

## **Response to Reviewers**

*The line numbers we refer to below are taken from the “clean” version of the manuscript we have submitted (manuscript.docx).*

### **Reviewer #1:**

**The manuscript by Lesniak and colleagues focuses on the protective role of fecal community transplant (FCT) against *Clostridioides difficile* infection (CDI). For this purpose, antibiotic-treated recipient mice were gavaged with fecal community from untreated control groups prior to *C. difficile* challenge. They indeed found that FCT was able to either prevent *C. difficile* colonization or cleared it later on, depends on the antibiotic group they tested. They highlighted that the effectiveness of FCT depends on the community structure after antibiotic challenge, thus provide a good rationale to use specific, defined, missing microbial community rather than whole fecal community in the future. Together, this paper offers a contribution to the literature and has potential impact how colonization resistance can be restored against *C. difficile*. However there are some specific points needs to be addressed:**

### **Major remarks:**

**-How was equal exposure to the cefoperazone and streptomycin ensured? Did drinking water consumption documented?**

*We thank the reviewer for being this issue in our methods to our attention. Antibiotic consumption was not measured. We were repeating the *C. difficile* model we had used previously as well as adapted one of the more common CDI mouse models. None of which measure the amount of antibiotic consumed. Additionally, our focus is not the effect of the antibiotic, it is on the community that results for the antibiotic perturbation. We have added clarification to the Methods and Discussion section as well as citations to the previous models. “Antibiotics were chosen and administered based on previous studies (14, 31, 32).” “Mice were given either cefoperazone, clindamycin, or streptomycin. Cefoperazone (0.5 mg/ml) and streptomycin (5 mg/ml) were administered via drinking water ad libitum for 5 days, beginning 9 days prior to *C. difficile* challenge.” (Lines 320-325).” We designed our experiments to closely match previous mouse models for CDI and added days prior to *C. difficile* challenge for the FCT treatment (14, 31, 32).” (Lines 290-292)*

**-The choice for the doses and routes for antibiotic administration needs to be better justified. It would be better to provide references for the doses or authors should justify the**

**calculations for dosing with a rationale.**

*Our response above should resolve this remark as well.*

**-It would be better to provide a reference for FCT preparation as well. After the homogenization step, is there any centrifugation step to use the supernatants?**

*We thank the reviewers for this recommendation. We have added a reference for FCT preparation and clarified the centrifugation step. “All fecal community dilutions were centrifuged at 7500 RPM for 60 seconds and the supernatants were used for inoculation.” “This method was adapted from our previous study (18).” (Lines 336-337, 341-342)*

**-Mice were gavaged with FCT once a day for 2 days. How efficient colonization ensured?**

*Colonization efficiency by the FCT is an interesting question. However, since we are adding back a community that is similar to the community the mice began with, it would be difficult to parse the efficiency of FCT engraftment compared to natural recovery. Furthermore, since our primary focus was on inhibiting *C. difficile* colonization, we focused on the effect of the FCT on the community and CDI.*

**-Why authors did not consider using an engineered *C. difficile* strain expressing a GFP protein to screen the colonization over time. It would have been interesting to see the colonization in vivo.**

*The *C. Difficile* strain was selected based on previous models. Engineering a strain to produce GFP could affect *C. difficile* colonization and metabolism, changing its interactions with the community. We are unaware of an established CDI that uses a GFP producing strain. We agree it would be interesting to observe colonization in vivo, however that is beyond the scope of this set of experiments. The growth of *C. difficile* from fecal pellets is sufficient to indicate the mice were colonized with *C. difficile*.*

**-It would have been interesting to see penetrating bacteria in colon tissue (using FISH, for example) to link the effect of FCT on intestinal permeability as well.**

*Future studies investigating the mechanism of the FCT will be necessary. In our study, we were investigating the differential effect of the FCT. Follow-up studies should investigate how the communities affect colonization and disease and whether there is variation to those as well. Those studies could investigate intestinal permeability. Here, we sought to test the effect of diluted fecal communities in different antibiotic treatments on CDI.*

**-How authors choose the antibiotic groups? FCT does not seem to have a contribution in clindamycin-treated mice, when compared to streptomycin and cefoperazone. The different activity spectrum of those antibiotics might be a strong contributor for this result. It may be beneficial to indicate this point in the Discussion.**

