*Clostridium difficile* infection differentially alters the structure and metabolic activity of distinct intestinal microbiomes to promote sustained colonization

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### Abstract

*Clostridium difficile* has grown to be the most common cause of hospital-acquired infection over the last decade in the United States. A problematic feature of this infection is that initial susceptibility to colonization by the pathogen is closely linked to previous antibiotic therapy. This connection is due to the impact that antibiotics have on the indigenous bacterial community in the gastrointestinal tract which, in a healthy state, possesses colonization resistance to *C. difficile*. However, perturbed communities are susceptible to colonization where the pathogen can subsequently multiply and produce toxin, leading to inflammatory diarrheal disease. Furthermore, certain patients are unable to clear the pathogen and remain persistently colonized. In this study, we explored the effect of *C. difficile* colonization on community-level gene expresion and metabolism using a murine model of antibiotic treatment and infection. We characterized multiple susceptible communities utilizing metagenome-enable metatranscriptomics supplemented by untargeted metabolomic mass-spectrometry across multiple classes of antibiotic treatment to begin to understand the depth of metabolic crosstalk and effect that *C. difficile* has on infected communities of bacteria. Our results demonstrate that the metabolic activity of microbiomes that *C. difficile* is more likely to colonize for longer periods of time are differentially impacted 18 hours post-infection. These changes in activity are reflected in the metabolic environment of the cecum and indicate restructured the nutrient-niche landscape particularly for those involving Stickland fermentation substrates and certain carbohydrates. This work underscores potential mechanisms by which the pathogen alters the ecology of the GI tract to promote persistence.

### Introduction

One of the many beneficial functions provided by the indigenous gut bacterial community is its ability to prevent infection by pathogens [1]. This attribute, termed colonization resistance, is one of the main mechanisms of protection from the gastrointestinal pathogen *Clostridium difficile* [2–4]. *Clostridium difficile* is the etiological agent of *Clostridium difficile* infection (CDI), a toxin-mediated diarrheal disease that has dramatically increased in prevalence over the last 10 years and results in an estimated 453,000 infections with 29,000 deaths in the US annually [5]. Antibiotics are a major risk factor for CDI and are thought to increase susceptibility to CDI as they disrupt the gut bacterial community structure, but it is still unclear what specific changes to the microbiota contribute to this sensitivity [6,7]. Associations between the membership and functional capacity of the microbiota as measured by the metabolic output suggest that antibiotics increase susceptibility by altering nutrient milieu in the gut to one that favors *C. difficile* metabolism [8–10]. An ongoing theory is that *C. difficile* colonization resistance is driven by competition for growth nutrients by an intact community of metabolic specialists and has been born out through animal model experimentation of the past several decades [11–13]. This line of reasoning has been carried through to the downstream restoration of colonization resistance in that, although community structure may not return to its precise original state, the functional/metabolic capacity rebounds and may be able to outcompete *C. difficile* for resources and clear the infection [10,14]. While most classes of antibiotics have been associated with initial susceptibility to CDI, fluoroquinolones, clindamycin, and cephalosporins in particular are linked to increased risk of recurrent or persistent *C. difficile* infection [15–17]. This raises questions about the groups differentially impacted both directly and indirectly by certain treatments and what ultimately means for these environments during infection by permitting long-term colonization.

Leveraging distinct antibiotic treatment regimes in a murine model of CDI [18], we and others previously shown that *C. difficile* adapts it's catabolism to distinct cecal microbiomes that resulted from separate classes of antibiotics [19]. This is supported by the hypothesis that each differentially sensitized gut environment possesses an alternative nutrient niche landscape and *C. difficile* is able to adjust its metabolism accordingly. Although it is well stablished that *C. difficile* is able to colonize these communities effectively, it is yet to be determined whether these differences in the metabolic capacity of communities following antibiotic treatment correlate with prolonged *C. difficile* colonization. Defining the functional status of the resident microbiota for any disease has been difficult and has led to a limited understanding of specific species interactions that occur with *C. difficile* during infection. To address this fundamental question we employed a conventionally-reared murine model of CDI in the context of treatment by distinct antibiotic classes and assessed the effect of CDI on the microbiome utilizing paired metagenomic-enabled metatransciptomics and untargeted metabolomics. This approach allowed us to not only characterize the metabolic output of the community, but also which subgroups of bacteria were differentially active under these conditions. Our data supports that *C. difficile* colonization indeed alters community-level gene expression, and that this degree of change was reflected in the metabolome of these communities. Furthermore, in spite of shifts species abundance, the metatrascriptome and metabolome changed very little in treatment groups that cleared the infection. This work highlights that a better appreciation of the effects of CDI on the gut microbiota may be needed to develop more successful targeted therapies that eliminate *C. difficile* after persistent colonization.

