Gastroenterology 2015;149:883–885

Proton Pump Inhibitors Alter Specific Taxa in the Human Gastrointestinal Microbiome: A Crossover Trial

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See Covering the Cover synopsis on page 827; see editorial on page 848.

We conducted an open-label crossover trial to test whether proton pump inhibitors (PPIs) affect the gastrointestinal microbiome to facilitate *Clostridium difficile* infection (CDI). Twelve healthy volunteers each donated 2 baseline fecal samples, 4 weeks apart (at weeks 0 and 4). They then took PPIs for 4 weeks (40 mg omeprazole, twice daily) and fecal samples were collected at week 8. Six individuals took the PPIs for an additional 4 weeks (from week 8 to 12) and fecal samples were collected from all subjects at week 12. Samples were analyzed by 16S ribosomal RNA gene sequencing. We found no significant within-individual difference in microbiome diversity when we compared changes during baseline vs changes on PPIs. There were, however, significant changes during PPI use in taxa associated with CDI (increased Enterococcaceae and Streptococcaceae, decreased Clostridiales) and taxa associated with gastrointestinal bacterial overgrowth (increased Micrococcaceae and Staphylococcaceae). In a functional analysis, there were no changes in bile acids on PPIs, but there was an increase in genes involved in bacterial invasion. These alterations could provide a mechanism by which PPIs predispose to CDI. ClinicalTrials.gov ID NCT01901276.

Keywords: *Clostridium difficile* Infection; Pharmacology; Gastroesophageal Reflux Disease; Acid Suppression.

Proton pump inhibitors (PPIs) have been associated with *Clostridium difficile* infection (CDI), but the mechanism linking PPIs and CDI is unknown. Broad-spectrum antibiotics are the most important risk factor for CDI and cause loss of diversity within the gastrointestinal microbiome.[1] There are also more specific changes within the microbiome that precede CDI. Increases in Enterococcaceae and decreases within key Clostridial taxa at the time of hospital admission are associated with increased risk for subsequent development of CDI.[2]

This study tested whether PPIs given in the absence of antibiotics alter the human colonic microbiome to predispose to CDI. Twelve healthy volunteers each donated a fecal sample at week 0 and week 4 of the study (see Supplementary Methods for complete description). They subsequently all took omeprazole 40 mg twice daily for 4 weeks and donated an additional sample (week 8). The subjects were then randomized 1:1 to stop PPIs or continue them for an additional 4 weeks, after which they donated a final sample (week 12) (Supplementary Figure 1). We excluded those who used antibiotics within 1 year or tested positive for the *C difficile* toxin B gene at week 0 (Supplementary Table 1 and Supplementary Figure 2). We used 16S ribosomal RNA gene sequencing to describe the fecal microbiome. Our a priori primary outcome was fecal microbial diversity, defined as the within-individual difference in Shannon's index of diversity comparing change during the 4-week baseline period to change during the 4-week period on PPIs. To focus on taxa predisposing to CDI, we prespecified taxa of interest referencing studies of lower gastrointestinal microbiome changes preceding CDI, and studies of upper gastrointestinal microbiome changes after PPIs (Supplementary Table 2).

We found no within-individual changes in diversity after 4 weeks of PPI treatment (Figure 1*A*). Two subjects received antibiotics between week 8 and week 12 for reasons unrelated to the study; for these subjects, the samples taken after antibiotics were excluded from the final analyses. In the remaining subjects that received 8 weeks of PPIs ($n = 5$), there was no difference between 8 weeks of PPIs compared with baseline ($P = .79$). On principal coordinates analyses, there was no distinct clustering of samples from before vs after PPI treatment (Figure 2). In sum, overall fecal microbial composition remained stable during use of PPIs.

However, PPI treatment for 4 weeks did induce significant within-individual increases in Enterococcaceae and Streptococcaceae, taxa that have been associated with exposure to antibiotics and increased risk for CDI (Figure 1*B*).[3–7] In a hospital-based study, patients who later

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Abbreviations used in this paper: CDI, *Clostridium difficile* infection; PPI, proton pump inhibitor.

