**Annotated reviewer comments:**

Part I - Summary

Reviewer #1: Jenior et al. present a manuscript for consideration for publication in PLOS Pathogens entitled “Clostridium difficile differentially alters the structure and metabolism of distinct cecal microbiomes to promote persistent colonization during infection.” This manuscript follows up on the results of their previous mSystems paper which described different nutrient niches are occupied by C. difficile in the ceca of mice depending on the antibiotics that are given to mice prior to infection with C. difficile. Specifically, this study sought to characterize the composition and metabolic output of the microbiome in response to antibiotics and/or C. difficile infection. They demonstrate that C. difficile burdens decrease over time in mice that are treated with clindamycin prior to infection but that when mice are given streptomycin or cefoperazone, the mice remain persistently colonized. They go on to show differences between the microbiome composition, transcriptome, and metabolome between these three antibiotic treated states and further show differences in the metabolome and transcriptome of mice under each antibiotic treatmen’t +/- C. difficile. This study opens a lot of interesting avenues for study but doesn’t follow up on any of them. Significantly more work is needed before it is acceptable for publication in PLOS Pathogens. Some specific suggestions for improvement are listed below:

A major shortcoming of the manuscript, is that the authors present a catalog of data without testing their hypothesis using molecular or genetic means, which results in a disjointed story with no mechanistic detail. For example, there is extensive discussion on the importance of Stickland fermentation for C. difficile infection, which is supported by the decrease in abundance of proline derivatives during infection. Does a proline-deficient C. difficile strain colonize mice? Several of the figures similarly point to interesting findings, but without experimental follow-up the study falls short of presenting cohesive insight.

A mutant in this pathway has been tested in vivo in a model of murine susceptibility by Bouillaut et. al. 2013. We initially only referenced the work in the introduction and discussion, but have now changed wording in those sections to make this point clearer. What the current study adds to this area of understanding is how apparently critical this form of metabolism is across infection models, and does not necessitate that the capability be removed in order to prove this point. Furthermore, we would point out that we have taken a systems approach to studying C. difficile colonization. Although there were not subsequent mouse studies, we layered a different and largely independent omics-based analysis to support our hypotheses. For example, we started with 16S rRNA gene sequencing, then metabolomics and metabolic modeling, and finally used metatranscriptomics to buttress our claims. Knocking in or out a single functionality certainly has its strengths as an approach, but it should not be discounted that applying multiple methods to a single experiment can have a similar ability to test the robustness of our results.

Reviewer #2: In this study, Jenior and colleagues studied murine model of C.difficile colonization, employing a multiomics approach to understand associated changes in composition and function. Use of multiple platforms is a sound and logically attractive approach to further understanding the biology of C.difficile, and it is quite welcome and sorely needed. However, there were some important overall concerns in the approach and execution that should be addressed, particularly with regard to the analysis and presentation of data.

Reviewer #3: This work from Jenior, Schloss and colleagues explores the underlying AbX-associated changes in microbial communities that predispose mice to colonization with Clostridium difficile. The authors pre-treat mice with Streptomycin, Cefoperazone and Clindamycin, which all increase the levels of C.diff cells and spores in the cecum, and vary in the levels of toxin production. The authors pose a central hypothesis that the alterations in the underlying microbial community associated with these various AbX treatments may all converge on metabolic alterations rather than changes in the specific community composition. Combining microbial metatranscriptomics, metabolomics and microbiome profiling, the authors demonstrate that both AbX treatment and C.difficile colonization may differentially modify the transcriptional and metabolic activity of distinct commensal microbes. These findings lay the groundwork for understanding the complexity of how different community structures may be more permissive to C.difficile colonization and how remediation may not simply be based on a census of microbes.

