Reviewer comments rebuttal

Line numbers refer to manuscript.docx

Reviewer #1 (Comments for the Author):

1. Clearance should be more precisely defined when used in the manuscript (e.g., Line 60-61). While the authors demonstrated that C. difficile levels were suppressed below the level of detection, they have not shown that mice are not colonized with C. difficile below the level of detection. Previous studies have shown that C. difficile can be suppressed below the level of detection but may be present at sufficient levels to allow recurrence following antibiotic challenge (e.g., Sun et al. 2011. Infect Immun. 79:2856-2864; Collins et al. 2015. Microbiome: 3:35; Seekatz et al. 2015. Infect Immun. 83:3838-46). Thus, it may be more accurate to state that active CDI has been cleared rather than C. difficile colonization has been cleared.

The reviewer points out the need to clarify clearance as there are different usages of this within the literature. We use clearance to describe reduction of C. difficile CFUs over time. We say the C. difficile has been cleared once it falls below the limit of detection. While it is possible that C. difficile persists in the community below detection, it hasn't been demonstrated in the mentioned references whether C. difficile persisted in the community below the limit of detection or if C. difficile was reintroduced through spores that persisted in their environment. Additionally, the reviewer suggests to state "active CDI has been cleared", however since we are not looking at any measures of infection, we cannot state that. We have removed "eliminate" and clarified clearance/cleared refers to reducing C. difficile below the limit of detection (Line 71) to clarify our usage.

2. Several key details about the animal studies that could potentially influence experimental outcomes are missing from the methods. These details include mouse strain background (C57BL/6J?), number of mice housed per cage, numbers of male and female animals, age of mice at time of antibiotic administration, number of independent experiments performed. These details should be included in the methods.

We thank the reviewer for pointing out these critical details necessary for replicating our experiments. We have expanded the methods section to better explain the experimental details and included sex, age, strain, cage numbers, n per treatment, replicates used (Lines 357-362).

3. The decision to aggregate data from different antibiotic doses of cefoperazone or streptomycin was not clearly explained. It appears that the decision to aggregate data from mice treated with different levels of cefoperazone may have contributed to the high levels of variation in alpha and beta diversity in cefoperazone mice that remained colonized. The authors should test whether variation in alpha and beta diversity measures observed in cefoperazone-treated mice was due to difference in amounts of antibiotics administered. Similarly, the decision to include two 0.1 mg/ml

cefoperazone-treated mice with single, low level CFU counts in the group that cleared C. difficile infection should be better justified. Given that the counts for these two mice were so close to the limit of detection, was abundance data verified using an alternative method?

Thank you for highlighting this area that would benefit from further clarification. We decided to aggregate the data from each antibiotic across different levels because we were hoping to capture the common differences associated with C. difficile colonization as opposed to the effects of the antibiotic. To be more transparent in this decision, we added a sentence to explain the aggregation (Lines 108-111). Also to ensure that the differences we observed were not merely differences due to antibiotic dosages, we added a supplemental figure (Figure S3) to show the alpha diversity by dose of cefoperazone. None of the comparisons were statistically significant, which we added a sentence to state this (Lines 148-149).

4. Given the authors' previous studies that point to different nutritional niches used by C. difficile in clindamycin, cefoperazone, and streptomycin-treated mice, it was surprising that all three types of treatments were used to build a machine learning model to identify microbiota configurations at the time of C. difficile challenge that would predict clearance. As noted by the authors, a single feature, Enterobacteriaceae OTU #8, had the highest odds ratio for clearance, but this was driven primarily by data from clindamycin-treated mice and not consistent with what was observed in cefoperazone-treated mice, where Enterobacteriaceae OTU #8 was higher in mice that remained colonized, or streptomycin-treated mice, where there appeared to be no differences between mice that remained colonized and mice that cleared C. difficile. Further, comparing data from Figures 5B and 5C, several of the features with high OR appear to be associated with clearance in clindamcyin-treated communities (OTU8, OTU36, OTU20, OTU14) or with cefoperazone/streptomycin-treated communities (OTU22, OTU9, OTU10, OTU13, OTU17), with relatively few predictive of clearance in both models. While other features of the model were still able to successfully predict outcomes, a stronger justification for how a single model will more effectively "offer a basis for hypotheses regarding the distinct combinations of bacteria that promote C. difficile clearance" (lines 194-195) rather than models based upon the unique microbiota signatures associated with clearance is needed, especially in light of the authors decision to present three independent models for clearance in the discussion.