Most current article

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2015.06.043

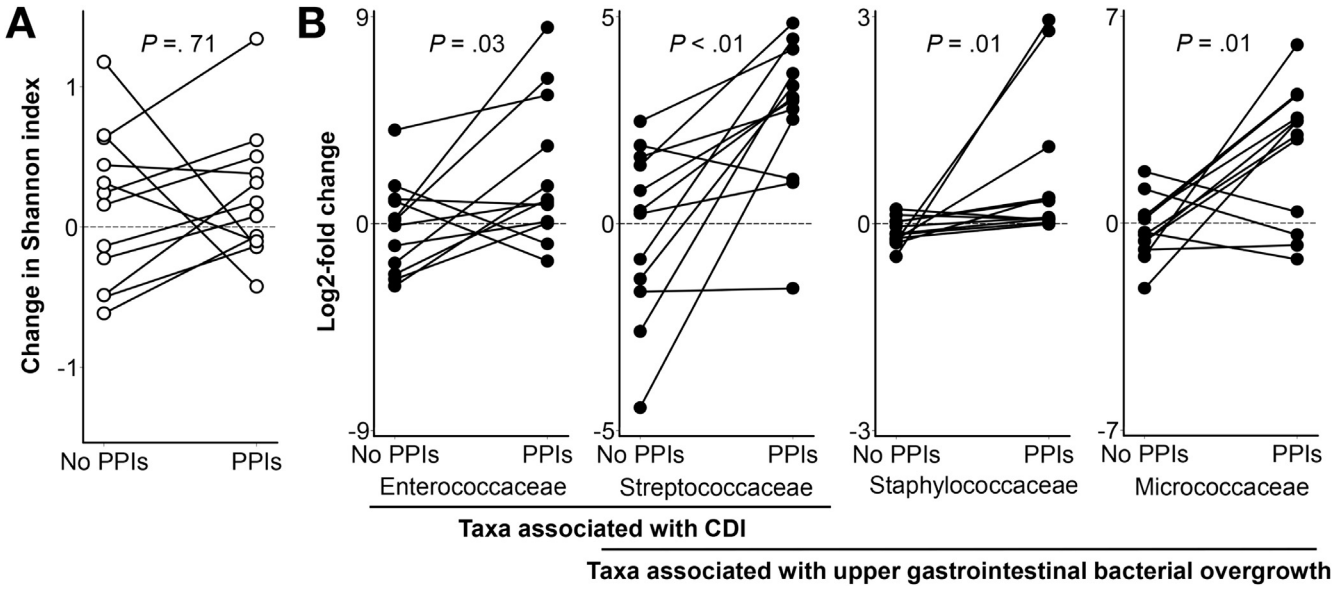

Figure 1. Changes in fecal microbiotal diversity and specific taxa throughout the study. (*A*) There was no significant change in overall diversity after 4 weeks of PPIs compared with 4 weeks at baseline. (*B*) Four weeks of PPIs induced within-individual changes in the relative abundance of prespecified taxa associated with CDI and with upper gastrointestinal bacterial overgrowth. Lines connect individuals.

developed CDI had low diversity and increased Enterococcaceae compared with control patients.[3] Enterococci are present in low abundance in human stool, but can rapidly expand after broad-spectrum antibiotics.[4] In mice, treatment with clindamycin is followed by proliferation of enterococci and CDI.[5] Dynamic modeling suggests that an increase in enterococci is a key step preceding *C difficile* colonization.[6] Streptococcaceae, which are predominantly upper gastrointestinal tract organisms, were increased >10-fold after PPIs. Gastric and small intestinal bacterial overgrowth with *Streptococcus* is an established consequence of PPIs, but the direct pH-raising effects of PPIs are attenuated by the distal duodenum.[8–10] Streptococcaceae are disrupted by broad-spectrum antibiotics and have been associated with CDI.[7] Our results are consistent with the hypothesis that PPI-induced hypochloryhydria causes increased gastric and fecal *Streptococcus*, leading to increased risk for CDI.

To identify additional changes caused by 4 weeks of PPIs, we looked across 97 bacterial families present in all samples and compared within-individual changes before

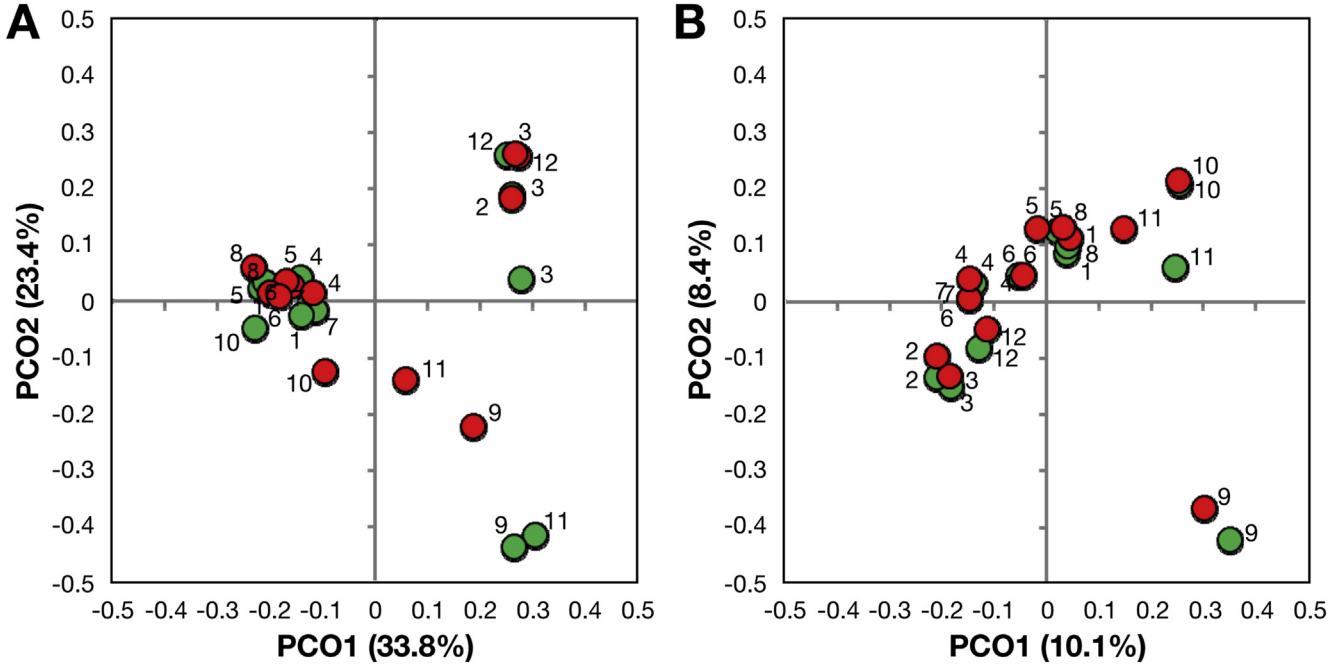

Figure 2. Principal coordinate analyses. Weighted (*A*) and unweighted (*B*) UniFrac analyses from immediately before and after 4 weeks of PPIs. *Circles* represent samples from before PPIs (*green*) or after PPIs (*red*); the corresponding subject's number is adjacent to each *circle*.

and after PPIs. We found a 44% median decrease in Clostridiaceae ($P = .03$). There were no further alterations in prespecified taxa in either arm of the study during the final 4 weeks of the study (Supplementary Figure 3). Stool polymerase chain reaction testing and culture for *C difficile* was performed on all samples. One subject had an equivocal toxin B test after 8 weeks of PPIs and also growth of *C difficile* in culture before and after PPIs.