Part II – Major Issues: Key Experiments Required for Acceptance

Reviewer #1: 1. The authors’ use of the word “clear” to describe their phenotype where C. difficile decreases below the limit of detection (100 cfu/g feces) in their plating assay is inappropriate. The authors should change the language to something like “suppress” or “reduce” because clearance implies that C. difficile is completely removed from the environment and these mice may be carrying C. difficile at low levels, as in Lawley et al. 2009 IAI. Can the high level shedding of C. difficile be re-established by giving a dose of clindamycin?

We acknowledge the reviewer’s point and have since changed the language accordingly. Additionally, we have tested this within our research group with a subsequent dose of clindamycin at 5 days after initial clearance and no detectable *C. difficile* was found. However, this was dependent on frequent cage changing during the period of clearance. This was performed for a separate project so it was not included. Furthermore we do realize that this clearance may be a function of microbiome present in mice in our specific colony.

2. How many animals were used in the study and how many times were the experiments repeated? It’s difficult for me to determine if the results presented throughout the paper are reproducible (e.g. reduction of C. difficile burdens, metabolic and transcriptional changes) because this information is not present in figure legends or in the methods section.

The authors apologize for this omission, and it has now been corrected as part of the methods (see lines 512 - 513). We have further included an analysis of variance within replicates in supplementary results (see lines 309 - 313, 980 - 991, and figure S5).

3. In line with comment #2, above, a major drawback of doing metagenomic/transcriptomic analysis on complex communities is that material (e.g. cecal contents) is limiting. The authors overcome this by pooling cecal contents from multiple mice transcriptomic analysis. However, by pooling multiple mice worth of cecal contents per treatment, it is impossible to tell whether we’re observing differences that are repeatable between mice within a treatment group or if the differences in transcription are driven by a couple of outlier mice. A revised manuscript, including validation of several transcripts (eg, by qPCR), is necessary for me to believe that their findings are not artifacts due to pooling and to show that there are statistically significant difference between conditions (for example, there are no error bars in Figure 5)

We agree that a significant limitation of metatranscriptomics is the need for a large amount of RNA. This was compounded by our desire to obtain RNA from *C. difficile*, which was relatively rare in these communities. That being said, in this study, we used our metatranscriptomics data to support the results from our metabolomics analysis, which was performed using replicates and presented with statistical test results (see lines 240 - 296). Furthermore, we do not agree that there is a large risk of outliers in our design. The amount that a single sample would need to contribute to the overall pool in order to generate a quantifiable impact is immense. Any sample providing an outlier would be considerably muted when pooled with non-outlier samples. Furthermore, the low levels of variability in the 16S rRNA gene sequence and metabolomic datasets suggests a similar low level of variation in the metatranscriptomic data.

4. The authors removed C. difficile 16S rRNA reads, and metagenomic/metatranscriptomic reads from their analyses because they wanted to focus specifically on the surrounding bacterial communities. Because C. difficile represents <3.54% of the community in any infection group, they expect this removal to not have much of an effect. Why not leave these reads in the analysis then? How are diversity, composition, and metatranscriptomic metrics different if C. difficile is left in?

There is no difference in any metric mentioned by this reviewer, therefore we did not include this analysis as there is already a substantial amount of data presented in the manuscript. With this in mind, it is just more technically correct to remove it as we sought to make claims about the community agnostic to *C. diffiicile*. Including those reads would confound out conclusions.

5. Line 213-214, lines 276-277, lines 303-305:

“These results demonstrated that each pretreatment lead to distinct susceptible metabolomic environments.”

“Together, our results supported that each susceptible environment was distinguishable from the others, with its own subset of enriched C. difficile growth substrates.”

“This supported the hypothesis that divergent community structures can ultimately share a convergent metabolic output despite changes to community structure and membership.”

The conclusions of this manuscript seem to be in line with their previous paper in mSystems. What is the novelty of this manuscript?

Never before has it been documented that *C. difficile* (let alone any pathogen) alters the activity of the microbiome during colonization differentially between models of susceptibility. The previous publication was about *C. difficile* adapting to its environment, the current study is about how the presence of this pathogen impacts the surrounding community. Again, we have changed language in the text to focus on this novelty (see lines 443 - 449).