We acknowledge that individual models would have allowed us to identify the OTUs most predictive of each treatment. While we treated them separately in previous comparisons, here with machine learning we felt it was best to model the data together. This way it would provide sufficient sample size to create a robust model and not overfit to one specific treatment. We have added a sentence to state our reasoning for modeling all treatments together (Lines 203-205).

5. There is relatively little discussion of the roles that the immune system might play in contributing to clearance of infection. This should be expanded in the discussion (lines 273-292), especially given that the C. difficile strain used in these studies (CD630) induces a much weaker immune response than other C. difficile strains (VPI10463, BI1; Theriot et al. 2011. Gut Microbes. 6:326-334) and that blooms of Enterobacteriaceae, a characteristic feature of all models you studied, likely induce, benefit, and perpetuate inflammatory conditions (e.g., Winter et al. 2013. EMBO Rep. 14:318-27; Zhu et al. 2018. Nature. 553:208-211; Byndloss et al. 2017. Science. 357:570-575 as well as ref 31 and 32).

Thank you for your suggestion. We see we could add more details to explain our previous statement, " more virulent strains, like C. difficile VPI 10463, may have a greater effect on the gut environment". We have added more details to what we were implying by "greater effects" with examples such as the one recommended by this comment by stating the more virulent strains drive a stronger immune response, increasing inflammation and inflammation-associated bacteria such as Enterobacteriaceae (Lines 326-330).

Additional comments:
Lines 111-112. The number of OTUs identified as required for recovery was 10/21. Recovery of ~50% of OTUs does not seem like a "small portion" as described; it would be more accurate to describe this as a "subset" or simply "portion"

We agree with you and have made this change by describing it as a "subset" (Line 132).

Lines 121-123: As Figure 1E only shows relative abundance of 12 genera, it does not provide strong support for the statement that the increased number of observed species was due to decreased levels of abundant populations and increased levels of low abundant populations. This could be better substantiated by including a supplemental figure that presents the number of observed species at low and high abundance at the time points indicated. Also, not all mice that remained colonized exhibit this increase in diversity.

Thank you for your suggestion. We understand that using Figure 1E isn't effective at demonstrating what this statement was describing. We have incorporated your comment by creating a supplemental plot (Figure S2) to show the distribution of OTU relative abundance in cefoperazone treated mice. The plot shows that the mice with increased alpha diversity have an increase in abundance of otus in lower abundance in all other samples. We revised the statement as well to describe this (Lines 143-145).

Lines 235-239 incorrectly characterize the results by Buffie et al (no data on C. difficile clearance, as C. difficile levels over time not reported) Reeves et al (no data on C. difficile clearance in mice pre-colonized with Lachnospiraceae, as mice were euthanized on day 2 following infection) and by Lawley et al (treatment with MixC suppressed C. difficile

shedding below the level of detection which would meet the definition of clearance). These lines should be revised.

We have reflected this comment by more explicitly describing the details and aspects of the referenced articles to state that these articles described bacteria associated with C. difficile colonization, and then described the outcomes when trying to use identified bacteria to affect C. difficile colonization (Lines 271-276).

