alteration of the intestinal microbiota. Many animal studies show that the inflammatory effects of high-saturated-fat diets are reversed by fish-oil feeding, and this appears to be due, at

least in part, to diet-microbial interactions. For example, fish oil in the diet counteracts the effects of a high-saturated-fat diet on increased proinflammatory Toll-like receptor signaling, increased intestinal permeability, and the nature of the intestinal microbiome (29, 30). In several different animal models, fish-oil feeding restored intestinal bacterial species with putative beneficial functions (31–34). In transgenic mice, endogenous conversion of tissue ω -6 to ω -3 FAs resulted in decreased adipose inflammation and endotoxemia that was mediated by changes in microbial composition (35). In persons at increased risk of colon cancer, we found that the dietary ω -3-to- ω -6 FA ratio was inversely related to serum LPS-binding protein concentrations, a marker of exposure to gram-negative intestinal bacteria (36).

Most studies aimed at investigating the role of the intestinal microbiome in health and disease quantified the intestinal microbiota in excreted stool of rodents or humans or in the ceca of rodents. The intestinal microbiota, however, do differ along the length of the intestine (37, 38). In the present study, colon biopsy and stool brushing samples near the colonic biopsy site were available for analysis. Bacteria adhering to the colonic mucosa have been shown to differ from those in the lumen and stool (39–43). The mucin layer provides protection to the colonic epithelium, but the outer mucin layer is also a niche for growth of bacteria (44, 45). We hypothesized that the bacteria localized in the mucin are in closer proximity with the colonocytes than bacteria in the lumen and therefore might have a greater impact on the biology of the colonic epithelium.

Methods

Subjects and samples

This was a secondary analysis of the Fish Oil Study (23). Briefly, a total of 47 eligible individuals, 17 men and 30 women, ages 26–75 years, completed 12 weeks of personalized ω -3 FA dosing. Questionnaires were administered as a part of the study to collect demographic information, health status, and agent adherence data. All study participants gave their written informed consent to participate. This study was approved by the University of Michigan Internal Review Board (HUM00051786) and was registered at www.clinicaltrials.org (NCT 01860352; Effects of Fish Oil on the Colonic Mucosa).

Eligibility criteria included having a BMI (kg/m²) of 18–40, aged 25–75 y, and in good general health (as defined by normal white blood cell, hemoglobin, and platelet counts; not taking insulin or hypoglycemic drugs; and normal renal and hepatic function determined within the last 28 d). Subjects were asked to avoid consuming fish with a high content of ω -3 FAs, steroids (with the exception of inhaled steroids for asthma), nonsteroidal anti-inflammatory compounds, fish oils, and other anti-inflammatory supplements while participating in the study. Two subjects took antibiotics but discontinued use ≥ 2 weeks before a study biopsy visit.

The primary goal of the study was to achieve a 50% reduction in colonic PGE₂ concentrations using personalized dosing of ω -3 FAs. This was accomplished using serum FA responses after short-term, low, and high doses of ω -3 FAs given for 2 weeks each. We used an ω -3 FA preparation highly enriched in EPA (EPA-Xtra from Nordic Naturals). The acute testing established the dose that each individual needed in order to attain a specific serum EPA-to-AA ratio that was predicted to reduce colonic PGE₂ by 50% using a mathematical model. The personalized dose was 2–10 g/d for each study participant (mean: 5.5 g/d), and this dose was provided for 12 weeks (23). Serum and colon samples were analyzed for FAs using GC, serum was analyzed for cholesterol using enzymatic assays, and PGE₂ in biopsy samples was analyzed using LC with mass spectral detection as previously described (23).

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Supplemental Tables 1 and 2 and Supplemental Figures 1–4 are available from the “Supplementary data” link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/>.

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Abbreviations used: AA, arachidonic acid; OTU, operational taxonomic unit; PCoA, principal coordinates analysis; PGE₂, prostaglandin E₂; post, post-supplementation with ω -3 fatty acids; pre, pre-supplementation with ω -3 fatty acids; rRNA, ribosomal RNA; θ_{VC} , Yue and Clayton community dissimilarity index.