### Results

**Distinct antibiotic treatments are associated with different patterns of clearance following primary infection.** Conventionally-reared SPF mice were treated with one of three different antibiotics to sensitize the animals to *C. difficile* colonization. The selected antibiotics were streptomycin, cefoperazone, and clindamycin (Table S1). Each drug was chosen not only due to its ability to reduce *C. difficile* colonization resistance in a mouse model [18], but also for their distinct and significant impacts on the structure and diversity of the cecal microbiota (all *p* < 0.001; Fig. S1a) as well as differences in ability of the community to recover and clear infection (Fig. 1B). Selection of this toxigenic *C. difficile* strain was based on its moderate clinical severity in mouse models [20], previous studies of *in vitro* metabolism [21], and well-annotated genome [22]. Briefly, mice were treated with the respective antibiotic and were subsequently orally gavaged ~1×103 *C. difficile* str. 630 spores (Fig. 1A). We then monitored for disease over the following 10 days and cultured *C. difficile* from stool to quantify colony forming units (cfu) per gram over time. The day after infection in each antibiotic treatment model, we observed equal high *C. difficile* colonization, however over the following 8 days only clindamycin treated mice cleared the infection (Fig. 1B).

***C. difficile* differentially expresses virulence factors across separate antibiotic treatments.** With the differential clearance results between antibiotic treatments, we hypothesized that each community presented separate metabolic challenges to *C. difficile* which could explain the clearance trends. It had been previously demonstrated that *C. difficile* virulence factor expression is regulated by availability of certain nutrients in the environment [21], so we first sought to measure sporulation and toxin production. Continuing with the same pretreatment and primary infection protocols used in the 10-day colonization experiments, we chose to focus our analysis on 18-hours post-infection to assess behavior of *C. difficile* directly prior to the beginning of clearance. This end point corresponded with a previous study where *C. difficile* reached maximum cecal vegetative cell density with few detectable spores [23]. Moreover, we also elected to take all further measurements from cecal content because it is more likely to be a site of active bacterial metabolism compared to stool. This also allowed for assessment of functional differences in the microbiota were apparent between antibiotic treatments early during infection and would correlate with the downstream clearance phenotypes. At 18 hours after infection, there were no significant differences in the number of vegetative cells between any antibiotic-treatment tested. All susceptible mice were colonized to ~1×108 vegetative cfu per gram of cecal content, while untreated mice maintained *C. difficile* colonization resistance (Fig. 1C). We also measured both sporulation and toxin activity as activation of both processes has been linked to environmental concentrations of specific growth nutrients [21]. Despite having similar amounts of vegetative *C. difficile* cells, varying levels of both spore cfu and toxin titer were observed across each of the antibiotic treatments. These results showed that *C. difficile* colonized different antibiotic-treated mice to consistently high levels, and the distinct treatments corresponded with moderate differences in the expression of *C. difficile* virulence factors.

**Initial *C. difficile* colonization levels were consistent in spite of significantly different community structures.** It has not yet been established whether *C. difficile* colonization impacts the structure of the gut microbiota during infection in susceptible mice. In order to evaluate changes in bacterial population abundances in response to perturbation and *C. difficile* colonization, we sequenced the V4 region of the 16S rRNA gene from the cecal content of both mock and *C. difficile*-infected mice across antibiotic treatment models. To focus out analysis specifically on the surrounding bacterial communities, we ignored all *C. difficile* rRNA gene sequences. We confirmed that each antibiotic treatment significantly impacted both cecal community structure (Bray-Curtis distances) and diversity (inverse-Simpson) compared to untreated control mice (all *p* < 0.001, Fig. S1A & S2B). We then confirmed that each antibiotic treatment induced distinct shifts in the community structure which were significantly different (all *p* < 0.001, Fig. S1C). The composition of streptomycin-treated communities was more variable between cages, but was generally enriched for members of phylum Bacteroidetes (Fig. 2A). Cefoperazone and clindamycin-treated cecal communities were consistently dominated by the families Lactobacillaceae and Enterobacteriaceae respectively (Fig. 2A). Despite variation in the community structures generated by streptomycin treatment, those communities were colonized evenly (Fig. 1B, 1C, & S1).