Line 256-260: The authors characterize the cefoperazone-treated mice that fail to clear infection as exhibiting a persistent increase in facultative anaerobes, yet their data (Figure 4A) shows that these four OTUs (OTU8, OTU29, OTU91, and OTU52) are only significantly different between mice that clear infection and mice that fail to clear infection at the time of C. difficile challenge. If this was a persistent increase in these OTUs, these OTUs should still be signficantly different at the end of the experiment. If the authors continue to propose that these OUTS persist, then the abundance of these OTUs over time in samples should be reported. If that data is not available, then the abundance at the end of infection should be reported or a more complete explanation of how lack of significant differences between groups at the end of infection fits this model should be offered.

We believe this figure 4 is being misinterpreted. Figure 4C, shows the significant differences for mice that remain colonized between initial community and time of challenge (where the increase in OTUs 8, 29, 91, and 52) OR between the time of challenge and the end of the experiment. It is possible the reviewer is under the assumption the comparison is between the end of the experiment and the initial, but it is not. In the OTUs mentioned in the comment, since there is no significant change after the initial decrease (Initial vs Time of challenge) there is not significant difference between the time of challenge and end of experiment. To try to be more clear about this, we considered plotting the non-significant abundances to show the unchanged or non-different abundances but this quickly crowds and complicates the plot. So we felt it was best to make it clear in the legend, stating only significant differences are plotted and the titles of each column stating the comparison being tested/shown.

Also, it is unclear how you reach the conclusion that multiple niches are available in cefoperazone-treated mice (lines 260-261) while limited niches are available in streptomycin-treated mice (lines 265-267). While the data in Jenior et al (ref 20) point to C. difficile utilizing more diverse substrates in cefoperazone-treated mice (mannitol, nicotinamide-beta-riboside, guanosine, inosine, dGDP, and bicarbonate) compared to clindamycin-treated mice (salicin-6P, L-alanine, succinyl-CoA, Dihydrolipoylprotein), a larger number of substrates are predicted to be used by C. difficile in streptomycin-treated mice (D-sorbitol, galactitol, starch, S-3-hydroxybutanoyl-CoA, 3-Indoleacetonitrile, benzonitrile) than those used in cefoperazone-treated mice.

This comment points out the number of differences in utilized substrates reported in previous studies in our lab. However, we believe niches available are not limited to the number of unique substrates metabolized, but also includes the amount of resources and space available. We added text to help clarify this meaning (Lines 300-302)

Lines 260-263: While multiple niches likely preclude effective treatment with a defined community of a small number of microbes that do not fill all available niches, it is a stretch to state that a single FMT from a healthy donor is unlikely to be effective. FMT failure and need for repeated in FMT is relatively rare in patients with rCDI (ref. 29; Seekatz et al. 2014. mBio 5:300893-14), who often exhibit extreme levels of microbiome disruption. Factors correlated with FMT failure (immunocompromised state, severity of CDAD) may be due to magnitude of microbiome disruption, but this has not been definitively demonstrated and limited data indicates a lack of correlation between FMT engraftment and clinical FMT failure (Seekatz et al 2014. mBio 5:300893-14). Further, a single FMT transplantation in 0.5 mg/ml cefoperazone-treated mice from the University of Michigan breeding colony is sufficient to clear C. difficile infection an prevent relapse (Seekatz et al. 2015. Infect Immun. 83:3838-3846). This section should be revised.

Thank you for your perspective, however we are unsure what this comment is requesting to be revised. We state that it is possible that an FMT may not be sufficient to recover a significantly disrupted microbiome, such as our hypothesis for the cefoperzaone-treated mice. It seems like this comment is stating since Seekatz et al 2015 showed FMT prevented relapse with cefoperazone that our hypothesis is incorrect. However, Seekatz et al 2015 have a much different experimental scenario, which used a different breeding colony and sensitized using cefoperzaone, challenged with C. difficile 630, 4 days later given a 5 day course of vancomycin, then two days later given two daily gavages of an FMT. After that there was a transient increase in CFU which remained below LOD until the mice were given an IP of clindamycin which caused another transient increase in CFU. So I feel the referenced manuscript is not directly applicable to lines 274-277. Additionally, the comment states the rarity of FMT failure (Seekatz et al. 2014). While an FMT is ~80-90% effective, this still leaves 10-20% of patients in which it does not work. So even if it is relatively rare, it still occurs.

