Proton pump inhibitor administration does not promote *Clostridium difficile* colonization in a murine model

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Abstract

Proton pump inhibitor (PPI) use has been associated with microbiota alterations and susceptibility to *Clostridium difficile* infections (CDIs) in humans. We assessed how PPI treatment alters the fecal microbiota and whether PPIs promote CDIs in a mouse model. Mice receiving the PPI treatment were gavaged with 40 mg/kg of omeprazole during a 7-day pretreatment phase, the day of *C. difficile* challenge, and the following 9 days. We found that mice treated with omeprazole were not colonized by *C. difficile*. When omeprazole treatment was combined with a single clindamycin treatment, one cage of mice remained resistant to *C. difficile* colonization, while the other cage was colonized. Treatming mice with clindamycin and no omeprazole followed by challenge resulted in *C. difficile* colonization. 16S rRNA gene sequencing analysis revealed that omeprazole had minimal impact on the structure of the murine microbiota throughout the 16 days of PPI exposure. These results suggest PPI treatment alone is not sufficient to disrupt microbiota resistance to *C. difficle* infection in mice that are normally resistant in the absence of antibiotic treatment.

Importance

Antibiotics are a major risk factor for *Clostridium difficile* infections (CDIs), but other factors may also contribute. In epidemiological studies, PPI use has been associated with CDI incidence and recurrence. Proton pump inhibitors (PPIs) have also been associated with alterations in the human intestinal microbiota in observational and interventional studies. We evaluated the effects of the PPI omeprazole on the structure of the murine intestinal microbiota and its ability to disrupt colonization resistance to *C. difficile*. We found the PPI treatment had minimal impact on the murine fecal microbiota and did not promote *C. difficile* colonization. Further studies are needed to determine whether other factors such as the composition of the starting bacterial community, comorbidities, and use of additional medications contribute to the association between PPIs and CDIs seen in humans or whether aspects of murine physiology may limit its utility to test these types of hypotheses.

Antibiotics have a large impact on the intestinal microbiome and are a primary risk factor for developing *Clostridium difficile* infections (CDIs) (1). It is less clear whether other human medications that impact the microbiota also influence *C. difficile* colonization resistance. Multiple epidemiological studies have suggested an association between proton pump inhibitor (PPI) use and incidence or recurrence of CDIs (2–5). There have also been a number of large cohort studies as well as interventional clinical trials that have demonstrated that specific alterations in the intestinal microbiome were associated with PPI use (4, 6). PPI-associated microbiota changes have been attributed to the ability of PPIs to increase stomach acid pH which may promote the survival of oral and pathogenic bacteria (4, 6). Human fecal microbiota changes with PPI use include increases in Enterococcaceae, Lactobacillaceae, Micrococcaceae, Staphylococcaceae and Streptococcaceae and decreases in Ruminococcaceae (6, 7). Several of these taxa have been associated with C. difficile colonization in humans (8)

Unfortunately, most of the studies suggesting a link between PPIs and *C. difficile* were retrospective and did not evaluate the microbiome (2, 3, 5). Thus, it is unclear whether the gastrointestinal microbiome changes associated with PPI use explain the association between PPIs and CDIs. Additionally, epidemiological studies have a limited capacity to address potential confounders and comorbidities in patients that were on PPIs and developed CDIs or recurrent CDIs (2, 5). Here, we evaluated the impact of a daily high dose PPI treatment on the murine microbiome and susceptibility to *C. difficile* colonization in relation to clindamycin, an antibiotic that perturbs the microbiome enough to allow *C. difficile* to colonize but is mild enough that *C. difficile* is cleared within 10 days (9).