Colonic mucosal biopsy samples and a stool brushing sample near the biopsy sites were obtained at baseline and after 12 weeks of personalized ω -3 FA supplementation using flexible sigmoidoscopy, without previous preparation of the bowels. The biopsy samples were collected in the colon 20–25 cm from the anal sphincter. Biopsy samples were flash frozen in liquid nitrogen exactly 5 s after harvesting and were frozen at -70°C until analysis. Of the 47 study participants, a stool brushing was not collected from one subject at baseline and one subject after supplementation, leaving 45 pairs of stool brushings. For the biopsy samples, 42 pairs were available for the present analysis of bacterial microbiota after other assays of biopsy tissues were completed for the primary study endpoints. For 10 of the biopsy samples analyzed (5 at baseline and 5 at 12 wk), a duplicate biopsy sample was analyzed for evaluating community variability between biopsy sites.

Dietary assessments

Subjects were asked to follow their usual diet while in the study. Dietary intakes of study subjects were assessed by 24-h dietary recalls that were conducted by the 5-pass method. Two recalls were done in person, one at the study screening visit and one at study baseline. Three recalls were done by unannounced telephone calls during supplementation: 4 weeks after starting the acute dosing protocol, one approximately halfway through the 12-week target dosing period, and one 1 week before the target dosing was concluded. The recalls were analyzed using the Nutrition Data System for Research (2013 version; University of Minnesota, Nutrition Coordinating Center). Food and nutrient intakes were obtained as an average of all recalls obtained for each subject.

Bacterial 16S ribosomal RNA gene sequencing

Sequence analysis of bacterial 16S ribosomal RNA (rRNA)-encoding gene amplicons was used to analyze the study samples. Our goal in the study was to examine possible changes in the community structure due to ω -3 FA supplementation, and this method can compare a large number of samples at reasonable cost (46, 47). DNA was isolated from samples with a PowerMag Microbiome RNA/DNA Isolation Kit (Mo Bio Laboratories, Inc.) using an epMotion 5075 liquid handling system. The V4 region of the 16S rRNA gene was amplified and sequenced as described previously using undiluted DNA, except for 2 samples that needed dilution (48). PCR products were visualized using an E-Gel 96 with SYBR Safe DNA Gel Stain, 2% (catalog no. G7208-02; Life Technologies).

Libraries were prepared using a dual-indexing strategy according to Illumina's protocol for Preparing Libraries for Sequencing on the MiSeq (part no. 15039740 Rev. D). Libraries were normalized using Life Technologies SequelPrep Normalization Plate Kit (catalog no. A10510-01) following the manufacturer's protocol for sequential elution. The concentration of PCR products in the pooled samples was determined using Kapa Biosystems Library Quantification kit for Illumina platforms (Kapa Biosystems KK4824). The sizes of the amplicons in the library were determined using an Agilent Bioanalyzer High Sensitivity DNA analysis kit (catalog no. 5067-4626). The final library consisted of equal molar amounts from each of the plates, and it was normalized to the pooled plate at the lowest concentration. If the library concentration was <1 nM, modified hybridization buffers were used for denaturation (49). The final load concentration was 4 pM with a 10% PhiX spike to add diversity. Sequencing reagents were prepared according to a published protocol (50, 51).

Sequencing was done on the Illumina MiSeq platform using a MiSeq Reagent Kit V2 500 cycles (catalog no. MS-102-2003), according to the manufacturer's instructions with published modifications that are available in a Web-based standard operating procedure for generating libraries (50, 51). The Accuprime High Fidelity Taq (Life Technologies, Carlsbad, CA, catalog no. 12346094) was used instead of Accuprime Pfx supermix. Control samples (a water blank, a mock bacterial community, and a rinse of the biopsy instrument that had been opened in the biopsy procedure room) indicated no problems with assay performance.

Bacterial sequence processing and analysis

FASTQ files were generated for paired end reads. The analysis of bacterial taxa was based on the procedures developed by the Schloss Laboratory (50, 51). The 16S rRNA gene sequence data were processed and analyzed using the software package "mothur" and the most recent MiSeq standard operating procedure (50, 52). After sequence processing and alignment to the SILVA reference alignment (53), sequences were binned into operational taxonomic units (OTUs) based on 97% sequence similarity. Sequence counts per OTU for each sample were calculated. Subsampling was done to 2330 sequences per sample, which eliminated 4 biopsy samples and none of the brush samples (leaving 38 pairs of biopsy samples for the statistical analyses). Genus-level taxonomic classifications were made using a modified version of the Ribosomal Database Project training set within mothur (54, 55).