Lines 267-271: As mentioned above, Jenior et al point to the nutritional niches used by C. difficile in streptomycin-treated mice as equal to or broader than those used by cefoperazone-treated mice, so it is not clear how the data from these mice were used to establish a third model with more limited disruption. While the number of OTUs that are different at the end of experiment in streptomycin-treated mice are fewer and the magnitude of differences is smaller, the potential phylogenetic diversity of these OTUs (Turicibacter, Alistipes, Lactobacillus, Porphyromonadaceae) is greater than that observed in cefoperazone-treated mice (10/12 OTUs are Porphyromonadaceae; Barnesiella, Alistipes). This section should be revised to better explain how these conclusions were reached.

Thank you for your suggestion. While the Jenior et al. experiments are similar to the ones here, there are differences that make direct application of those findings to these difficult. Those experiments looked at activity at peak infection, whereas we are looking at the taxonomic changes associated with clearance. There may be overlap but there also could be differences taxonomically as well as nutritionally through the clearance process. Also this comment associates phylogenetic diversity with functional diversity. We acknowledge that we were not clear about the limited disruption. We believe it is possible that specific OTUs have specific functions not shared by other species/OTUs of the same genus. We clarify that we believe, based on the changes we observed in figures 3 and 4, that only a few specific bacteria are necessary to clear colonization in those communities (lines 310-312, 336-338).

Figure 1 legend: Methods include the challenge dose of C. difficile spores (103); it would be helpful to have this number in the legend.

We have reflected this comment by adding spore info to the figure legend (Line 576).

Figure 1D-F: A better explanation of how taxa are grouped in Figure 1 is needed. Line 517 indicates the 12 most abundant genera are displayed, but four of the taxa listed are bacterial families (Lachnospiraceae, Ruminococcaceae, Enterobacteriaceae, Porphyromonadaceae), and one is a bacterial order (Clostridiales). Were these higher-level taxa a grouping of all sequences not classified at the genus level for the indicated family or order? Or, were they a single OTU that was classified at this level? More details are needed. In addition, the number of mice listed in the figure labels (n=6), figure (n=4) and figure legend (n=5) for mice treated with 0.5 mg/ml cefoperazone do not match. Similarly, the number of mice listed in the figure legend for 0.3 mg/ml and 0.1 mg/ml cefoperazone (n=9 and n=2) do not match the number of mice in the figure (n=13 and n=6, respectively).

Thank you for bringing these inconsistencies to our attention. We have corrected the language describing the taxonomic data to explain that "twelve most abundant taxonomic groups, labeled with the lowest level of classification" (Lines 578-580). We have removed the incorrect N numbers and removed them from the figure to allow more room in the text box of the plot.

Figure 3 legend: Lines 540-543 contains a sentence fragment rather than a complete sentence; simple fix would be to end the sentence with "were identified", but it could also be helpful to include the method used for identification of significant OTUs in this sentence. The term "bold points" on line 543 is a little confusing; "larger points" might be less confusing.

We agree that description of the points may not be correctly interpreted so we added to their descriptions to ensure they will be correctly interpreted "Dark larger points in foreground are median relative abundance and transparent smaller points in background

are relative abundance of individual mice." (Lines 601-602). Also we have completed the first sentence with "were identified" (Line 600-601)

Figure 4: Panels A and B lack individual mouse data present in panels C and D.

Thank you for identifying this missing information. We have added the individual points back to panels A and B.

Figure 4 legend: As with Figure 3 legend, lines 552-555 contain a sentence fragment. Comment about "bold points" is also true for this legend as well as Figure 3.