Murine fecal microbiomes were minimally affected by PPI treatment To test whether PPI treatment alters the microbiome and promotes susceptibility to CDIs, we gavaged mice with 40 mg/kg of omeprazole for 7 days before *C. difficile* challenge (Figure 1A). A principle coordinates analysis (PCoA) of the Bray-Curtis distances over the initial 7 days of treatment revealed the bacterial communities of PPI-treated mice remained relatively unchanged relative to animals that did not recieve the PPI (Figure 1B). We observed no significant fluctuations in the relative abundance of those taxa previously shown to respond to PPI treatment throughout the course of the 16-day experiment (Figure 1C, Figure S1A-B). We also observed no significant fluctuations in relative

abundances at the family and genus level over the beginning, day of *C. difficile* challenge, and end of the experiment for the PPI-treated mice (Figure 1D, Figure S1C). Next, the fecal bacterial communities of PPI-treated mice were compared to a group of mice that received a single 10 mg/kg dose of clindamycin and a group that received both PPI and clindamycin. In contrast to the dynamics of mice only receiving omeprazole, the microbiomes from the 2 groups of mice that received clindamycin started to shift away from the PPI-treated mice and the samples from previous days one day after treatment (Figure 1D). These results demonstrated that the PPI treatment alone had a minimal impact on the murine fecal bacterial community relative to the impact of clindamycin treatment.

PPI-treatment did not promote susceptibility to *C. difficile infection* in mice Next, we examined whether PPI treatment altered susceptibility to *C. difficile* infection in mice. After PPI treatment or clindamycin treatment, mice were challenged with *C. difficile* 630 spores. While *C. difficile* colonized the clindamycin treated mice, it was unable to colonize the PPI-treated mice (Figure 2A). Interestingly, only 1 cage of mice that received both the PPI and clindamycin were colonized, while the mice in other cage wwere resistant (Figure 2A). The greatest shifts in bacterial communities occurred in the clindamycin-treated mice (Figure 2B, Figure S2). Regardless of whether the mice became colonized, all of the mice had cleared *C. difficile* within 5 days (Figure 2A), suggesting that omeprazole did not affect the rate of clearance. Our results suggest that PPI treatment had no effects on bacterial community resistance to *C. difficile* colonization in mice. Instead most of the differences between our 3 treatment groups appear to be driven by clindamycin administration (Figure 2C-D) and included previously described decreases in *Alistipes*, *Barnesiella*, Porhyromonadaceae, Ruminococcaceae (1). These findings demonstrated that high dose PPI treatment did not promote susceptibility to *C. difficile* colonization.

Conclusions

We found the PPI omeprazole did not impact the gut microbiota and did not promote *C. difficile* infection in mice. Our findings that PPI treatment had minimal impact on the fecal microbiome were comparable to another PPI mouse study that indicated PPIs had more of an effect on the small intestinal microbiota compared to the fecal microbiota (10). The same group demonstrated PPI

treatment increased the stomach pH in mice (10), which may improve survival of bacteria passing through the stomach. We did not find significant changes for the taxa observed to be significantly impacted by PPI use in human studies. However, 3 of the human-associated taxa were absent or at low abundance in our mice. Additionally, some of the significance of PPI associations in human interventional trials appears to be driven by a handful of specific taxa with overall differences on a PCoA plot difficult to distinguish (11). The PPI-treated mice fecal communities did not change overall and no differences in the relative abundances of individual families or genera were observed over time. While our fecal microbiota findings are comparable to what's been shown in another mouse study, whether PPI-induced changes in specific bacterial abundances observed in humans play a role in CDIs remains to be determined.

Although there have been a few *C. difficile* mouse model studies that have demonstrated PPIs have some effect on CDIs with or without additional antibiotic treatment (13–15), there were some key methodological differences between these studies and our own. One group administered 0.5 mg/kg of the PPI lansoprazole daily for 2 weeks to mice and then challenged with *C. difficile* demonstrated that PPI treatment alone resulted in detectable *C. difficile* in the stool 1 week after challenge, but also showed there was detectable *C. difficile* in mice not treated with antibiotics (13, 14). The presence of *C. difficile* in mice not treated with antibiotics may be partly attributed to the higher dose of vegetative cells (10^8 CFU) used, but nevertheless makes it difficult to interpret the extent to which PPI treatment contributed to *C. difficile* colonization in their mouse model (13, 14). In contrast, we have previously shown mice from our colony that were not given antibiotics were resistant to *C. difficile* 630 when challenged with 10^3 spores (16) and previous work by our collaborators demonstrated mice not given antibiotics were resistant to 10^5 vegetative cells of *C. difficile* VPI 10463 (17). The other PPI CDI mouse study also used a higher dose of 10^6 spores or 10^7 vegetative cells to challenge antibiotic-treated mice or mice treated with both antibiotics and the PPI esomeprazole (40mg/kg dose) for 2 days and demonstrated the antibiotic/PPI-treated mice developed more severe CDIs (15). We tested the same high 40 mg/kg PPI dose and expanded pre-treatment to 7 days before challenge to test the impact of PPI treatment alone on our CDI mouse model.