Calculation of diversity indexes

The Shannon Diversity Index (H) and Inverse Simpson Index were calculated as previously described (56). The community dissimilarity index (θ_{YC}) was calculated using the method of Yue and Clayton that accounts for the proportions of both shared and nonshared species, producing a "Nonparametric Maximum Likelihood Estimator" index, with values of 0 indicating complete similarity and 1 indicating complete dissimilarity (increased distance) between the communities present (57). The mean θ_{YC} was calculated at baseline and post-supplementation to evaluate the similarity between paired biopsy and stool brushing by averaging the θ_{YC} for all pairs of values at each time point. The θ_{YC} values for each pair of biopsy or brush samples before and after 12 weeks of supplementation were also generated to estimate similarity pre- and post-supplementation in the 2 types of samples.

Statistical analyses

Linear discriminant analysis effect size methods of Segata et al. (58) were used to identify bacterial OTUs that differ pre- and post-supplementation. Analysis of molecular variance was conducted in mothur using the method of Anderson (59). Principal coordinates analysis (PCoA) were used to visualize the θ_{YC} values between samples. PCoA axes were calculated in mothur software. Results were plotted using the *pca3d* package with the R program (60, 61). All other analyses were performed with SPSS software, version 24 (IBM Corporation). Correlations were evaluated using Spearman's rank correlation coefficient (ρ). Comparisons between means were conducted using 2-sample t tests or paired t tests. Comparisons between proportions were made using chi-square tests. Adjustment for false discovery rates was done using the method of Benjamini and Hochberg (62).

Results

Bacterial sequencing success and subject characteristics

The sequencing of colonic mucosal bacteria yielded ≥ 2330 sequences in all but 4 of the biopsy samples, 2 at baseline and 2 after supplementation. This resulted in 38 pairs of pre and post biopsy pairs after subsampling from the 42 biopsy pairs submitted for bacterial analysis. For the stool brushings, none of the submitted samples were removed upon subsampling. The demographic characteristics of the 9 subjects from whom bacterial sequencing data were not available for all biopsy samples (either due to the lack of sample or due to elimination on subsampling) were similar to the 38 other study subjects (Supplemental Table 1).

For 10 subjects, an additional biopsy was sequenced to evaluate differences in biopsy bacterial communities between 2 different biopsy sampling sites. Five of those biopsies were from baseline and 5 were after 12 weeks of supplementation. The θ_{YC}

values for 9 of those biopsy pairs obtained from the same subject at the same time point, but at a different site, ranged between 0.001 and 0.094, except for one pair of biopsy samples obtained at baseline for which the θ_{YC} was 0.509.

Associations of subject characteristics with bacterial populations diversity at baseline

We first evaluated associations of bacterial diversity indexes with BMI and dietary fiber and fat intakes at baseline, given the growing literature on the role of the microbiome as one important mediator of the health effects of obesity (63). Associations of diversity indexes with diet and BMI were explored using correlative analyses as shown in Table 1. Diet was an average of 5 unannounced recalls that were completed during the 12-week intervention (a mean of 4.8 recalls/subject were successfully completed; range: 3–5 recalls). Both BMI and percentage of energy from dietary fat were negatively associated with the Shannon and Inverse Simpson diversity indexes, whereas dietary fiber was positively correlated with these indexes. The diversity measures in biopsy samples correlated more strongly with BMI than with diversity in the stool brushing (Table 1), but P values were not quite significant in each case ($0.05 < P < 0.08$).

Effects of ω -3 FA supplementation on bacterial abundance and diversity indexes

Changes in bacterial composition post-supplementation were not large. The bacterial community composition of the 30 most abundant OTUs before and after ω -3 FA supplementation (Supplemental Figures 1 and 2) for both the biopsy samples and luminal stool brushings. Using linear discriminant analysis effect size analyses, the only OTUs to differ significantly pre- and post-supplementation (pre/post) were an OTU classified to the *Blautia* genus in stool brushings (relative abundance decreased from 4.6% to 4.1%; $P = 0.012$) and an OTU classified to the *Clostridium* XIVa genus in biopsy samples (relative abundance decreased from 3.0% to 2.9%; $P = 0.03$). The relative abundance of major phyla, families, and Shannon and Inverse Simpson indexes did not change appreciably pre- and post-supplementation (Table 2). There were, however several differences between brush and biopsy samples, with higher diversity, lower *Bacteroides*-to-*Prevotella* ratios and higher relative abundance of Actinobacteria, *Coriobacteriaceae*, and *Bifidobacteriaceae* being found in the brush samples than in the biopsy samples (Table 1).