We made the same changes to Figure 4 legend as we did to Figure 3.

Figure 5 legend: Line 567-568 specify what the different lines mean (Dashed line equals AUROC, dotted lines equal IQR?)

We agree with you, it was not clear how to differentiate or identify the lines mentioned in the legend. We changed IQR to a light green band and AUROC to a dark green line to make them more easily differentiated from each other and boxplots and updated figure legend to match. (Line 620-622)

Figure 6: Would be nice to include a key that indicates how line thickness relates to strength of correlation and how node size correlates with median relative abundance.

Thank you for your suggestion. We have added the strength values to all edges and added legend for node size to Figure 6.

Figure S1: If relative abundance is plotted from each individual mouse from a single fecal sample prior to antibiotic treatment, from which repeated measures is the mean calculated? Was the fecal sample sequenced more than once? This is not clear from the methods.

You have asked an important question. Fecal samples were only sequenced once. No mean abundance was calculated. Figure S1 depicts the actual relative abundances of individual mice. In subsequent analyses mice are compared to their own communities only. This plot is allowing the reader to see what the taxa plotted in figure 1 were to begin.

There were also a few typos in the body of the paper:
In line 100, it starts with "were are", get rid of the "are".

Thank you for identifying these typos. We have removed "are" to be "the mice were also able to clear". (Line 119)

In line 152, C. difficile is misspelled.

We corrected the misspelled “difficle” to “difficile” (Line 179).

In line 159, colonization should be colonized

We corrected the “colonization” to “colonized” (Line 186).

In line 165, a word is missing between "those" and "had".

We added “that” to state “and those that had been cleared” (Line 194).

References are inconsistently formatted and should be copy-edited. Clostridium difficile is not capitalized in many references nor is "Italian" (line 379) or "United States" (line 373). At least two titles lack spaces "Health careAssociated" line 376, "ofClostridium difficileInfection" (line 438).

Thank you for identifying these issues in our references. We edited the reference input to ensure proper formatting for all of our references to have appropriate spacing, capitalization and italics. (Lines 434, 436, 478)

Reviewer #2:

Major comments:
1) The figures in this manuscript are mostly unreadable unless highly zoomed in the digital version. For those that print the articles, it's difficult to read such fine print.

Thank you for your suggestion. We understand some of the text is small. We did our best to keep the data and text equally visible without them obscuring each other. We increased font sizes and adjusted the futures to be more legible within the restrictions of plotting the complex data set.

2) Cefoperazone is not used by all of the C. difficile community. The cocktail of antibiotics described by Chen & Kelly 2008 is frequently used by the field. It would be of significant value if this antibiotic cocktail could be added to the analysis. This would provide a good resource for the community by showing the networks that form in this model.

We acknowledge cefoperazone is not one of the major models for CDI; however, our intention was not necessarily for replicating CDI models. It was to generate different murine gut communities permissive to C. difficile colonization. We didn’t need the cocktail to get C. difficile to colonize. In other models, the cocktail seems to be

necessary for vegetative cells to colonize not spores, as we used. Although, we agree this additional perturbation would be interesting to explore in future experiments to see how the overlapping effects of the antibiotics affect the community changes that would allow clearance and how those changes compare with our results presented here.But those additional experiments are beyond the scope of this study.

Minor comment:
1) The discussion section nicely summarizes the findings and the authors have hypothesized that there are likely multiple niches that C. difficile colonizes - depending on the antibiotic. What is missing is the "who's there vs. what's there" in the gut. Though it would be a significant / costly undertaking, metabolomics for this exact analysis would have the potential to identify common players in the susceptibility / resistance game.

This comment raises an important point which we are interested in and we agree that it is a significant undertaking, that is outside the scope of this project. We discussed the importance of understanding the relevant functions in the manuscript (Lines 336-340). Determining “who’s there” vs “what’s there” is important but with this data set we were unable to obtain metabolites. Future experiments based on these results will need to address these questions.