Our study extended the previous work examining PPIs and *C. difficile* in mice by evaluating the contribution of the intestinal microbiota. We found the PPI omeprazole had no significant impact on

bacterial taxa within the murine intestinal microbiota over the 16-day experiment and in contrast to previous work, PPIs did not alter *C. difficile* colonization resistance. 16S rRNA sequencing suggested that *Streptococcus* and *Enterococcus* are rare genera in our C57BL/6 mouse colony and variation in these 2 genera have been observed across other facilities and vendors (18, 19). These genera could be important contributors to the associations between PPIs and CDIs in humans, and could be a contributing factor to our observation that PPI treatment had no effect on *C. difficile* colonization in our CDI mouse model.

Beyond microbiome differences, factors such as age, body mass index, comorbidities, and use of other medications in human studies may also be contributing to the association between PPIs and CDI incidence or recurrence. This study addressed the impact of PPIs with or without antibiotics on a murine model of CDI, and found PPIs did not promote *C. difficile* colonization. The epidemiological evidence linking PPIs to CDIs is primarily from observational studies, which makes determining causality and whether other risk factors play a role challenging (20). Future studies are needed to determine whether age, other comorbidities and bacterial strains that are less common in mice can increase the risk of CDIs or recurrent CDIs when combined with PPI treatment.

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Materials and Methods

Animals All mouse experiments were performed with 7- to 12-week-old C57BL/6 male and female mice. Each experimental group of mice was spit between 2 cages with 2-3 mice housed per cage and male and female mice housed separately. All animal experiments were approved by the University of Michigan Animal Care and Use Committee (IACUC) under protocol number PRO00006983.

Drug treatments Omeprazole (Sigma Aldrich) was prepared in a vehicle solution of 40% polyethylene glycol 400 (Sigma-Aldrich) in phosphate buffered saline. Omeprazole was prepared from 20 mg/mL frozen aliquots and diluted to an 8mg/mL prior to gavage. All mice received 40 mg/kg omeprazole or vehicle solution once per day through the duration of the experiment with treatment starting 7 days before *C. difficile* challenge (Figure 1A). One day prior to *C. difficile* challenge, 2 groups of mice received an intraperitoneal injection of 10 mg/kg clindamycin or sterile saline vehicle. All drugs were filter sterilized through a 0.22 micron syringe filter before administration to animals.

***C. difficile* infection model** Mice were challenged with *C. difficile* 630 seven days after the start of omeprazole treatment and one day after clindamycin treatment. Mice were challenged with 10^3 spores in ultrapure distilled water. Stool samples were collected for 16S rRNA sequencing or *C. difficile* CFU quantification throughout the duration of the experiments at the indicated timepoints (Figure 1A). Samples for 16S rRNA sequencing were flash frozen in liquid nitrogen and stored at -80℃ until DNA extraction, while samples for CFU quantification were transferred into an anaerobic chamber and serially diluted in PBS. Diluted samples were plated on TCCFA (taurocholate, cycloserine, cefoxitin, fructose agar) plates and incubated at 37℃ for 24 hours under anaerobic conditions to quantify *C. difficile* CFU.