We also evaluated the Yue and Clayton dissimilarity index (θ_{YC}) for pairs of samples. The θ_{YC} similarity indexes for pre and post biopsy pairs and pre and post brush pairs were correlated with Spearman's $\rho = 0.674$ ($P < 0.001$), indicating that subjects with high similarity pre/post in biopsy samples also had higher similarity in pre/post brush samples. The θ_{YC} was, however, higher (i.e., lower similarity) in bacterial communities between brush and biopsy pairs of samples post-supplementation than that at baseline (Table 3). Using analysis of molecular variance, the θ_{YC} between brush and biopsy pairs was significantly different post-treatment ($P = 0.015$) but not pretreatment ($P = 0.205$). There was lower similarity for pre/post pairs than for biopsy/brush pairs at a single time point, as indicated by the higher θ_{YC} dissimilarity index (Table 3). Overall, this indicates that there was a change in the distribution of bacterial populations after ω -3 FA supplementation.

PCoA of θ_{YC} values

The distribution of θ_{YC} values for bacterial communities in both biopsy samples and brushings was visualized using PCoA plots of θ_{YC} values (Supplemental Figures 3 and 4). The PCoA analyses identified a group of outlying samples that ordinated on axis 1 with values >0.2 : these were enriched in the relative abundance of *Prevotella*. This included 8 subjects with outlying values for both their brush and biopsy samples and 2 subjects with outlying values for a stool brushing sample only. In 5 of those 10 subjects, both the pre and post sample was an outlier.

The 10 subjects who had a biopsy and/or brush sample as an outlier on the PCoA plot showed a high abundance of *Prevotella* in both types of samples as compared with the other subjects (Supplemental Table 2, Supplemental Figures 3 and 4). There were no significant differences in age (53 compared with 46 y), BMI (28.9 compared with 26.3), dietary fat as percentage of energy (37.6% compared with 35.3%), or dietary fiber (10.5 compared with 12.6 g/1000 kcal) using 2-sample t tests ($P > 0.12$ in each case). There also was no significant difference by gender using chi-square tests. However, the outliers were more likely to have a high waist circumference by National Heart, Lung, and Blood Institute clinical criteria for obesity (>102 cm for men and >88 cm for women, with $P = 0.018$) by the chi-square test (64). After supplementation, PGE₂ increased a mean of 12% in the 10 outliers with high *Prevotella* in a luminal brushing sample compared with a mean decrease of 36% in the 37 other subjects ($P = 0.021$; Supplemental Table 2). Both the Shannon and Inverse Simpson diversity indexes were lower in the 10 outliers, and some of these differences were significant (Supplemental Table 2), but the number of high *Prevotella* outliers on the PCoA plots was small. The highly significant differences between outliers and nonoutliers in the diversity indexes and in the extent of decreases achieved in colonic PGE₂ after supplementation, however, indicated that diversity indexes should be evaluated as predictors of changes in colonic PGE₂.

Effects of bacterial composition on colonic PGE₂ concentrations

The mean changes in bacterial composition after ω -3 FA supplementation were small, but there was considerable interindividual variability, especially in the Inverse Simpson Index (Table 2), and the θ_{YC} values for brush biopsy pairs differed pre- and post-supplementation (Table 3). We therefore evaluated correlations between changes in diversity indexes and changes in colonic PGE₂, as shown in Table 4. These correlations were very similar when using either fold change in colonic PGE₂ or the absolute difference in colonic PGE₂. Change in colonic PGE₂ was negatively correlated with diversity measures in brush samples (not biopsy samples) and with changes in colonic EPA-to-AA ratios (Table 4).

To evaluate the relative contribution of bacterial diversity in brush samples to change in colonic PGE₂, linear regression models were constructed. In these models, colonic PGE₂ post-supplementation was the dependent variable, and square root transformation was needed to achieve a normal distribution. Baseline colonic PGE₂ accounted for 20% of the variability in square root of PGE₂ post-supplementation, with higher baseline PGE₂ predicting higher PGE₂ post-supplementation (Table 5). Higher baseline BMI also predicted higher PGE₂ post-supplementation, but this had a modest effect and was not significant ($P = 0.245$). Change in colonic EPA:AA

TABLE 1 Spearman correlation coefficients (ρ) between microbiome diversity indexes in brush and biopsy samples with the characteristics of study participants at baseline¹