One type of analysis that was omitted was the integration of the current data (which is community focused) with the previous data (which is C. difficile focused). Furthermore, a demonstration that the differences in metabolites actually matter for colonization is important but is left out of the story. For example, the authors should be able to manipulate the metabolite pool in mice and show some effect on C. difficile (eg, does feeding clindamycin-specific metabolites to cefoperazone-treated mice result in a reduction of C. difficile burdens?)

The reviewer brings up a great point, but would require repeating all animal experimentation and is therefore outside of the scope of the current study. This point will likely be the subject of future studies. In the current study, we took an alternative approach of using multiple, semi-independent methods, applied to the same experiment to support our claims.

6. Line 708-709: Do the differences in toxin expression and cfus hold up if the authors set “undetectable” levels at the limit of detection versus half the limit of detection? Their assumption that undetectable=0.5x is generous.

The authors apologize for the confusion this caused. Undetectable points were excluded from statistical analysis so any differences we report were calculated without them. This was a remnant from a very early draft and was correctly labeled in the figure 2 legend (see line 880), but was incorrectly labeled in the Statistical Methods section. It has now been corrected (see line 658 - 659).

7. In figure 5A, the authors show the top 5 KEGG pathways with the largest amount of change between Mock and C. difficile infected states within each antibiotic pretreatment. And, in figure 5B-5D, they show the top 10 genes with the largest difference in expression within each indicated antibiotic pretreatment group. The values of each KEGG pathway and each gene in this figure should be shown for all treatment groups analyzed to give a clear picture of whether the genes are uniquely up-regulated in one condition or of whether the genes are upregulated in all conditions. For example, as the figure is presented now, I am tempted to think that oxidative phosphorylation is over-represented exclusively in the streptomycin treatment and that it is unchanged under the other conditions. This ambiguity should be removed for all of the KEGG pathways/genes represented in this figure.

We agree that panel A of figure 5 may not add much to our discussion so we have since removed it entirely. However, we do disagree that showing the same genes in each condition would present a more interpretable figure to the reader. Showing genes with the largest disparity provides a much easier standard of comparison between metatranscriptomes as the pool of species and available genes differs so greatly between conditions.As there are so many genes with large changes in the dataset, with only limited space to present meaningful figures, we felt that our approach gave the best representation to our data and conclusions.

Reviewer #2: (1) While the study design and incorporation of multiple omic platforms is promising and appears to be a massive effort, a major overall issue concerns the analysis performed. Numerous analyses were performed to detect differences in various communities, as well as the specific impact of C.difficile’s presence. The questions these analyses were designed to answer overlapped greatly, testing and re-testing at different levels, and utilizing a different method for each level/platform. This led to a lot of conflicting results that were very difficult to mentally assemble, at least for this reviewer. A few examples of this are listed as follows:

---In overall testing, CDI-infected communities were “only modestly impacted by the presence of C.difficile” (Fig 2), but testing within each antibiotic pretreatment showed C.diff differences in 2 out of 3 groups (Fig S1D-F).

This is the difference between community structure (16S) and metabolome. The reviewer points out 2 separate figures that are displaying distinct datasets as conflicting, which is entirely inappropriate. CDI indeed did not seem to affect the overall abundance of bacterial taxa in the microbiota, however there were large changes in the metabolites in the environment of the cecum. We feel that this point is sufficiently made clear in the text, but have since made additional effort to convey the correct message (see lines 165 - 177).

---The metabolomic profile in C.diff-infected vs. uninfected was not significantly different overall (Fig S3a), but individual testing at the metabolite level identified compounds such as 5-aminovalerate, which were correlated with C.diff infection.

That is correct. When testing for global differences in large datasets, it is not uncommon for significant differences in small subsets of features to be swamped out by negligible differences in the rest of the data. This is the reason we have included machine learning in order to quickly discern those metabolites that do indeed experience significant change in concentration between conditions.