*We thank the reviewer for highlighting this gap in reasoning. We chose the antibiotics based on our previous set of experiments and publications which generate different communities and different activities by *C. difficile* when colonizing and clearing. We have added this explanation to our Methods as well as citations. “Antibiotics were chosen and administered based on previous studies (14, 31, 32). Cefoperazone, clindamycin, and streptomycin treatment produced diverse communities and responses to CDI.” (Lines 320-322).*

**-Pls see Line 126-what is the exact meaning of 'simplified fecal communities' after dilution? Dilution may randomly reduce OTU abundance. And, diversity seems similar after dilutions. So what 'simplified' refer to?**

*We understand the confusion in what “simplified” is describing since some of the community measures maintain diversity. We have switch to use “reduced” which more accurately describes the differences between the diluted communities. (Lines 43, 60, 66, 75, 124, 126, 210, 221, 223, 258, 260, 301, 306, 312).*

**-An important aspect of this study is the focus on the protective role of FCT before CDI. It would have been advantageous to include a later *C. difficile* challenge arm in the study to see the long-term protective effect of FCT in streptomycin-treated group.**

*This suggested set of experiments to test how the time between the FCT treatment and the *C. difficile* challenge would affect the protective effect in the streptomycin-treated group would be an interesting follow-up to this study. Those experiments could refine our understanding of the FCT. For this study, we chose the timing to best replicate the previously established CDI model with the addition of the FCT. We have added this explanation to help clarify our*

reasoning. “This time frame was designed to closely replicate the previous mouse model (14, 31, 32), with the insertion of a day (clindamycin) or two (cefoperazone and streptomycin) for inoculating the mice with the fecal communities.” (Lines 353-355)

**-It may be beneficial to include limitations of the study in the last paragraph of Discussion.**

*We have added discussion of limitations. “Further investigation into the heterogeneity of CDI will help to elucidate the niche range C. difficile and the interventions to eliminate them. Here we were limited by our experimental design and methods to refining our understanding of colonization resistance restoration in streptomycin-treated mice. Future studies can expand beyond the presence and abundance of the bacterial groups and investigate the metabolites and host immune response. A refined understanding of the bacteria, metabolites and host response can help develop more targeted therapies to restore C. difficile colonization resistance. Additionally, building up experimental design to incorporate more FCT treatment variations or inoculation regimens could expand our understanding of the necessary components for colonization resistance for each antibiotic treatment. We designed our experiments to closely match previous mouse models for CDI and added days prior to C. difficile challenge for the FCT treatment (14, 31, 32). It may be possible to restore colonization resistance to clindamycin or cefoperazone if the antibiotic treatment, recovery period, and FCT treatment were modified to allow the FCT to have an effect. Other methods could be used to make the mice susceptible to CDI and then tested for the effectiveness of the FCT treatment (18, 33). Further modification and characterization of the fecal communities could reduce the necessary community members and metabolites to promote colonization resistance. The results from these additional studies could expand upon our limitations and reveal specific bacterial communities that could restore C. difficile colonization resistance for each susceptibility.” (Lines 281-300).*

**Minor Remarks:**

**Line 299- Pls revise 'mice we housed with'**

*We have corrected “we” to be “were”. (Line 317)*

**Line 362-Pls include the reference from Huttenhower group for Lefse**

*We have added the LEfSe reference. (Lines 383).*

**Line 246 - unable to colonize**

*We have made the correction and inserted "to". (Line 244)*

**Figure S5 - labels are too small**

*We have adjusted the figure dimensions for the labels to be legible. (See Figure\_S5.tiff)*

**Reviewer #2:**

**The authors have designed a mouse study to study the effect of antibiotics and fecal community transplant (FCT) on *Clostridium difficile* colonization. Unfortunately, the absence of a critical timepoint at day -2 hindered the analysis in separating the contribution of the FCT from the antibiotic. Moreover, the use 16S rRNA gene sequencing and the identification of most taxa at the family level impedes a fine understanding of the taxonomy of the bacteria. Whilst the phenotypic result showing *C. diff* colonization is valid, the lack of a clear impact of the FCT or higher-resolution microbiota identification leave much more to be desired.**

**Here are my fine points:**

**"For the streptomycin-treated mice, the FCT pre-treatment resulted in either no detectable *C. difficile* colonization (8 of 14) or an infection that the community cleared within 5 days (Figure 2)."**