Next, we measured the effect that *C. difficile* colonization had on overall community structure and composition for each of the antibiotic groups. We found that the structure of both streptomycin and clindamycin-treated infected communities were significantly different from their corresponding uninfected controls (streptomycin *p* = 0.014, Fig. S1D; clindamycin *p* = 0.003, Fig. S1F). Cefoperazone-treated communities did not significantly differ from their control group (Fig. S1E). We then sought to identify specific OTUs that significantly differed in abundance between mock and *C. difficile*-infected communities within each treatment group. Utilizing LEfSe differential abundant feature detection [24], we identified 16 OTUs that discriminated between infected and uninfected communities in at least one antibiotic treatment (Fig. 2B-2D); however, these OTUs were generally near the limit of detection and had an inconsistent abundance profile across pretreatment groups.

**Distinct antibiotic classes lead to alternative markers of *C. difficile* colonization susceptibility.** Several groups have demonstrated that treatment with antibiotics not only alters the structure of the resident microbiota, but also has a dramatic impact of the intestinal metabolome [8–10]. To test the metabolic features of the altered communities, we performed untargeted metabolomic analysis on separate aliquots of the cecal contents that were also utilized in the 16S rRNA gene sequencing. A total of 727 distinct metabolites were identified through a combination of several liquid chromatography and mass spectrometry techniques. First, we characterized the differences between the metabolomes of the mock-infected communities to measure the impact of antibiotic treatment that generated *C. difficile*-susceptible conditions (Fig. 3). All of the antibiotic treatments significantly altered the cecal metabolome compared to untreated, *C. difficile*-resistant mice as quantified by Bray-Curtis dissimilarity (*p* < 0.001; Fig. 3a). When metabolites were mapped to KEGG pathways, it was clear that the differences between resistant and susceptible metabolomes were the result of widespread physiological effects (Fig. S4). Similar to the differences between resistant and susceptible states, the patterns of specific metabolite concentrations diverged depending on the antibiotic treatment (Fig. S5). These results demonstrated that each antibiotic treatment lead to distinct susceptible metabolomic structures.

We were then interested in identifying those metabolites with the highest degree of change between resistant and susceptible groups groups. Due to the large number of individual metabolites with significant differences in relative concentration between groups, we employed a machine learning approach using random forest [25] to highlight those metabolites that most easily differentiated the antibiotic groups. In order to further limit the analysis to only the most informative metabolite, we ranked metabolites in order of highest Mean Decrease Accuracy (the amount in which their removal negatively impacts correct sample classification) and reported the top 7 in each analysis (Fig. 3C). These lists primarily included precursors or intermediates of carbohydrate fermentation such as chiro−inositol, malonate, erythritol, 4−guanidinobutanoate, and soyasaponin II, the majority of which were increased in susceptible conditions. Decreases in these metabolites *in vivo* have previously been associated with a reduction in the normal levels of polysaccharide fermentation present in the intact microbiota [9]. Furthermore, N−methylpipecolate was the only metabolite we identified that consistently decrease during antibiotic treatment. This molecule is byproduct of amino acid catabolism and is typically recycled under normal conditions [26]. These data support that amino acid catabolism is disrupted at a community-level following antibiotic treatment and may suggest open nutrient niches for *C. difficile*, a known fermenter of peptides [27], to colonize. We then applied this technique to identify metabolites that distinguish antibiotic treatment groups from each other(Fig. 3d). Interestingly, several additional carbohydrate or amino acid catabolism byproducts were highlighted by these means including hydroxyisocaproate, methylvalerate, glutamylmethionine, and N−carbamoylaspartate [9]. These data suggested that the populations responsible for normal chains of fermentation may be differentially effected by distinct classes of antibiotics. It also supported that the distinct antibiotics allowed for different forms of nutrient catabolism to survive the treatment and potentially implied alternative profiles of metabolic competition for *C. difficile* to cope with upon colonization. Additionally, sucrose is a *C. difficile* growth substrate [28] which further promoted the hypothesis of differentially vacated nutrient niches due to antibiotic treatment that are now accessible to *C. difficile*. After following this unsupervised approach for identification of susceptibility markers, we also measured differences in relative concentration of metabolites previously connected to potential *C. difficile* colonization susceptibility [10]. In this way, we assessed differences in bile acids, deeply connected to the life cycle of *C. difficile* [29] and whose bioconversion by the microbiota has been implicated as a driver of colonization resistance [30]. We found that there was no persistently increased bile acid across the chosen antibiotic treatments (Fig. S2a). A similar trend was also seen in amino acids (Fig. S2b). This suggested that despite varying efficiencies, the fact that *C. difficile* can recognize a subset of these molecules in any of the observed contexts appears to be sufficient to allow for sufficient germination and outgrowth to occur. For carbohydrates (Fig. S2b), we found that several were significantly increased across all antibiotic groups which included arabitol/xylitol, ribitol, and sucrose. Together, our results supported that each susceptible environment was distinguishable from other groups with its own subset of enriched *C. difficile* growth substrates. This could be an indication that particular competitors were eliminated during antibiotic treatment, or those community members normally responsible for the consumption of these metabolites have altered their metabolic program to exploit alternative nutrient sources.