Baseline characteristics	Brush ($n = 46$)		Biopsy ($n = 38$)	
	Shannon Index	Inverse Simpson	Shannon Index	Inverse Simpson
		Index		Index
Age, y	0.102	0.096	-0.073	-0.086
BMI, kg/m ²	-0.185	-0.150	-0.316	-0.302
Dietary fiber, g/1000 kcal	0.360*	0.365*	0.317	0.352*
Dietary fat, % of energy	-0.334*	-0.286	-0.360*	-0.349*
Colonic PGE ₂ , ng/mg protein	-0.120	-0.152	-0.081	-0.056
Colonic total FAs, μ g/mg protein	0.015	-0.044	-0.112	-0.110
EPA:AA				
Colonic	0.308*	0.346*	0.138	0.197
Serum	0.002	0.032	0.048	0.027
Serum cholesterol, mg/dL	-0.121	-0.205	-0.145	-0.185

¹Positive correlation coefficients indicate increased bacterial diversity is associated with higher values of any given subject characteristic. *Significant ($P < 0.05$), although no longer significant after correction for false discovery rates. AA, arachidonic acid; FA, fatty acids; EPA, eicosapentaenoic acid; PGE₂, prostaglandin E₂.

also improved the model slightly without being significant. Absolute change in the Inverse Simpson Index for brushes predicted an additional 8% of the variance in colonic PGE₂ post-supplementation after including the other variables, and the final model predicted 31% of the variance in PGE₂ post-supplementation (Table 5). In models using change in the Shannon Index instead of the Inverse Simpson Index, the predictive value of the overall model was similar but

slightly weaker—6% of the variance in colonic PGE₂ post-supplementation after including the other variables, with the final model predicting 29.5% of the variance in colonic PGE₂ post-supplementation.

Both models were highly significant ($P = 0.001$), and the negative β -coefficients indicate that increased bacterial diversity at baseline in luminal brush samples predicted lower colonic PGE₂ after supplementation. Changes in bacterial diversity

TABLE 2 Bacterial microbiome diversity indexes and relative abundance of major phyla, families, and genera in colon mucosal biopsy tissues and luminal stool brushings before and after ω -3 FA supplementation of healthy volunteers¹

Measure	Biopsy samples		Brush samples	
	Pre	Post	Pre	Post
Diversity indexes and abundance ratios				
Shannon Diversity Index	3.50 \pm 0.49	3.40 \pm 0.61	3.66 \pm 0.41*	3.65 \pm 0.41*
Inverse Simpson Index	20.3 \pm 11.6	18.2 \pm 10.6	23.4 \pm 11.1	22.9 \pm 11.3*
Firmicutes:Bacteroidetes	2.86 \pm 1.61	2.80 \pm 2.18	2.09 \pm 1.01	1.94 \pm 1.00
<i>Bacteroides:Prevotella</i>	142 \pm 202	97 \pm 202	111 \pm 190*	67 \pm 143
Relative abundance of major phyla (mean abundance >0.1)				
Firmicutes	61.0 \pm 14.6	56.6 \pm 18.3	55.9 \pm 9.7	53.5 \pm 0.1
Bacteroidetes	27.3 \pm 13.0	29.8 \pm 16.2	31.4 \pm 10.8	31.9 \pm 9.8
Actinobacteria	2.2 \pm 1.8	2.1 \pm 2.1	5.3 \pm 3.6*	5.8 \pm 4.2*
Proteobacteria	4.5 \pm 9.6	6.9 \pm 14.0	2.6 \pm 2.2	3.0 \pm 2.1
Fusobacteria	0.1 \pm 0.5	0 \pm 0.1	0.1 \pm 0.7	0.1 \pm 0.0
Verrucomicrobia	3.8 \pm 7.7	3.7 \pm 6.4	3.7 \pm 4.8	4.5 \pm 7.2
Unclassified	1.1 \pm 1.5	0.8 \pm 1.0	1.1 \pm 1.3	1.1 \pm 1.8
Relative abundance of major families (abundance >2)				
<i>Lachnospiraceae</i>	33.1 \pm 13.1	30.0 \pm 14.5	26.4 \pm 9.2*	26.0 \pm 8.1
<i>Bacteroidaceae</i>	15.4 \pm 8.7	16.1 \pm 11.3	18.0 \pm 9.5	18.2 \pm 9.8
<i>Ruminococcaceae</i>	15.3 \pm 7.1	12.1 \pm 6.9	16.3 \pm 6.2	14.2 \pm 6.8
<i>Prevotellaceae</i>	7.2 \pm 14.1	9.8 \pm 17.8	6.9 \pm 12.2	6.9 \pm 11.9
<i>Verrucomicrobiaceae</i>	3.8 \pm 7.6	3.7 \pm 6.4	3.7 \pm 4.8	4.5 \pm 7.2
<i>Porphyromonadaceae</i>	2.2 \pm 1.2	2.6 \pm 2.7	3.1 \pm 2.1	3.6 \pm 2.5
<i>Rikenellaceae</i>	2.0 \pm 3.3	1.7 \pm 2.2	3.2 \pm 4.2	3.1 \pm 3.2
<i>Coriobacteriaceae</i>	0.9 \pm 0.7	1.0 \pm 1.0	2.9 \pm 2.2*	3.3 \pm 2.9*
<i>Acidaminococcaceae</i>	1.8 \pm 2.0	2.6 \pm 3.1	2.3 \pm 2.1	2.7 \pm 3.0
<i>Bifidobacteriaceae</i>	0.9 \pm 1.4	0.9 \pm 1.4	2.0 \pm 2.6	2.2 \pm 2.4*