Bacterial production of secondary bile salts can play an important role in CDI by inhibiting *C difficile* spore germination. In cirrhotics given PPIs, there were decreased levels of urinary dimethylamine, which is produced by the bacterial metabolism of bile salts; subjects also had increased fecal Streptococcaceae after PPIs, as was seen in our study.[11] Cluster XIVa Clostridia including *Clostridium scindens* actively produce secondary bile acids.[12] In our study, there was a decrease in Clostridiaceae after PPIs, but we were unable to directly map *C scindens* within our reference library. Instead, we performed real-time polymerase chain reaction for the *baiCD* gene, which encodes the rate-limiting enzyme in the production of secondary bile acids.[12] We found no change in *baiCD* gene copy number after PPIs ($P = .79$) and, to further investigate bile acids, we then used liquid chromatography and mass spectrometry to directly assess bile acid levels in our samples. There was no change after PPIs in any of 10 dominant human primary and secondary bile acids (Supplementary Figure 4).

Next, we used PICRUSt to impute the metagenome from our 16S sequencing results.[13] We found no changes in the KEGG pathways for bile acid biosynthesis after PPIs (Supplementary Figure 5*A*). We then performed an unbiased metagenomic analysis across all KEGG pathways, assessing for within-individual differences after PPIs compared with the baseline period. After 4 weeks of PPIs, there was a significant increase in the pathway corresponding to genes for *Staphylococcus aureus* infection, which includes genes for antimicrobial lectins (Supplementary Figure 5*B*). After 8 weeks of PPIs, there were significant increases in the pathways corresponding to genes for bacterial invasion of epithelial cells and for the renin-angiotensin system (Supplementary Figure 5*C*); these pathways include genes for antibacterial peptides and maintenance of epithelial integrity. Together, these results imply that PPIs do not increase risk for CDI by altering fecal levels of secondary bile acids, but rather that PPIs might be important after *C difficile* sporulation, by lowering colonization resistance.

Antibiotics cause CDI and reduce the diversity and overall size of the microbiome.[14] We did not find a reduction in fecal microbial diversity after PPIs, but loss of diversity may represent an epiphenomenon that often accompanies the key changes within specific taxa that are permissive for CDI. One prior human study found a small but statistically significant reduction in total bacterial operational taxonomic units after PPIs, but did not identify changes in Shannon diversity or within specific bacterial taxa.[15] In contrast, we did not find changes in operational taxonomic unit counts after PPIs ($P = .12$).

In conclusion, 4 weeks of high-dose PPIs did not change fecal microbial diversity beyond baseline variability, but significantly affected certain taxa including Streptococcaceae and Enterococcaceae. PPIs may increase risk for CDI by altering crucial taxa involved in colonization resistance to *C difficile*.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.06.043.

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Received January 29, 2015. Accepted June 29, 2015.

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Acknowledgments
The authors thank Justin Cross for assistance in measuring primary and secondary fecal bile acid levels.

Author contributions: DEF and JAA were involved in all aspects of the study. NCT was involved in acquisition, analysis, and interpretation of data, and revision of the manuscript. SPC and SW were involved in acquisition of the data and revision of the manuscript. AJR was involved in acquisition and analysis of the data and revision of the manuscript. TCW was involved in study concept, interpretation of the data, and revision of the manuscript. HHW was involved in study concept, acquisition, analysis, and interpretation of data, and revision of the manuscript.

Conflicts of interest
The authors disclose no conflicts.

Funding
Daniel E. Freedberg was funded by the National Center for Advancing Translational Sciences (NIH KL2 TR000081). Harris H. Wang was funded by the National Institutes of Health Director's Early Independence Award (1DP5OD009172-02) and the National Science Foundation CAREER Award (MCB-1453219). Sway P. Chen was funded by the Columbia University's Medical Scientist Training Program. The content of this manuscript is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or other funding organizations.

Supplementary Materials and Methods

Subjects

Participants considered for the study were healthy volunteers 18 years or older lacking the following exclusion criteria: use of systemic antibiotics within 1 year, use of proton pump inhibitors (PPIs) or histamine-2 receptor antagonists within 1 year, new medications within 1 month, chronic gastrointestinal mucosal disease, abnormal bowel frequency (minimum of 3 bowel movements per week, maximum of 3 per day), use of medications with potential interactions with PPIs, pregnancy, and travel planned outside of the United States during the study period. Subjects were instructed to avoid probiotics and major dietary shifts during the study period.

Study Design

The study had a randomized, crossover design. At the initial study visit, information was gathered regarding medical history, diet, use of medications, and anthropomorphic data, including height, weight, and waist and hip circumference. Stool was tested for the *C difficile* toxin B gene by polymerase chain reaction (PCR) at all study visits and those testing positive at baseline were excluded. All subjects were observed for 4 weeks and subsequently block randomized to 4 vs 8 weeks of omeprazole 40 mg twice daily (Supplementary Figure 1). Study visits were scheduled 0, 4, 8, and 12 weeks after enrollment. At each study visit, subjects provided a stool sample, answered questions regarding interval history, and completed a food frequency questionnaire derived from the National Health Interview Survey and validated for assessment of fat and fiber intake over the preceding 4 weeks.[1,2] Omeprazole compliance was assessed via pill counts and by performing mass spectrometry for omeprazole and its metabolites, which were detected at week 8 in all subjects and at week 12 in all subjects randomized to 8 weeks of PPIs. All authors had access to the study data and approved the final manuscript. This study was approved by the Institutional Review Board of Columbia University and registered at ClinicalTrials.gov (ID NCT01901276).

