Editor Comments:

1. Both reviewers commented about the appropriateness of strain that was used in your experiments. Please provide a justification for why this strain was chosen.

> We have responded to the reviewers' comments below regarding why we chose to test *C. difficile* 630 and have added the following sentence to the discussion to acknowledge the strain choice as a potential limitation: "The type of *C. difficile* strain type could also be an important contributing factor, our study was limited in that we only tested *C. difficile* 630 (ribotype 012)."

2. Both reviewers commented on the low number of mice used in the experiments, perhaps making it hard to pick up any small differences. Please add some additional discussion around this point.

> We have responded to the reviewers' comments below and added the following sentence to the discussion regarding the low number of mice used: "One limitation of our study is that there were only 4-5 mice per group, which may have limited our ability to identify PPI-induced changes in specific bacteria genera."

3. Lastly, from my own reading of the paper, I recall that rodent gut microflora consist mainly of Gram-positive organisms, whereas human gut flora is mainly Gram-negatives and anaerobes. One would presume that any treatment that disrupts the microbiome would interact very differently with these two environments, and that the rodent microbiome may not accurately reflect what happens in the human gut. This caveat is hinted at in lines 106-107 of your manuscript, but I would like you to make this point more clearly.

> We have added an additional sentence to precede the sentence from lines 106-107 to draw attention to the differences in intestinal microbiome composition between mice and humans: "*While the intestinal microbiomes of both humans and mice are dominated by the Bacteroidetes and Firmicutes phyla, there are significant differences in the relative abundances of genera that are present and some genera are unique to each mammal (Hugenholtz & de Vos Cell. Mol. Life Sci. 2018), differences that may partly explain our results.* The microbiota and physiological differences between humans and mice may limit the usefulness of employing mouse models to study the impact of PPIs on the microbiota and CDIs."

Reviewer #1 (Comments for the Author):

In their manuscript Tomkovich et al. address the question whether the correlative association of proton pump inhibitors with the occurence of CDI is due to the changes in the microbiome following PPI administration as it is speculated in the field. This correlation drawn mainly from epidemiological studies and typically retrospectively, lacks experimental evaluations. Hence, the authors used the mouse colonization model of C. difficile. They checked the changes in the microbiome and the colonization efficiencies in mice treated either with the PPI, omeprazole or clindamycin or with a commbination of both. They show that efficient colonization is dependent on clindamycin treatment prior to infction and that omeprazole alone has no significant effect on the diversity of the microbiome, and does not result in effective colonzation by C. difficile. As this is the first attempt to experimentally address a question arising from medical practice and observation, the study and its findings are interesting. Neverteheless, the study has some points that need to be addressed at least in the discussion before publication

1. Is it correct that the study was perfomed on max. 4 mice/treatment group? This might be too low a number, especially in case of the colonization studies to draw conclusions.

> The reviewer is correct that the number of mice per group was low. We state the exact number of mice per treatment group in Figure 1A (5 mice each for 2 of the treatment groups and 4 mice for the 3rd treatment group). We agree that this is a low number, but would argue that the low microbiome variation across laboratory mice means less mice are needed compared to

human epidemiological studies when examining *C. difficile* colonization. Previous *C. difficile* infection mouse model studies from our group and a close collaborator have used 3-5 mice per treatment group (see Koenigsknecht et al. Infection and Immunity 2015 and Theriot et al. Gut Microbes 2011) and were still able to draw meaningful conclusions. We have added the following sentence to the discussion to acknowledge the low number of mice used for these experiments as a limitation: "One limitation of our study is that there were only 4-5 mice per group, which may have limited our ability to identify PPI-induced changes in specific bacteria genera."

2. The authors should address the fact that they have used only C. difficile str. 630 for their experiments. This is a ribotype 012 strain that is not predominant in the clinical setting. Which ribotypes are typically observed in PPI associated CDI, is there a tendency towards a certain ribotype? Could this have an impact on the interpretation of the data derived from the model described in this manuscript?

We thank the reviewers for pointing out that the type of *C. difficile* strain could be an important factor influencing the association between PPIs and CDIs observed in human epidemiological studies. Unfortunately, the studies to date seldom examine the *C. difficile* strains found in PPI users that develop CDIs. The studies that do discuss *C. difficile* strains merely note the type of *C. difficile* frequently found in the region that the study was conducted (Roughead et al. Expert Opinion on Drug Safety 2016; McDonald et al. JAMA Internal Medicine 2015; Chitnis et al. JAMA Internal Medicine 2013; Vardakas et al. International Journal of Infections Diseases 2012; Dalton et al. Alimentary Pharmacology & Therapeutics 2008). Our lab prefers using *C. difficile* 630 for experiments because we are interested in colonization and clearance. *C. difficile* VPI 10463 administration to mice results in a more agressive disease that leads to mortality. Using *C. difficile* 630 after clindamycin administration results in an intermediate phenotype, where the mice are colonized but able to clear infection within 10 days. The intermediate phenotype with *C. difficile* 630 allows us to examine whether the addition of a drug exacerbates or prevents infection and track the mice for a longer period of time. In addition to our lab's prior experience with the *C. difficile* 630 infection mouse model, we also decided to use *C. difficile* 630 because of a recent study that demonstrated three proton pump inhibitors had no effect on *in vitro C. difficile* 630 growth (Maier et al. Nature 2018). We have added the following sentence to the discussion to acknowledge that the type of *C. difficile* strain is another potential contributing factor to the association between PPIs and CDIs seen in human epidemiological studies: "The type of *C. difficile* strain type could also be an important contributing factor, however our study was limited in that we only tested *C. difficile* 630 (ribotype 012)."

3. As the manuscript is pretty concise and has in total 4 figures, the supplementary figures can be integrated into the main body.