16S rRNA gene sequencing DNA for 16S rRNA gene sequencing was extracted from 10-50 mg fecal pellet from each mouse using the DNeasy Powersoil HTP 96 Kit (Qiagen) and an EpMotion 5075 automated pipetting system (Eppendorf). The ZymoBIOMICS^{TM} Microbial Community DNA Standard (Zymo, CA, USA) was used as a mock community (21) and water was used as a negative control. The V4 hypervariable region of the 16S rRNA gene was amplified with Accuprime Pfx

DNA polymerase (Thermo Fisher Scientific) using previously described custom barcoded primers (22). The 16S rRNA amplicon library was sequenced with the MiSeq (Illumina). Amplicons were cleaned up and normalized with the SequalPrep Normalization Plate Kit (ThermoFisher Scientific) and pooled amplicons were quantified with the KAPA library quantification kit (KAPA Biosystems).

16S rRNA gene sequence analysis Mothur (v1.40.5) was used for all sequence processing steps (23) using the previously published protocol (22). In brief, forward and reverse reads for each sample were combined and low-quality sequences and chimeras were removed. Duplicate sequences were merged, before taxonomy assignment using a modified version (v16) of the Ribosomal Database Project reference database (v11.5) with an 80% cutoff. Operational taxonomic units (OTUs) were assigned with the opticlust clustering algorithm using a 97% similarity threshold. To adjust for uneven sequencing across samples, all samples were rarefied to 3000 sequences, 1,000 times. PCoAs were generated based on Bray-Curtis distance. R (v.3.5.1) was used to generate figures and perform statistical analysis.

Statistical Analysis To test for differences in relative abundances in families and genera across our 3 different treatment groups (Clindamycin, Clindamycin + PPI, and PPI) or within the PPI treatment group across 3 timepoints (Day -7, 0, and 9), we used a Kruskal-Wallis test with a Benjamini-Hochberg correction for multiple comparisons.

Code availability The code for all sequence processing and analysis step as well as an Rmarkdown version of this manuscript is available at https://github.com/SchlossLab/Tomkovich_PPI_XXXX_2019.

Data availability The 16S rRNA sequencing data have been deposited in the NCBI Sequence Read Archive (Accession no. PRJNA554866).