Sample Preparation

Stool specimens were captured in standard collection containers and brought promptly (<1 hour) or temporarily frozen. At study visits, specimens were mixed and aliquoted in a sterile manner, and frozen at −80°C. At the end of the study, batched DNA extraction was performed using the PowerFecal DNA Isolation Kit (Mo Bio, Carlsbad, CA). Polymerase chain reaction was performed targeting the V4 hypervariable region of the 16S ribosomal RNA gene with primers derived from the human microbiome project.[3] Samples were pooled and purified with the QIAquick PCR kit (Qiagen, Valencia, CA) and library quantification performed using a KAPA Library Quantification Kit (Kapa Biosystems, Wilmington, MA).

16S Ribosomal RNA Gene Sequencing

Sequencing of the 16S ribosomal RNA gene V4 region was performed using the Illumina MiSeq 300PE platform (Illumina, San Diego, CA). Singleton reads were discarded and read pairs were merged, trimmed, and filtered for quality using *mothur*.[4] Subsequently singleton contigs were discarded to yield 24,187,741 total sequence contigs or an average 484,000 reads per sample. Greengenes[5] was used as a reference database with additional sequences of interest retrieved from the National Center for Biotechnology, including sequences corresponding to *C difficile*. Clustering of taxonomic units was made at 97% sequence similarity using USEARCH[6] and taxonomic assignments were made using *mothur*.[5] FastTree2[7] (version 2.1.7) was used to generate a phylogenetic tree of the contigs.[7,8] Using *mothur* and the phylogenetic tree, we calculated α- and β-diversity indices including weighted and unweighted UniFrac distances.

Outcomes

The a priori primary outcome was a change in fecal microbial diversity, defined as the within-individual difference in Shannon's index of diversity comparing the change during the 4-week period before PPIs to the change during the 4-week period on PPIs. To assess for a change in fecal microbial composition, we additionally compared Bray-Curtis indices corresponding to the 4-week period before PPIs and the 4-week period on PPIs. To focus on taxa predisposing to the development of CDI, we prespecified taxa of interest referencing 2 types of prior studies: studies of change within the lower gastrointestinal microbiome preceding CDI,[9,10] and studies of change within the upper gastrointestinal microbiome after PPIs[11,12] (Supplementary Table 1). A post-hoc analysis was performed among all family-level assignments to examine for significant changes, comparing the period after with the period before PPI exposure.

Clostridium difficile *Polymerase Chain Reaction and Culture*

Fresh aliquots of stool samples were tested via commercial PCR for the *C difficile* toxin B gene according to the manufacturer's protocol (BD GeneOhm, Sparks, MD). For anaerobic culture, aliquots of frozen stool specimens were thawed and inoculated under anaerobic conditions onto Brucella 5% sheep's blood agar containing hemin and vitamin K1 and agar with cefoxitin, cycloserine, and fructose (Remel, Lenexa, KS). Presumptive Clostridial isolates were identified using the RapID ANA II System (Remel).[13]

Reverse Transcription Polymerase Chain Reaction for baiCD *Gene*

To quantify the relative abundance of the *baiCD* gene in fecal samples, we measured *baiCD* and 16S ribosomal RNA by quantitative PCR using the CFX96 Real-Time System and C1000 Touch Thermal Cycler (Bio-Rad). *BaiCD* was amplified using primers *baiCD*_f (5′-GGWTTCAGCC CRCAGATCTTCTTTG-3′) and *baiCD*_r (5′-TGTGWGYGCATG GAATTCWACTGC-3′). These primers were designed to

amplify a 160-bp region of the aligned sequences of bile acid-inducible operons from *Clostridium hiranonis* TO-931 and *C scindens* VPI 12708. 16S quantitative PCR was performed using previously described primers, which amplify a 172-bp amplicon.[14] PCR reactions were performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) with a template of 10 ng genomic DNA and 0.5 uM of each primer in a total volume of 20 uL. Genomic DNA from *Escherichia coli* K-12 MG1655 and *C scindens* (DSM 5676) were used as templates for negative and positive controls, respectively. The PCR program consisted of an initial step of 98°C for 2 minutes, followed by 40 cycles of 98°C denaturation for 5 seconds and 62°C annealing/extension for 30 seconds, and 65°C−95°C melting for 3 minutes.

Fecal Bile Acids

Methods used to quantify fecal bile acids were similar to those described in Buffie et al.[15] In brief, samples were homogenized, corrected to a final concentration of 0.5 mg/10 μL, and then sonicated. After adding an internal standard (d4-chenodeoxycholic acid), we performed 2 methanol extractions and transferred filtered samples to a mass spectrometry vial containing a reduced volume glass insert. Bile acids were then separated using an Agilent 1290 HPLC and Cogent C18 column (2.1 mm × 150 mm, 2.2 μm; MicroSolv, Eatontown, NJ). Mobile phase A was water + 0.05% formic acid; mobile phase B was acetone + 0.05% formic acid, run at a flow rate of 0.35 mL/min. The injection volume was 5 uL and the liquid chromatography gradient was 25%−70% B in 25 minutes. Bile acids were detected using an Agilent 6550 Q-TOF mass spectrometer with JetStream source, operating in negative ionization mode. Acquisition was from m/z 50 to 1100 at 1 spectra/s, gas temperature 275°C, drying gas 11 L/min, nebulizer 30 psig, sheath gas 325°C, sheath gas flow 10 L/min, VCap 4000 V, fragmentor 365 V, and Oct1 RF 750 V. Bile acids were confirmed by alignment to authentic standards (Steraloids Inc, Newport, RI or Sigma Aldrich, St Louis, MO). Final abundances and normalization for 10 dominant human bile acids (Supplementary Table 3) was performed using the ProFinder and Mass Profiler Professional software (Agilent Technologies, Santa Clara, CA).