**It is not possible to discuss the effect of the FCT pre-treatment because there is no timepoint at day -2.**

*We sought to determine the effect of FCT on *C. difficile* colonization. Therefore, we compare mice given FCT to mice given PBS. If we were to compare the community on day 0 to day -2, it would only tell us how the community changed with the FCT but not the effect of FCT on *C. difficile* colonization. We could have tested challenging mice with *C. difficile* at day -2 and another group at day 0 but then the group on day 0 also has two additional days of recovery from antibiotics. Thus, our comparison group of PBS treatment replicates the timing and recovery from antibiotic treatment without the supplemented FCT.*

**"The streptomycin-treated mice pre-treated with FCT as dilute as 1:10<sup>2</sup> had no *C. difficile* CFU detected throughout the length of the experiment."**

**I am slightly confused. Isn't there at least 1 mouse with a detectable CFU at figure 3B right panel?**

*We thank the reviewer for identifying this unclear statement. We intended to say that no CFU was a possible outcome for the mice given FCT dilution 10<sup>2</sup>. We now understand that this conveys that all mice given the 10<sup>2</sup> were not colonized. We have clarified the statement. "Some streptomycin-treated mice pre-treated with FCT as dilute as 1:10<sup>2</sup> had no *C. difficile* CFU detected throughout the length of the experiment." (Lines 119-121).*

**The fonts are too small in figure S2.**

*We have adjusted the figure dimensions for the fonts to be legible. (See Figure\_S2.tiff)*

**"The effect of FCT was not large enough at the time of *C. difficile* challenge to be detected in the community diversity"**

**The effect of the FCT cannot be determined because there is no timepoint at day -2.**

*We have clarified this statement to more accurately represent our intent to communicate that the communities treated with FCT remained significantly different than the initial communities but still were able to inhibit *C. difficile* colonization. "Thus, the less dilute FCT treatments did not result in restoration of pre-antibiotic treatment community diversity at the time of *C. difficile* challenge but were sufficient to affect *C. difficile* colonization." (Lines 148-150)*

**" $\alpha$ -diversity, measured by Sobs (A) and Inverse Simpson (B), prior to beginning antibiotic treatment (day -9), after fecal community transplant on the day of *C. difficile* challenge (day 0) and at the end of the experiment (day 10)."**

**Is there a timepoint at the first day of the FCT (day -2) so that we can calculate the**

## **diversity after antibiotic treatment but prior to FCT?**

*We have added the time point prior to FCT treatment (day -2) text to the figure legends for Figures 4 and S3. “before fecal community transplant (day -2),” (See Figure\_4.tiff and Figure\_S3.tiff, Lines 602-603, 650-651)*

**"Communities resistant to *C. difficile* colonization had more abundant populations of OTUs related to Akkermansia, Clostridiales, Olsenella, and Porphyromonadaceae and less abundant populations of an OTU related to Enterobacteriaceae."**

**Many bacteria are at the family level and a few are at the genus level. Is there any at the species level? How important is it to look at strain level as seen in some fecal microbiota transplantation papers?**

*OTUs were clustered with sequences with 97% identity, which could potentially assign a taxonomy at the species level, and then taxonomy was assigned using the RDP training set version 16. The taxonomic classifications reported are the lowest level available. Many of the sequences lowest taxonomic level that they are classified at is family or genus. In regards to how this affects the analysis in comparison to other FMT papers, based on this paper as well as our previous work Lesniak et al. mSphere 2021, some of the inhibition of *C. difficile* may be specific to the susceptible community or the inhibitory function may be present in higher taxonomic levels and not just the strain level. Either way, this paper suggests further studies are needed to understand the specificities of the susceptibilities and to test whether it is the family-specific or strain-specific function that is sufficient to eliminate exposed niches that *C. difficile* has colonized.*

**"Multiple OTUs related to Lachnospiraceae and Porphyromonadaceae (N = 14 and N = 5, respectively) were significant and accounted for greater portions of the community (more than 10%)."**

**There are so many Lachnospiraceae. How informative is this?**

*As stated in the concluding sentence, “Thus, as more of the gut bacterial members returned to their initial abundance, there was a greater likelihood of clearing *C. difficile*.” (Lines 185-187), many OTUs that were initially present have returned in the communities that cleared *C. difficile*. Furthermore, since many populations of Lachnospiraceae associated with clearance could indicate that clearance is not mediated by an OTU-specific function of a single*

*Lachnospiraceae* population but by a family level function present in all of those individual populations.