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Figures

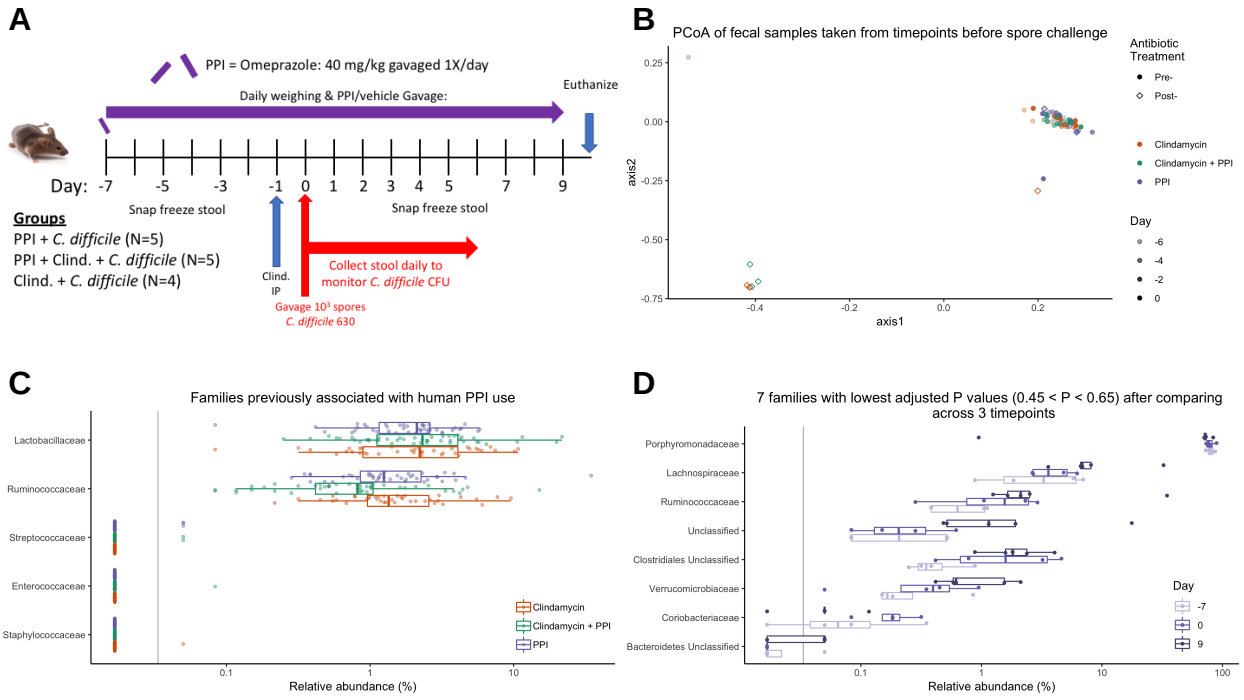


Figure 1. PPI treatment had minimal impact on the murine fecal microbiota. A. Mouse experiment timeline and logistics. The PPI omeprazole was administered throughout the duration of the experiment. Clindamycin was administered 1 day before *C. difficile* challenge on Day 0. Stools for 16S rRNA sequencing analysis were collected on the days that are labeled (Day -7, -5, -3, -1, 0, 1, 2, 3, 4, 5, 7, 9). *C. difficile* CFU in the stool was quantified daily through 6 days post-infection by anaerobic culture. B. Principal Coordinates Analysis (PCoA) of Bray-curtis distances from stool samples for all treatment groups during the intial 7 days of the experiment. Each symbol represents a stool sample from an individual mouse on a given day. Transparency of the symbol corresponds to treatment day. Circles represent samples before antibiotic treatment (administered on Day -1), while open diamonds represent samples from the day after antibiotic treatment (Day 0). Mice treated with the PPI omeprazole or vehicle cluster together and do not change much over time while mice that receive clindamycin have samples that start to separate from the rest of the samples the day after antibiotic treatment. C. Relative abundances of families previously associated with PPI use in humans do not change much over the 16-day course of treatment with PPIs (7 days before *C. difficile* challenge through 9 days post *C. difficile* challenge). D. Boxplots showing the relative abundances of 7 familes within PPI-treated mice that had the lowest adjusted P values

after comparing across 3 timepoints. There were no significant differences acoss time for any of the identified families (All $P > 0.05$) after analysis by Kruskal-Wallis test with a Benjamini-Hochberg correction for multiple comparisons. See Fig. S1C for genera with lowest adjusted P values (All $P > 0.05$). For C-D, the grey line indicates the limit of detection.