Imputed Metagenomic Analysis

To impute the metagenome, we used PICRUSt, a freely available predictive strategy that uses 16S sequences as input data.[16] In brief, QIIME[17] (v1.8.0) was used to perform closed-reference OTU picking at 97% similarity of the preprocessed 16S reads within our dataset against the Greengenes[5] reference OTU database (May 2013, 99% OTU clustering). The resulting OTUs were used to predict functional composition with PICRUSt and the predicted functions were collapsed into KEGG pathways. Differential pathway abundance analysis was performed using phyloseq[18] combined with DESeq2.[19]

Statistical Analysis

Within-individual differences in Shannon indices were tested using paired *t* tests (normally distributed data). Changes in relative abundance of specific taxa were tested using paired *t* tests for normally distributed data or Wilcoxon signed rank tests. Data was analyzed using STATA 12 (StataCorp, College Station, TX). To determine our population size, we referred to studies that assessed the effects of antibiotics on diversity and powered our study to detect a difference of half that magnitude.[20,21] Significance tests for prespecified taxa of interest were not adjusted for multiple hypothesis testing (Supplementary Table 2); for other tests, the Benjamini-Hochberg method was used to adjust for multiple hypothesis testing. All significance tests were performed 2-sided and at the $\alpha = .05$ level.

Primer Sequences and Polymerase Chain Reaction Protocol

Primer	Sequence
515_f1	GAGTTCAGACGTGTGCTCTTCCGATCT GTGCCAGCMGCCGCGGTAA
806_r1_N3	CCTACACGACGCTCTTCCGATCT NNN GGACTACHVGGGTWTCTAAT
806_r1_N4	CCTACACGACGCTCTTCCGATCT NNNN GGACTACHVGGGTWTCTAAT
806_r1_N5	CCTACACGACGCTCTTCCGATCT NNNNN GGACTACHVGGGTWTCTAAT
806_r1_N6	CCTACACGACGCTCTTCCGATCT NNNNNN GGACTACHVGGGTWTCTAAT
P5_r2	AATGATACGGCGACCACCGAGATCT ACAC TCTTTC CCTACACGACGCTCTTCCGATCT
P7_bc01	CAAGCAGAAGACGGCATACGAGAT CAGGTT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc02	CAAGCAGAAGACGGCATACGAGAT TCACAA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc03	CAAGCAGAAGACGGCATACGAGAT ACATCA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc04	CAAGCAGAAGACGGCATACGAGAT AGCGCA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc05	CAAGCAGAAGACGGCATACGAGAT CATCAA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc06	CAAGCAGAAGACGGCATACGAGAT GCTATT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc07	CAAGCAGAAGACGGCATACGAGAT TAGATC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc08	CAAGCAGAAGACGGCATACGAGAT CATGAC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc09	CAAGCAGAAGACGGCATACGAGAT GAATCG GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc10	CAAGCAGAAGACGGCATACGAGAT TCTTCT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc11	CAAGCAGAAGACGGCATACGAGAT ATTCCG GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc12	CAAGCAGAAGACGGCATACGAGAT GGAATT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc13	CAAGCAGAAGACGGCATACGAGAT ACGGTG GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc14	CAAGCAGAAGACGGCATACGAGAT CTCAGC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc15	CAAGCAGAAGACGGCATACGAGAT TCCGGT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc16	CAAGCAGAAGACGGCATACGAGAT TGCAGT GTGACTG GAGTTCAGACGTGTGCTCTTC

Continued

Primer	Sequence
P7_bc17	CAAGCAGAAGACGGCATACGAGAT TTCATA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc18	CAAGCAGAAGACGGCATACGAGAT ATACAC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc19	CAAGCAGAAGACGGCATACGAGAT CGTTAT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc20	CAAGCAGAAGACGGCATACGAGAT CTCGGA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc21	CAAGCAGAAGACGGCATACGAGAT TGTGTG GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc22	CAAGCAGAAGACGGCATACGAGAT ACCGCG GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc23	CAAGCAGAAGACGGCATACGAGAT GATCGG GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc24	CAAGCAGAAGACGGCATACGAGAT TCACGG GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc25	CAAGCAGAAGACGGCATACGAGAT ATTACT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc26	CAAGCAGAAGACGGCATACGAGAT CTTAGA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc27	CAAGCAGAAGACGGCATACGAGAT GCAGCT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc28	CAAGCAGAAGACGGCATACGAGAT TCCTCC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc29	CAAGCAGAAGACGGCATACGAGAT GAACTA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc30	CAAGCAGAAGACGGCATACGAGAT ACAACC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc31	CAAGCAGAAGACGGCATACGAGAT GGTAAC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc32	CAAGCAGAAGACGGCATACGAGAT GTGGTC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc33	CAAGCAGAAGACGGCATACGAGAT CCGCGT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc34	CAAGCAGAAGACGGCATACGAGAT CTGACA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc35	CAAGCAGAAGACGGCATACGAGAT CCGAAT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc36	CAAGCAGAAGACGGCATACGAGAT AGCCGC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc37	CAAGCAGAAGACGGCATACGAGAT TAGCGC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc38	CAAGCAGAAGACGGCATACGAGAT TGACCT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc39	CAAGCAGAAGACGGCATACGAGAT CTTATC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc40	CAAGCAGAAGACGGCATACGAGAT GTAGCC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc41	CAAGCAGAAGACGGCATACGAGAT CCATAG GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc42	CAAGCAGAAGACGGCATACGAGAT GAGGCA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc43	CAAGCAGAAGACGGCATACGAGAT AATTGA GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc44	CAAGCAGAAGACGGCATACGAGAT ACTCAC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc45	CAAGCAGAAGACGGCATACGAGAT AAGTTG GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc46	CAAGCAGAAGACGGCATACGAGAT TACGAT GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc47	CAAGCAGAAGACGGCATACGAGAT CACCAC GTGACTG GAGTTCAGACGTGTGCTCTTC
P7_bc48	CAAGCAGAAGACGGCATACGAGAT GCATTC GTGACTG GAGTTCAGACGTGTGCTCTTC