---OTU biomarkers were detected with LEFSE, metabolomics biomarkers were detected with Random Forest feature selection, and metatranscriptomic biomarkers appear to be selected simply based on top 5 highest magnitude of association.

The implementation of feature selection algorithms in this study was to rapidly identify those markers that best distinguish groups. This means that while occasionally they magnitude of change is large, we have particular interest in those signals with the highest consistency of difference between groups. This process is more robust than performing several thousand corrected-pairwise tests and is far more sensitive to differences in features that may not reach a strict cutoff of significance,

---C.diff infection seems to impact OTU community in streptomycin and clindamycin but not cefoperazone (no overall test performed), but in terms of metabolomic structure, it impacts streptomycin and cefoperazone but not clindamycin (overall test performed but negative).

That is also correct. As this seems to be a point of confusion in our discussion of the results, we have reworded this section to be clearer on this point (Lines - ). In addition, the revised manuscript includes the results of the statistical tests supporting these statements (Lines - ).

---No inferential statistics of any kind were performed in the analysis of transcriptomic data, apparently(!).

Because there was a limited amount of bacterial mRNA biomass in the cecum and we needed additional RNA to obtain the transcripts from C. difficile, it was unfortunately necessary to pool the RNA extracts from multiple mice. This precluded our ability to perform inferential statistics with the metatranscriptomic pool. We believe that our statements regarding this analysis are cautious. Furthermore, we used the metatranscriptomic data to support our 16S rRNA gene sequence and metabolomic data where it was possible to obtain replicates and perform inferential statistics. Our observation that the variation in these other data was low gave us confidence that if we had been able to analyze the RNA pools separately the level of variation would have been similarly low.

While the authors pose some valid questions, the approach of repeated overlapping hypothesis testing with inconsistent methodology each time came across in the manuscript as unfocused. In light of this, some of the interpretations of the data seem too liberal, such as concluding that “C.diff manipulates the niche landscape” to stay colonized, when the authors actually seemed to find very few differences relating to C.diff, and instead found vastly more differences due to antibiotics. It would be too easy for a skeptical reader to dismiss their findings/conclusions as spurious and over-reaching, in a multi-omic study where concerns of overfitting and false positivity are exceedingly high. The authors might do very well to revamp their analysis into answering a focused set of questions more rigorously.

The authors disagree with this reviewer’s point. In fact, it is the reason why we implemented these experiments across multiple susceptibility models of CDI and with multiple methods was to buttress claims from each method and to be able to better generalize our results across multiple rather than single antibiotic models. Since we found a similar trend across highly distinct gut environments, and applied the appropriate statistical rigor whenever possible, we maintain that the phenomena which we report here is robust to replication in other settings.

(2) The authors’ finding that clindamycin pretreated mice clear C.difficile infection more quickly is a stark contrast to much of the published literature. In clinical circles, clindamycin is well-known to carry the highest relative risk of CDI compared with all other antibiotics. In mouse models of CDI, clindamycin has been shown to induce a long-lasting abnormal dysbiotic state, often dominated by Enterobacteriaceae, which the authors did seem to find. The authors may want to discuss this further in the manuscript, add additional support for the finding, or reconsider their conclusion.

The reviewer is referring to Buffie et. al. 2012. *Infect. and Immun.* where the authors found that the same clindamycin pretreatment regime utilized here resulted in detectable. C. difficile colonization for at least 28 days. In the current study, clindamycin indeed induced initial susceptibility, but these mice appeared to clear infection during the following week. Our group has published this trend associated with clindamycin in the past (Schubert et al., 2014), and we have never observed long-lasting susceptibility to C. difficile in any of our experiments. We have also observed initial enrichment of Enterobacteriacea, but this community composition does not persist. This is most easily explained by differences in starting communities in the gut microbiota of separate mouse colonies across institutions. In short, collections of distinct species react differently to perturbation and subsequent recovery of colonization..