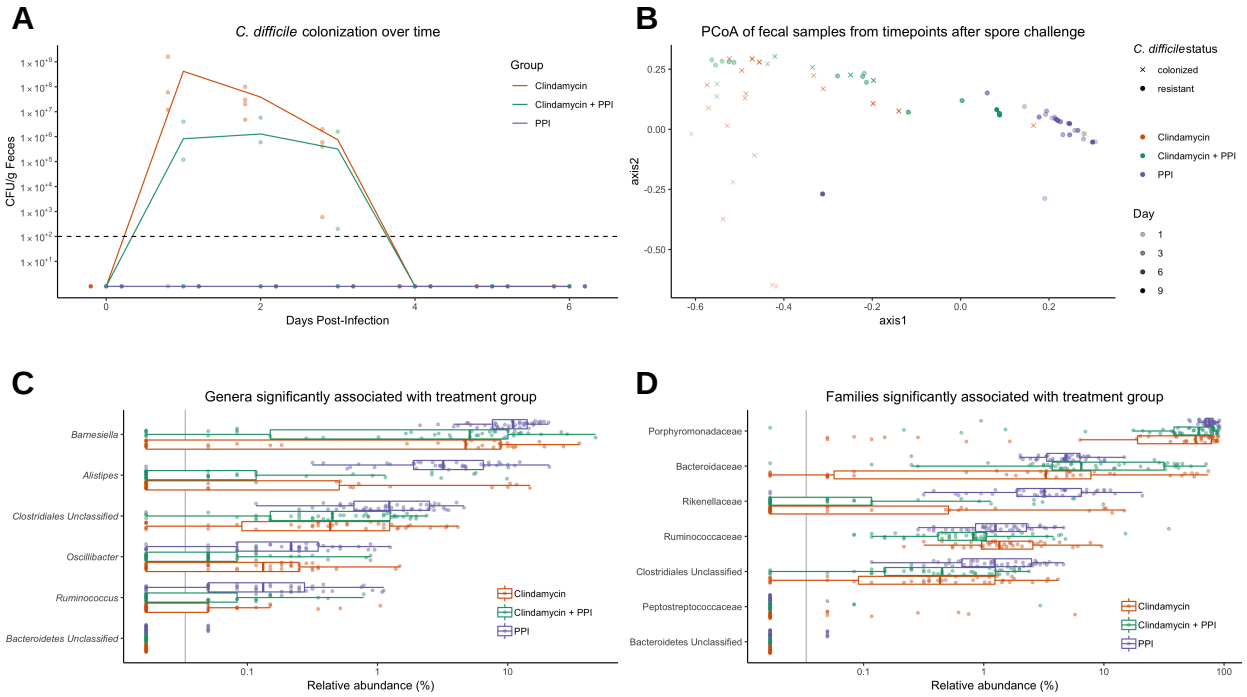


Figure 2. PPI treatment alone does not promote CDIs in mice. A. *C. difficile* CFUs/g stool measured each day post *C. difficile* challenge for clindamycin, clindamycin/PPI, and PPI-treated mice. Lines represent the mean CFU/g for each treatment group while points represent CFU/g for individual mice within each group. The black dashed line indicates the limit of detection. B. PCoA of of Bray-curtis distances from stool samples collected after antibiotic treatment (last 9 days of the experiment). Transparency of the symbol corresponds to treatment day. Symbols represent the *C. difficile* colonization status of the mice measured 2 days post-infection. Circles represent resistant mice (*C. difficile* was undetectable in stool samples), while X-shapes represent mice that were colonized with *C. difficile*, although all mice cleared *C. difficile* within 5 days of infection. PPI-treated fecal samples primarily cluster together throughout the experiment. C. Genera significantly associated with treatment groups of stool samples across all sequenced timepoints. D. Families significantly associated with treatment groups of stool samples across all sequenced timepoints. C,D data were analyzed by Kruskal-Wallis test with a Benjamini-Hochberg correction for multiple comparisons. For C-D, the grey line indicates the limit of detection.