Polymerase Chain Reaction Protocol

Low-cycle PCR was performed using KAPA SYBR FAST qPCR (Kapa Biosystems, Wilmington, MA) per the manufacturer's instruction. Nested PCR cycles were performed using the following protocol on a Bio-Rad CFX96 Touch real-time PCR instrument (Bio-Rad, Hercules, CA).

Polymerase Chain Reaction 1

Starting template: 20 ng DNA.
Primer sets: 515_f1 and 806_r1_N3-6.
Step 1 at 95°C for 3 minutes;
Step 2 at 95°C for 10 seconds;
Step 3 at 60°C for 30 seconds;
Repeat steps 2 × 3−30 cycles;
Step 5 at 68°C for 5 minutes;
Step 6 at 4°C on hold.

Polymerase Chain Reaction 2

Starting template: 1 μL of PCR product.
Primer sets: P7_bc01-48 and P5_r2 with P5/P7 adaptors.
Step 1 at 95°C for 3 minutes;
Step 2 at 95°C for 10 seconds;
Step 3 at 60°C for 30 seconds;
Repeat steps 2 through 3 × 10 cycles;
Step 5 at 68°C for 5 minutes;
Step 6 at 4°C on hold.

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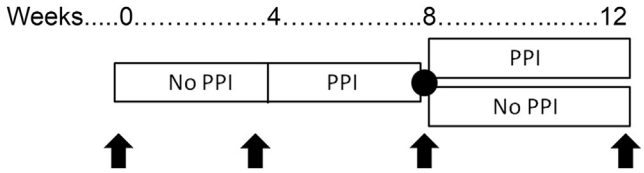
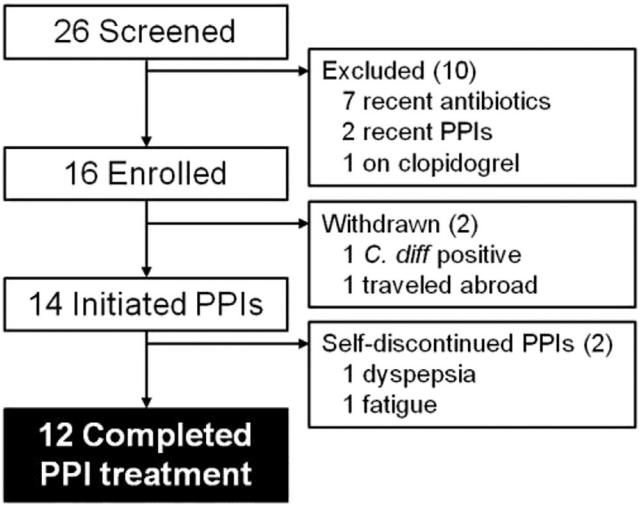
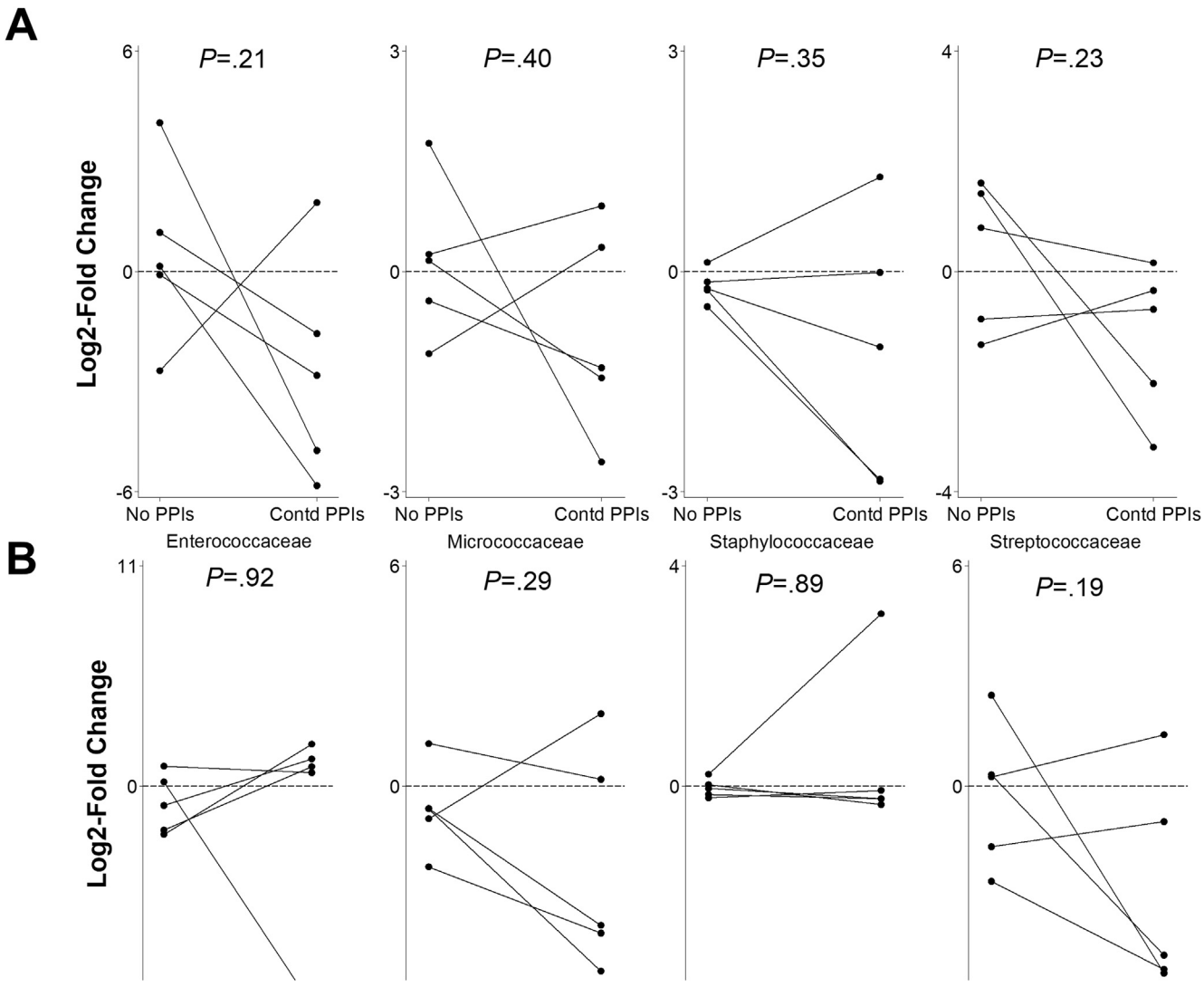
Author names in bold designate shared co-first authorship.