¹Values are means \pm SDs at each time point. There were 45 brush and 38 biopsy pre/post pairs. Paired t tests indicated no significant differences pre- and post-supplementation ($P \geq 0.05$ in each case) in any listed variable. Only 2 pre/post differences were found when the abundance of individual operational taxonomic units was evaluated (*Blautia* in brushing samples and *Clostridium XlVa* in biopsy samples both decreased, $P < 0.05$; see Results). *Different from their paired biopsy samples collected at the same time point, $P < 0.01$ (after correction for false discovery rates). Post, post-supplementation; Pre, pre-supplementation.

TABLE 3 θ_{YC} Microbiome dissimilarity index for brush and biopsy pairs and pre- and post-supplementation pairs of samples from healthy volunteers supplemented with ω -3 FAs¹

Paired samples	Number of pairs	θ_{YC} Index
Pre and post biopsy pairs	38	0.53 \pm 0.28
Pre and post stool brushing pairs	45	0.42 \pm 0.21
Biopsy and brush pairs at baseline	38	0.22 \pm 0.22
Biopsy and brush pairs post-supplementation ²	40	0.32 \pm 0.23

¹Values are means \pm SDs unless otherwise indicated. AMOVA, analysis of molecular variance; post, post-supplementation; pre, pre-supplementation; θ_{YC} , Yue and Clayton community dissimilarity index.

²AMOVA analyses indicated that the θ_{YC} index differed significantly between brush and biopsy samples post-supplementation ($P = 0.015$ post-supplementation) but not pre-supplementation at baseline ($P = 0.205$ at baseline). The θ_{YC} values for pre and post biopsy samples did not differ ($P = 0.248$) nor did the θ_{YC} values for pre and post brush samples ($P = 0.957$).

measures in biopsy samples were not significant in these models, contributing to no more than 1% of the variance. Outlier status from the PCoA analyses, either in biopsy or brush samples, also was not significant, perhaps because there was a small number of outliers.

Discussion

This study utilized samples from a clinical trial that aimed to achieve a reduction in the colonic proinflammatory state using personalized dosing of ω -3 FAs. The dosing strategy was targeted to achieve serum EPA:AA that would reduce colonic PGE₂ by 50% in each individual based on a mathematical model. Despite the personalization of the ω -3 FA dose, there was a wide range of changes in colonic PGE₂ observed (23). Here, we explored the role of the colonic bacterial microbiome in the anti-inflammatory effects of ω -3 FA supplementation using

TABLE 4 Spearman correlations (ρ) of fold change in colonic PGE₂ concentrations with changes in bacterial diversity measures and colonic FAs in healthy volunteers supplemented with ω -3 FAs¹

Change variable ²	Number of observations	Correlation coefficient, ρ
θ_{YC} index		
Biopsy	38	0.263
Brush	45	0.141
Inverse Simpson Index		
Biopsy	45	-0.002
Brush	45	-0.503*
Shannon Index		
Biopsy	38	0.045
Brush	45	-0.400*
EPA:AA	47	-0.442*

¹*Significant after correction for false discovery rates, $P < 0.020$. AA, arachidonic acid; EPA, eicosapentaenoic acid; PGE₂, prostaglandin E₂; post, post-supplementation; pre, pre-supplementation; θ_{YC} , Yue and Clayton community dissimilarity index.