Reviewer #3: Some experiments are performed using stool, especially longitudinal studies. However other studies are performed on cecum samples. Could the authors provide greater context for how well stool represents colonization and whether other sites such as small or large intestine are also colonized in this mouse model? E.g. at 18 hours post infection, is C.diff in cecum, SI, LI, stool?

The authors apologize for not making this point clearer as the only experiment we performed that included longitudinal sampling of stool was for the preliminary experiment displayed in figure 1B. Everything else was done in cecal content in order to focus the analysis on a biogeography in which *C. difficile* is actively growing. Regarding the reviewer’s following up question, Koenigsknecht et al. (2015) explored this and demonstrated that *C. difficile* is present in all of those mentioned sites at 18 hours post-infection. They went on to show that the detectable *C. difficile* in the cecum is exclusively vegetative cells and were therefore more likely to be undergoing active metabolism as opposed to the larger amounts of spores found in other sites.

Do these antibiotics effect the physiology of the cecum? For some of the AbX cocktails the cecum becomes tremendously enlarged.

This enlarging effect is evident in all 3 antibiotic pretreatment groups described here. The authors are actually not aware of any antibiotic that doesn’t share this effect shortly after administration in mice.

Beyond KEGG pathways, can the authors perform any analysis of the greater number of ORF, function unknown genes which are up or down regulated?? What percent of the data are the authors analyzing? Are 10%, 50% or 90% of the transcripts mapping to a known pathway?

Our analysis included both those genes that mapped to genes from known KEGG pathways as well as those that mapped to genes that are as yet unannotated. This was the advantage of using the shotgun metagenomic data as a reference for the metatranscriptomic data. We found that ~30% of our transcripts mapped to a known pathway. Unannotated genes that recruit transcript during mapping are still informative in the sense that one can compare these abundances between conditions for an overall degree of difference between metatranscriptomes agnostic to function. That being said, we have since included a supplemental figure (figure S6) that addresses this point.

Figure 1C is exactly the same data as Fig 1 in mSystems from the same authors, simply rearranged. Transcriptome and metabolomics data also seems to be used in both papers. Perhaps authors could provide delineation of what data and analysis is shared between the two papers. It seems like the two papers use the same dataset but mSystems paper focuses more on building a metabolic model. I’m struggling because the Abstracts are so similar, especially the first half.

The reviewer is correct that these studies were both derived from the same very large dataset, however the ultimately focus and conclusion of each study are entirely different. The first study focused on the behavior of *C. difficile* during infection, while this current submission assesses the response of the microbiota to pathogen colonization. The reviewer themselves note the difference in focus between the two investigations. The mSystems paper explores the response of the pathogen to its environment, while this study investigates how varied microbiomes respond to infection. This is too broad of a scope for a single publication and together represents the entirety of the first author's dissertation work. Therefore, the authors do not understand how this issue can be considered a major concern. In spite of the confusion, we have made more of an effort in the text to acknowledge the complementarity of the studies.

Part III – Minor Issues: Editorial and Data Presentation Modifications

Reviewer #1: 8. In line with comment #4 above, how were the C. difficile 16S reads detected and removed? Details of this should be included in the methods section.

The authors agree with this comment and have added a portion to this section of methods on this point located along with cDNA normalization. Briefly, it was performed using Bowtie2 with strict mapping parameters against the *C. difficile* str. 630 16S full length gene. Reads that failed to map were carried through to the rest of the analysis.

9. Line 196-198: Does C. difficile discriminate between infected and uninfected animals if it is left in this analysis?

Yes, because when there *C. difficile* DNA and RNA it makes distinguishing groups by any other signal irrelevant. The fact that one can remove all traces of the pathogen and still observe significant changes is the main crux of the study.

10. Line 256: In Table 1 of Nakamura et al. 1982, sucrose is not a growth substrate for 82 C. difficile isolates. Does C. difficile 630 break this rule?