**"We observed antibiotic-specific changes associated with *C. difficile* clearance. The data presented here complement those observations."**

**Because there was no timepoint at day -2, it is impossible to tease apart the microbiota contributions from the antibiotic and the contributions from the FCT.**

*Day 0 is the more important timepoint to compare between FCT and PBS to understand the effect of the FCT on the community. Comparing day -2 to day 0 will tell us how the community has responded to antibiotic recovery and the gavage treatment. But comparing across gavage treatments (FCT, FCT dilutions, and PBS) we are comparing the effect of the a fecal community treatment on the mouse gut community recovering from the same antibiotic treatment. Therefore differences we see in *C. difficile* colonization or community we attributed to the FCT since the more dilute FCT dilutions and PBS we also undergoing similar recovery of the gut community from antibiotic treatment.*

**"This observation supports our previous report, indicating that the cefoperazone-treated community is more sensitivity to the amount of FCT it receives since cefoperazone reduced many bacterial groups and associations (Figure S6)."**

**I am slightly confused. Wouldn't this statement require data from day -2?**

*For this statement, we are comparing the effect of the FCT, which resulted in clearance, to the effect of the 1:10 dilution of the FCT, which resulted in persistent colonization. This comparison tells us the effect of the more concentrated FCT. Comparing post-FCT treatment (day 0) to pre-FCT treatment (day -2) is missing the natural recovery of the community and also includes the changes that don't have an effect on outcome, as observed in the more dilute treatments. Comparing across treatments on day 0 indicates the critical differences due to the increased concentration of the FCT on the outcome of the *C. difficile* challenge. We have added a citation for previous discussion. "This observation supports our previous discussion (14)," (Lines 248)*

**Fig 3: points are stacked above one another. Not able to count how many mice in many**



**cases.**

*Counts have been added to the figure legends of Figure 2 and 3. “(Clindamycin - PBS n = 4, FCT n = 7; Cefoperazone - PBS n = 2, FCT n = 2; Streptomycin - PBS n = 10, FCT n = 14)” “(Cefoperazone - 1:10 n = 2, 1:10<sup>2</sup> n = 2, 1:10<sup>3</sup> n = 3, 1:10<sup>4</sup> n = 2, 1:10<sup>5</sup> n = 2; Streptomycin - 1:10 n = 12, 1:10<sup>2</sup> n = 14, 1:10<sup>3</sup> n = 5, 1:10<sup>4</sup> n = 4, 1:10<sup>5</sup> n = 5)”(Lines 591-592, 597-599).*

**"We have demonstrated that a simplified bacterial community can restore colonization resistance"**

**The authors have only identified differentially abundant bacteria between FCT and non-FCT groups. They have not constructed an actual simplified bacterial community to place into gnotobiotic mice.**

*We have adjusted the language “simplified” to “reduced” to better represent the FCT dilution treatment (Lines 43, 60, 66, 75, 124, 126, 210, 221, 223, 258, 260, 301, 306, 312). Additionally, we have demonstrated restoring resistance to a susceptible community, specifically streptomycin-treated C57Bl/6 mice, and do not make any claims beyond this set of antibiotic treatments, such as gnotobiotic mice, which is stated with the rest of the quoted text “We have demonstrated that a reduced bacterial community can restore colonization resistance but the effect of the community and the bacteria that colonized was dependent on the specific changes to the community that were caused by each antibiotic.” (Lines 301-303)*

**"We rarefied samples to 2,480 sequences per sample to limit uneven sampling biases"**  
**This level is kind of low for stool community. It will affect the statistical tests.**

*To reduce the risk of low abundant taxa affecting statistical tests, the rarification performed sub-samples the data 1000 times and averages those 1000 sub-samplings. We have clarified this in our methods. “We averaged 1000 sub-samples of 2,480 sequences per sample, or rarefied, to limit uneven sampling biases.” (Lines 381-382)*

**"During the experiment, mice we housed with two or three mice per cage."**

**Mice in a cage do coprophagy and transmit C. diff among themselves. How important is it**

**to make sure that the detected C.diff is due to outgrowth from the gut and not transmitted from other mice?**

*While mice could potentially be recolonized via coprophagy, we don't have evidence this is an issue in our experiments. We collected stool directly from the mice. Thus, any significant level of C. difficile CFU detected in the stool would have been from outgrowth in the gut of the mouse and not spores that were consumed via coprophagy. Furthermore, mice that cleared the colonization but remained co-housed with other colonized mice remained uncolonized. This supports that the gut community is recovering colonization resistance and prevents C. difficile spores in fecal pellets from germinating and recolonizing the gut.*