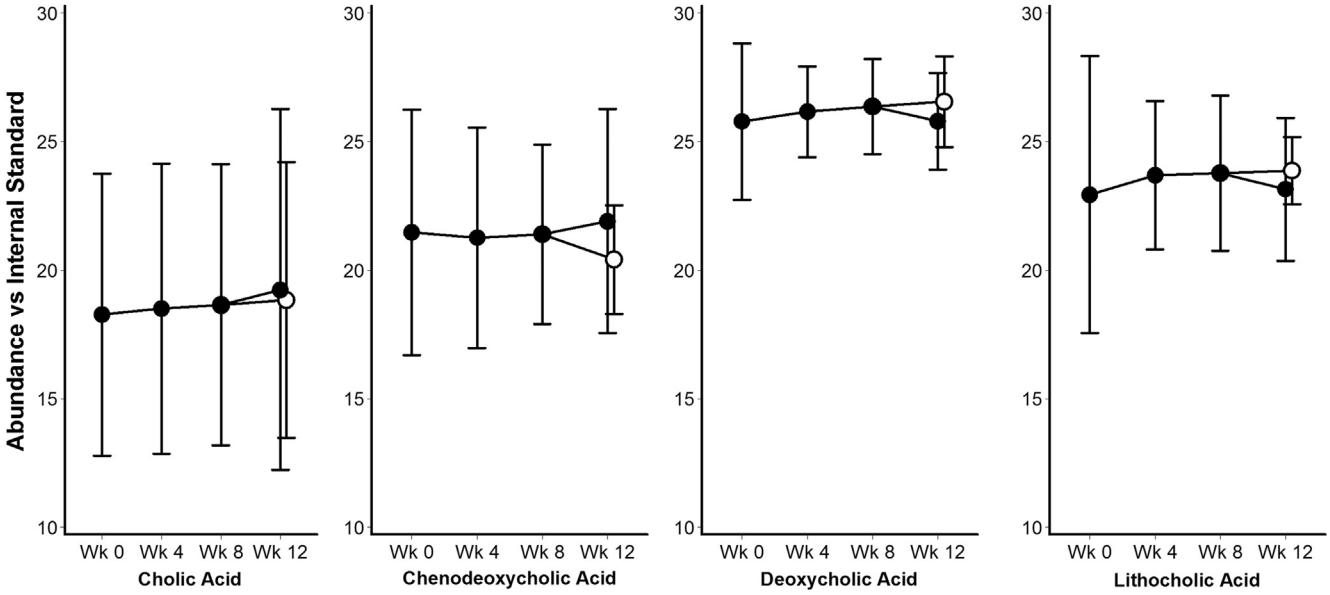
Supplementary Figure 1. Design of the study. Time in weeks is given at top. *Black circle,* randomization. *Black arrowheads*, study visits.



Supplementary Figure 2. Flow of patients into the study.



Supplementary Figure 3. Week 12 data. Log2-fold change in relative abundance, comparing change during the baseline period to change during the period corresponding to weeks 8 to 12 of the study. This is shown for subjects who continued PPIs during weeks 8 to 12 (*A*) and for subjects who completed 4 weeks of PPIs and then were off PPIs during Weeks 8 to 12 (*B*).