²For biopsy and brush θ_{YC} , the change variable is the θ_{YC} dissimilarity index between pre and post paired samples. The other change variables shown are the post-supplementation value minus the baseline value; percentage change was not used because very low baseline EPA-to-AA ratios result in a large percentage change despite a modest absolute change.

16S rRNA sequencing analysis of both colon biopsy samples and colon luminal brushings. Bacterial communities in biopsy samples from different sites have been shown to be similar (65–67), justifying the use of only biopsy for bacterial sequencing. Although ω -3 FA supplementation might not be expected to have a large effect on the colonic bacteria, host metabolic factors have been previously shown to affect the microbiome: host immune status has a bidirectional interplay with the intestinal bacteria (2–5). In particular, increased diversity of the intestinal microbiome has been associated with beneficial effects such as lower inflammation and insulin resistance (68).

In the present trial, increased bacterial diversity at baseline, as measured by the Shannon and Inverse Simpson indexes, was positively correlated with dietary fiber intakes and negatively correlated with fat intakes, as might be expected if increased diversity is beneficial to the host (Table 1). The dietary supplementation with ω -3 FAs had minimal effects on mean changes in the abundance of bacterial taxa and on diversity indexes (Supplemental Figures 1 and 2, Table 2). There were, however, significant differences between luminal brushing samples and biopsy samples both pre- and post-supplementation. Relatively higher diversity and abundance of Actinobacteria and *Coriobacteriaceae* were present in brush compared with biopsy samples (Table 2). This is consistent with published data showing that mucosal and fecal bacterial populations differ in both normal and diseased states (65, 69, 70). One other study found higher Proteobacteria in the colonic mucosa than in lumen brushings, but these samples were from patients with colorectal cancer (71). We also evaluated the θ_{YC} dissimilarity index. The θ_{YC} for brush and biopsy pairs was higher after supplementation than that at baseline, indicating that there was some change in the bacterial microbiome after supplementation (Table 3).

There also was a small subgroup of 10 subjects who had high *Prevotella* abundance in either brushing or mucosal samples, as identified from PCoA analyses of the θ_{YC} index (Supplemental Figures 3 and 4). These subjects with outlying samples had lower bacterial diversity and did not exhibit a decrease in colonic PGE₂ after ω -3 FA supplementation (Supplemental Table 2). This is consistent with data showing that high *Prevotella* abundance is associated with higher blood cholesterol and inflammatory conditions (72, 73). On the other hand, a high-fiber diet was shown to increase the *Prevotella*-to-*Bacteroides* ratio (74). Wu et al. (75) suggested that the *Prevotella*-to-*Bacteroides* ratio is strongly associated with long-term diets, particularly diets rich in protein and animal fat (*Bacteroides*) compared with carbohydrates (*Prevotella*). Other work suggests that this is an artefact of sparsity of samples with a high *Prevotella* abundance (76). In our study, only 10 subjects had samples with high *Prevotella*. In linear regression analyses, the effect of having high *Prevotella* on predicting colonic PGE₂ was minimal. Larger studies will be needed to obtain more data on individuals with high *Prevotella* abundance.

Our supplementation study with a highly purified fish-oil supplement did not find a large impact, on average, on changes in bacterial communities in either the colonic mucosa biopsy samples or luminal brushings other than the increased θ_{YC} dissimilarity index for brush and biopsy pairs post-supplementation (Table 3). This could be due to the fact that fats are largely absorbed in the small intestine, leaving little to reach the colon. In a review of studies, diets higher in fiber, legumes, and fruit generally were associated with higher diversity; and Western diets decreased Firmicutes abundance, likely due to the

TABLE 5 Predictors of colonic PGE₂ after ω -3 FA supplementation in healthy volunteers from linear regression analyses¹

Predictor	Adjusted R ²	Standardized regression coefficient β in final model	Significance, P	
			F-change	Final model
Model 1				
Baseline PGE ₂	0.200	0.638	0.001	—
Plus baseline BMI	0.207	0.127	0.245	—
Plus change in colonic EPA:AA	0.231	−0.171	0.139	—
Plus change in Inverse Simpson Index brush samples	0.310	−0.313	0.022	0.001
Model 2				
Baseline PGE ₂	0.200	0.665	0.001	—
Plus baseline BMI	0.207	0.177	0.245	—
Plus change in colonic EPA:AA	0.231	−0.209	0.139	—
Plus change in Shannon Index brush samples	0.295	−0.283	0.035	0.001

¹Colonic PGE₂ was square root transformed to achieve normality. Gender and age were not significant predictors ($P > 0.7$ in each case) and decreased the adjusted R². Change variables were expressed as absolute change (difference between post-supplementation and baseline). The adjusted R² indicates the fraction of the variance in colonic PGE₂ explained by the model, the sign of the regression coefficient indicates whether the association is positive or negative, and the significance is given as a P value. AA, arachidonic acid; EPA, eicosapentaenoic acid; PGE₂, prostaglandin E₂.

low fiber content (74). We did, however, expect that changes in colonic biology due to the fairly high dose of ω -3 FAs used in our study, namely 6 g/d on average, conceivably might affect at least the mucosal bacterial populations via its anti-inflammatory effects on the host.