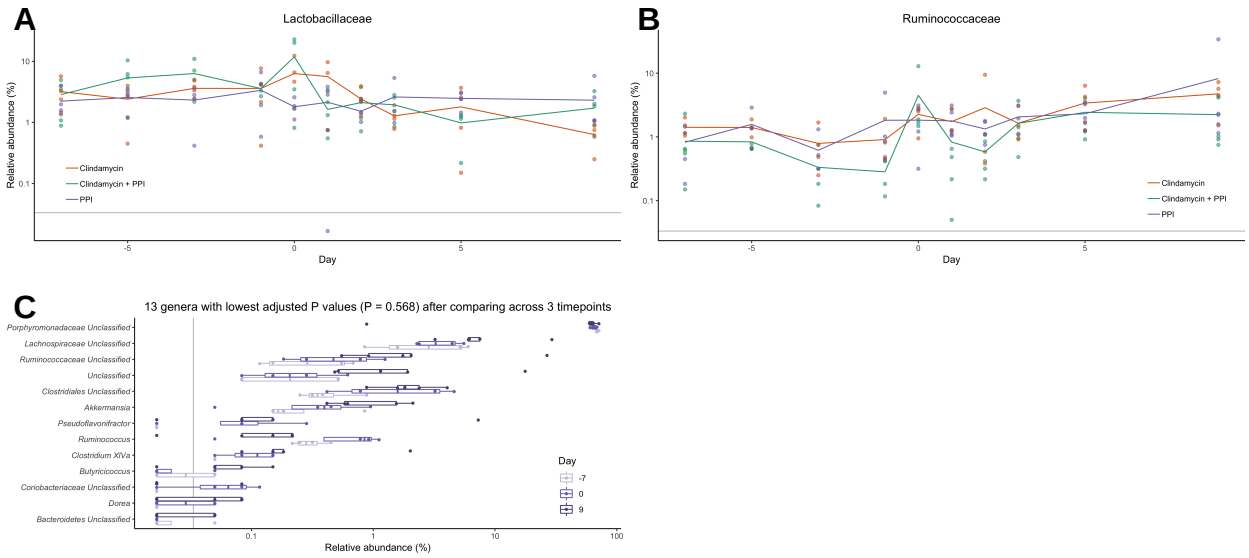
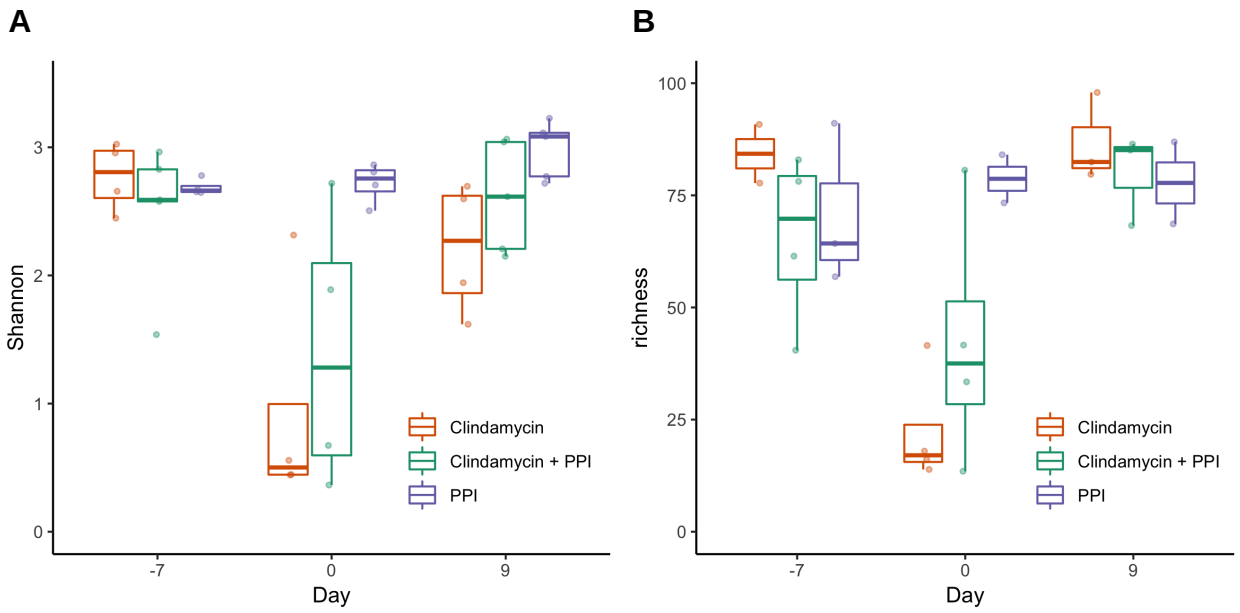


Figure S1. Taxa within PPI treated mice fluctuate over time with no overall trend in either direction. Relative abundance over time for Lactobacillaceae (A) and Ruminococcaceae (B), 2 of the PPI-associated familes from human PPI studies across all 3 treatment groups. Each point represents the relative abundance for an individual mouse, while the lines represent the mean relative abundances for each treatment group of mice. C. Boxplots showing the relative abundances of 13 genera within PPI-treated mice that had the lowest adjusted P values after comparing across 3 timepoints. There were no significant differences acoss time for any of the identified families (All P = 0.568) after analysis by Kruskal-Wallis test with a Benjamini-Hochberg correction for multiple comparisons. For A-C, the grey lines indicate the limit of detection.



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307 **Figure S2. Microbiota diversity and richness decrease with antibiotic treatment but remain**
308 **relatively constant with PPI treatment.** A. Boxplots of the Shannon Index (A) and richness (B)
309 values for each group of mice over 3 timepoints (Day -7, 0, and 9). Each circle represents the value
310 for an individual mouse.