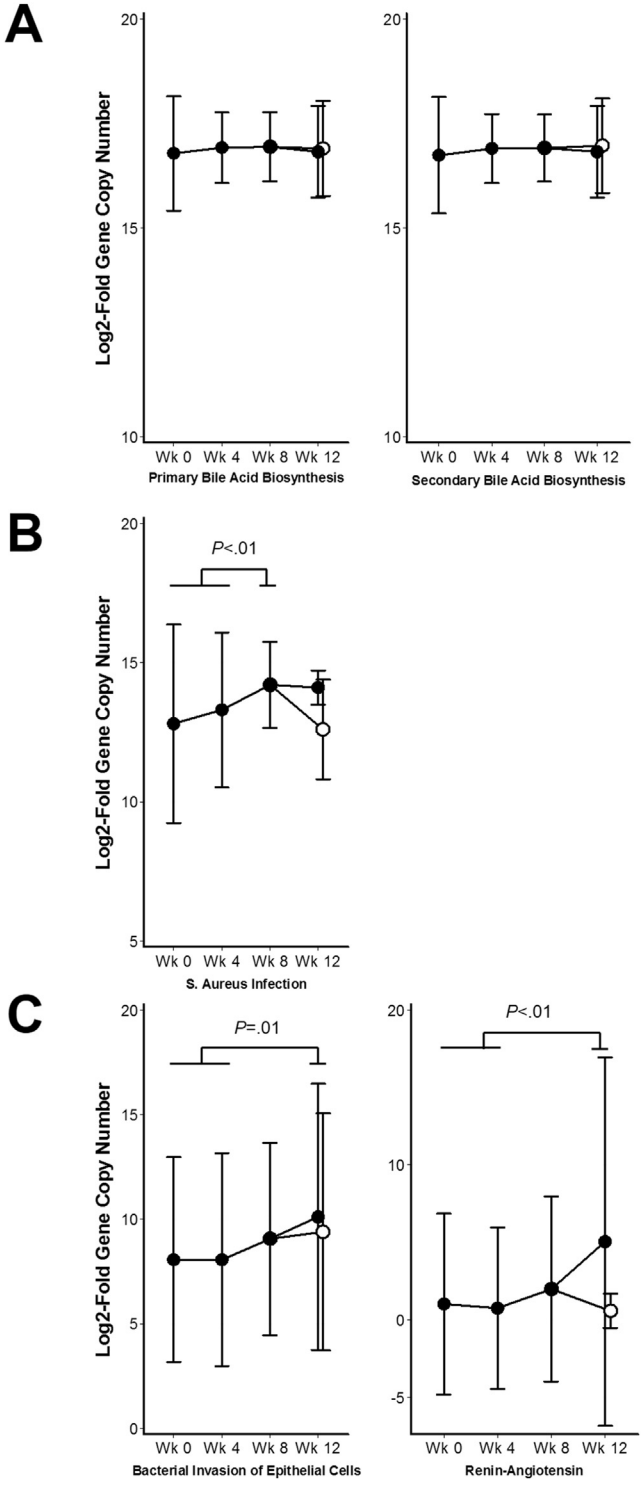


Supplementary Figure 4. Bile acid levels. Combined data for the 2 main primary bile acids (cholic acid and chenodeoxycholic acid) and for the 2 main secondary bile acids (deoxycholic acid and lithocholic acid). Comparing the baseline period (weeks 0 to 4) to the period on PPIs (weeks 4 to 8), there were no differences in levels of these bile acids or any of the other 6 dominant human primary and secondary bile acids. *Vertical lines* are 95% confidence intervals; subjects randomized to discontinue PPIs after the week 8 visit are shown as *open circles*.

Supplementary Table 1. Baseline Characteristics of Subjects

Variable	Median (IQR)
Age, *y*	39.5 (29.0–51.5)
Sex	
Male	3
Female	9
Anthropomorphics	
BMI	28.4 (21.3–34.5)
Waist: hip ratio	0.89 (0.83–0.97)
Diet	
Calories as fat, %	34.7 (32.1–38.2)
Daily grams fiber	14.5 (12.7–18.9)

BMI, body mass index; IQR, interquartile range.



Supplementary Figure 5. Imputed metagenomic changes. PICRUSt was used to perform an unbiased estimatation of within-individual metagenomic changes. There were no changes in the genes for the pathways corresponding to primary bile acid biosynthesis or to secondary bile acid biosynthesis (*A*). After adjusting for multiple hypothesis testing using the Benjamini-Hochberg method, there was a significant increase in genes within the KEGG pathways for *Staphylococcus aureus* infection after 4 weeks of PPIs (*B*) and for bacterial invasion of epithelial cells and renin-angiotensin after 8 weeks of PPIs (*C*). For all pathways, *vertical lines* show 95% confidence intervals. Subjects randomized to discontinue PPIs after the week 8 visit are shown as *open circles*.

Supplementary Table 2. Taxa of Interest

Taxon	Taxonomic level	Primary reference, first author
Associated with *Clostridium difficile* infection		
Bacteroidetes	Phylum	Vincent[9]
Enterococcaceae	Family	Vincent[9]
Tissiarellaceae	Family	Vincent[9]
Streptococcaceae	Family	Rosen[12]
Blautia	Genus	Stein[10]
Coprobacillus	Genus	Stein[10]
Akkermansia	Species	Stein[10]
Associated with bacterial overgrowth		
Enterobacteriaceae	Family	Pyleris[11]
Micrococcaceae	Family	Rosen[12]
Staphylococcaceae	Family	Rosen[12]
Streptococcaceae	Family	Rosen[12]
Veillonellaceae	Family	Rosen[12]
Serratia	Genus	Pyleris[11]

Supplementary Table 3. Bile Acids Tested

Name	Molecular formula	Molecular weight, *g/mol*	Retention time, *min*
Primary bile acids			
Cholic acid	$C_{24}H_{40}O_5$	408.57	15.6
Chenodeoxycholic acid	$C_{24}H_{40}O_4$	392.57	23.5
Glycocholic acid	$C_{26}NH_{43}O_6$	465.63	15.0
Glycochenodeoxycholic acid	$C_{26}NH_{43}O_5$	449.62	19.4
Taurocholic acid	$C_{26}H_{45}NO_7S$	515.70	12.4
Taurochenodeoxycholic acid	$C_{26}H_{45}NO_6S$	499.70	16.5
Secondary bile acids			
Lithocholic acid	$C_{24}H_{40}O_3$	376.57	28.7
Deoxycholic acid	$C_{24}H_{40}O_4$	392.57	24.0
Ursodeoxycholic acid	$C_{24}H_{40}O_4$	392.57	19.6
Hyodeoxycholic acid	$C_{24}H_{40}O_4$	392.57	20.0