Other studies also have found minimal change in bacterial composition using dietary interventions. In a study in 10 healthy men, 4 weeks of a high-fat, high-calorie diet did not result in a change in the Firmicutes-to-Bacteroidetes ratio, the *Prevotella*-to-*Bacteroides* ratio, or in any specific OTU in the fecal bacteria (77). There were, however, correlations of changes in bacterial abundance with metabolic parameters: negative correlations between IFN- γ with changes in the Shannon Index and positive correlations between changes in metabolic rate, platelet count, and energy expenditure with the *Prevotella*-to-*Bacteroides* ratio (77). Other studies that targeted changes in dietary patterns found that fecal bacterial populations either were minimally affected, such as after a dietary intervention with the New Nordic Diet (73), or that different types of diets, Mediterranean or low-fat, high-complex-carbohydrate, had contrasting effects on the fecal microbiome despite similar metabolic benefits on improving insulin sensitivity (78). Our study did not collect fecal samples, but previous work has shown that fecal and rectal swab microbiota are highly similar (79).

Because we observed a high degree of variability in the reduction in colonic PGE₂ in our supplementation study, we evaluated whether the bacterial microbiome was predictive of the reduction in colonic PGE₂ using linear regression analyses. Surprisingly, changes in diversity of the bacteria in the luminal brushing samples, but not in the biopsy samples, were significant predictors of colonic PGE₂ concentrations post-supplementation (Table 5), after including baseline PGE₂, baseline BMI, and changes in colonic EPA:AA in the models. It is possible that the luminal bacteria are more susceptible to dietary changes than the bacteria associated with the mucin. The mucin layer provides a niche for bacterial growth that can shelter the microbiome from changes in the lumen (80). Fecal samples were not collected in our study, but because there are similarities in the microbiome composition between feces and rectal swabs (79), this indicates that analysis of feces may be sufficient for some types of dietary studies. The contribution of changes

in brush diversity to changes in colonic PGE₂ in the present study was modest but significant, with an increase in diversity predicting a larger decrease in colonic PGE₂. Whether or not dietary and other lifestyle practices that increase gut bacterial diversity could work in concert to augment the preventive effects of ω -3 FAs is not known.

There are several limitations of our clinical trial. Our use of 16S rRNA-encoding gene amplicon analysis provides robust data with regard to potential changes in community structure; this method does not provide analysis of potential changes in community function. Metagenomic and meta-transcriptomic methods could provide this functional information and could be used in subsequent studies. Second, although we had a strong rationale for targeting a reduction in PGE₂ as the primary outcome with the EPA-enriched formulation, it is likely that the profile of lipid metabolites as a whole is important for the inflammatory state of the colonic tissue. There are many forms of ω -3 FAs; this trial utilized a TG form naturally found in fish due to its excellent bioavailability (compared with that of ethyl esters) and stability (compared with that of FFAs) (82). The supplement used was highly enriched in EPA, but it also contained 16% DHA and 20% other ω -3 FAs, all of which can form bioactive metabolites (81). It is possible that other formulations that are more highly purified or in a different form could yield different effects. For example, feeding rodents fish oil that was high in DHA or krill oil that was high in EPA resulted in similar changes in the 8 most abundant bacterial genera in feces, but diets with the same dose but differing ratios of the fish and krill oils changed the relative abundance of several of the genera in opposite directions (83). Third, the background diet of the study participants was not controlled. This makes it more difficult to detect an effect of dietary supplementation, as opposed to that in rodents where the same background diet is used for all groups. In rodent studies, the diets typically also are provided for a larger percentage of the life span, and often start at an earlier developmental age, compared with that in humans. Future studies could be designed to increase gut bacterial diversity by addressing the background diet to determine if this would work in concert with increased ω -3 FA intakes to maximize the anti-inflammatory effects on colonic tissue.

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