Actually, Nakamura et al. (1982) state that some strains of *C. difficile* are known to ferment sucrose, just not the strains they tested (see first paragraph of Discussion, page 110). Additionally, this ability may have been gained by 630 specifically growth in broth culture during the subsequent 35 years.

11. Lines 347-349: Why is it not possible to isolate the source of changes in metabolism? A more substantive discussion of the limitation of current assays and computational approaches would be helpful.

This is a great point and we have expanded specifically on this topic in the discussion (lines 466-489).

12. Line 569-570: It’s unclear if males and females were mixed in the same cages for the mouse experiments done.paragra

We have added a line to the methods denoting separate gender housing (line 512).

13. Line 599-603: Was a standard curve used?

Yes, the publication we cite (Leslie et al., 2015) in the text to which this reviewer is referring describes the standard curve thoroughly.

14. Line 683: “for a” not “or a”

We have corrected this typo.

15. Line 738-740: Cannot differentiate black from gray stars

We noted this difficulty and have since switched the grey color to bright green.

16. The metabolites that are discussed in the manuscript all have the units of “scaled intensity.” Is it possible to quantify these metabolites (at least relative to each other?). This point is raised because it’s very interesting that the taxonomic groups that were represented as outliers in the normalized metatranscriptomes were rare in the cecal community (discussed in line 489-491 of the manuscript). Are the metabolites that differentiate conditions (inter- or intra- antibiotic treatment) also low abundance?

Unfortunately using the mass-spectrometry techniques presented here, it is not possible to quantify concentrations relative to each other. We do agree that this point is very interesting and have made more effort to address it in the revised manuscript. From what we observed in the data we collected, it seems like the changes in the metabolome are much more pronounced than in the relative abundance of species. This led us to believe that subtle changes in species abundance or activity are amplified at the metabolomic level where they are much more evident. We hope to follow up on this line of research in future studies.

17. Taxa assignment from metatranscriptomes in Fig. 6 do not correspond well with dominant taxa in 16S data presented in Fig. 2. Can the authors explain this apparent discrepancy?

The authors apologize for not making this discussion point clearer, as we also thought it was an interesting feature of the data. We do discuss the biological interpretation of this at length in the discussion section, as well as the normalization strategy to metagenomic coverage in the methods. We have since made this more prominent in the Discussion (lines 429-442) and Abstract (lines 33-37).

Reviewer #2: Minor points:

---Line 113-117: This sentence refers to results from Figure 1B to justify the rationale for performing the experiment in the first place. That logic is circular, this should be re-written.

The panel we reference in these lines are from early experiments where we tracked C. difficile load in stool over time. All of the subsequent data was collected from cecal content, which you need to kill the mouse to obtain. Therefore, the preliminary experiments absolutely fit in where they currently are and demonstrate an important downstream effect of the different pretreatments.

---Line 172: “Fig S2B” should probably be “Fig S1B”

The authors apologize for not catching this error sooner, and we have since fixed this.

---Line 213: “Fig S3” should probably be “Fig 3”

We fixed this error as well.

---Fig S1B-F: Slightly different versions of the data are shown repeatedly here. Fig S1C-F convey little additional data to Fig S1B. It would be more elegant to provide a single overall plot, with an adjacent ANOVA-like table containing all the ANOSIM R and p-values.

The authors respectfully disagree with the reviewer on this point. Displaying all data points on a single ordination is a chronic problem in the field and results in interpretation of edge effects as quantifiable differences between groups.

---Table S3 and S4 are incredibly large…. There are over 8100 rows of data, between the 2 of them. This reviewer made the mistake of trying to print it before realizing its immense size. This is perhaps an easy example of the “information overload” that occurs throughout the manuscript. Unless the authors feel these are somehow important for the reader, would remove or reference a different way.

The complete read abundance tables were included to promote the reproducibility of our study, and we feel that this is an important inclusion for transparency.

Reviewer #3: (No Response)