We have submitted this manuscript as an Observation, which is limited to 2 figures total. Additionally, in response to Reviewer #2, we have expanded the size of all the panels in our figures, limiting our ability to include additional panels for the 2 main text figures. Because of the changes to the sizes of the figures, we have opted to keep the supplementary figures as supplemental.

5. There are some studies that looked into the effect of PPIs on microbiomes in cats and infants, which seem to be in accordance with the findings in this manuscript. Hence, the authors should include these in their discussion: - Schmid SM, et al.,2018 - Castellani C, et al., 2017

Thank you for the suggestion, we have added the following sentence to the discussion referring to these studies to indicate that other groups have also observed minimal changes in the mammalian microbiome after proton pump inhibitor treatment: "Interestingly, other groups examining fecal microbiota communities before and after PPI administration to healthy cats and infants with gastroesophageal reflux disease, found PPIs have minimal effects on fecal bacterial community structures, although there were a few significant changes in specific genera (Schmid et al. Frontiers in Veterinary Science 2018; Castellani et al. Frontiers in Cellular and Infection Microbiology

2017)."

6. It is becoming more and more accepted that Clostridium difficile, due to its biology, is a separate genus, resulting in Clostridioides difficile as the accepted name. The authors should give credit to this and at least state it as Clostridium (Clostridioides) difficile in the text the first time they use it.

We appreciate the suggestion and have changed "*Clostridium*" to "*Clostridioides*" in the title, abstract, importance, and introduction sections of our manuscript.

Reviewer #2 (Comments for the Author):

The manuscript by Tomkovich et al tests if the PPI omeprazole can influence the fecal microbiome of mice and promote C. difficile infection. The study is based on epidemiological findings in humans that associate increased PPI use with CDI as well as a few mouse studies that implicate PPIs in CDI. The data presented here show no significant impact of omeprazole on the microbiome of mice after 7 days of treatment and the mice did not become susceptible to C. diff colonization. This is a small study but adds to the literature in that it conclusively shows no impact of omeprazole on the microbiome.

1. The size of the figures and the fonts are very small and should be improved in a resubmission. It makes it difficult to see individual points in the graphs.

We have adjusted the sizes of all figures and increased the sizing of the fonts and points in the graphs.

2. The choice of CD630 and dose may have a big impact on the results and comparison with previous studies. In ref. 15 the authors used a more virulent strain (VPi) and at a 10,000 fold increased concentration. The authors do a good job of citing other factors that may influence the results (like the increased pH of the mouse stomach) but this should also be noted for readers.

We thank the reviewer for pointing out *C. difficile* strain type as an additional factor that could explain the association between proton pump inhibitors and CDIs in human studies, which was also pointed out by Reviewer # 1. In response, we have added the following sentence to the discussion: "The type of *C. difficile* strain type could also be an important contributing factor, however our study was limited in that we only tested *C. difficile* 630 (ribotype 012)."

3. Please provide evidence for how the dose of 40mg/kg of OMZ corresponds to what humans get in a dose.

Omeprazole is prescribed to adults in 20 mg or 40 mg doses that are taken once per day. The 40 mg/kg dose administered to the mice in our study is higher than what is administered to humans translating to ~ 3.25 mg/kg (compared to ~ 0.33-0.66 mg/kg for the prescribed human dose) based on a reference body weight of 60 kg for humans and 0.02 kg for mice (converted according to the calculation described in Nair & Jacob J Basic Clin Pharm. 2016). However, previous studies have justified administering omeprazole at higher doses to mice because the drug administered to the mice lacks an enteric coating (Llorente et al. Nature Communications 2017) and is eliminated more quickly compared to humans (plasma elimination half life of 5-15 minutes compared to 1 hour for humans, see Regardh et al. Scandinavian Journal of Gastroenterology 1985). The 40 mg/kg dose chosen for our study is in line with what was used by one of the previous studies that examined the proton pump inhibitor esomeprazole and *C. difficile* in mice (see Hung et al. Journal of Infectious Diseases 2015). We have added the following sentence to the methods section to account for the higher dosing: "Although the omeprazole dose administered to mice is higher than the recommended dose for humans, omeprazole has a shorter half-life in mice compared to humans (Regardh et al. Scandinavian Journal of Gastroenterology 1985) and lacks an enteric coating (Llorente et al. Nature Communications 2017)."

4. Although OMZ did not have a major impact on community structure by itself, in figure 2 it seems that it did have an impact on a few groups that were treated with clindamycin. Although the P-values aren't significant, this could be mainly due to the low number of mice that were used in the study as a few of the genera showed 10-fold changes. I can see why it isn't commented on due to the lack of stat significance, but it is pretty striking as presented in the figure.

We agree with the reviewer regarding Figure 2C, where the relative abundances for specific taxa in the Clindamycin + omeprazole treated mice were typically in between the clindamycin-treated and omeprazole-treated mice. Beyond the P-values not being significant, we decided not to comment on this because we suspect that the majority of the differences in bacterial relative abundances across groups is driven by the clindamycin treatment. We have added the following sentence to the discussion to acknowledge the low number of mice used as a potential limitation in detecting omeprazole-induced changes in the fecal microbiota: "One limitation of our study is that there were only 4-5 mice per group, which may have limited our ability to identify PPI-induced changes in bacteria genera."

5. The final sentence of the importance section is a run-on sentence and needs to be broken up or simplified.

To simplify the sentence, we have removed the part that elaborates on what other factors ("composition of the starting bacterial community, comorbidities, and use of additional medications") might be contributing to the correlations between PPIs and CDIs seen in humans.

6. There are many typos and missed italics in the references.

Thank you for pointing these errors out, typos have been corrected and italics have been added to the references.