***C. difficile* colonization alters each susceptible cecal metabolome distinctly.** Following the changes to the cecal metabolome in response to antibiotic treatment, we assessed the degree to which *C. difficile* colonization altered the cecal metabolome of susceptible animals. We hypothesized that the introduction of a new competitor, *C. difficile*, would impact the metabolome either through signatures of its own metabolism or by causing a shift in the metabolism of other members in the surrounding community. First, in a similar approach to identifying susceptibility markers, we observed the global Bray-Curtis dissimilarities of mock-infected and *C. difficile*-infected metabolomes within each antibiotic treatment group separately. First we performed this analysis at a global level and compared cecal metabolomes from all mice across treatment groups, and were unable to detect a consistent difference between groups in this way (*p* = 0.075; Fig. S3a). We moved on to individual antibiotic groups and found that both streptomycin (*p* = 0.039) and cefoperazone (*p* = 0.016) treated metabolomes deviated significantly from that of mock infection (Fig. 4a-4b). However, clindamycin treated cecal metabolomes were not significantly altered by the presence of the pathogen (*p* = 0.127; Fig. 4c). These results diverged from what was seen in the paired OTU relative abundance results where instead the community structure of cefoperazone was unchanged and clindamycin was significantly different (*p* = 0.003; Fig. S4e, S5f, & 5c). Interestingly, streptomycin-treated microbiomes were significantly altered by *C. difficile* infection at both the OTU and metabolomic levels (Fig. S4d & 4a). These data indicated that large shifts of populations in the cecal microbiota was not implicitly associated with concordant shifts in the metabolome. This supported the hypothesis that divergent community structures can ultimately share a convergent metabolic output despite changes to community structure and membership.

We then sought to identify changes those metabolites that were potential markers of *C. difficile* infection. To accomplish this task, we applied the same random forest that was used earlier to differentiate infected and uninfected metabolomes overall and within each treatment group separately. We were able to distinguish those microbiomes infected with *C. difficile* from those that were not, and reported the 10 metabolites with the greatest MDA (Fig. S3b). The strongest single predictor and only metabolite among the top 10 that was increased during infection was 5-aminovalerate. This amino acid analog is a known byproduct of D-proline fermentation in *C. difficile* [31]. *C. difficile* is able to catabolize proline along with glycine through a set of paired biochemical reactions known as Stickland fermentation [32]. Additional prominent signatures of infection across our metabolomic datasets were the significant decreases to the concentration of 4 individual proline-containing amino acids which were each highly abundant in the absence of infection (all *p* < 0.001). These combined results demonstrated that while distinct metabolic challenges may exist in each susceptible metabolome, Stickland fermentation could perhaps be a preferred energy acquisition pathway for *C. difficile* *in vivo*. In agreement with the ordination analysis from Figure 4a-4c, random forest was only able to reliably classify infected mice in streptomycin and cefoperazone treatment while clindamycin maintained a high out-of-bag error (OOB = 44.44%). Metabolites that distinguished infection conditions within each treatment group had a high level of variation with only a few shared metabolites including acetylarginine, dimethylguanine, and adenine however none were in top 5 of all groups. Despite a moderate amount of conserved metabolic signatures across infections these data support our hypothesis that not only does each antibiotic treatment create different microbiota community structures with different metabolic potentials, but also that the metabolism of these distinct communities responds to *C. difficile* colonization in a unique manner. However, it is not possible from these data to distinguish changes to the metabolome that were a result of altered community metabolism, altered host metabolism, or from *C. difficile* directly.

***C. difficile* colonization induced shifts the expression of several catabolic pathways and nutrient acquisition systems across susceptible communities.** Our combined 16S rRNA gene sequencing and metabolomic results demonstrated that antibiotic treatments resulted in distinct bacterial communities which likely led to the altered metabolite profiles in each; however, it does not preclude the possibility that the host or *C. difficile* itself (only during infection) were responsible for the metabolomic differences. We hypothesized that the altered metabolic function of the microbiota as a product of antibiotic perturbation drove the changes seen in the metabolomes of the respective antibiotic treatment groups. To gain a more specific understanding how the microbiota is shaping the metabolic environemtn in each treatment group, we employed a metagenomic-enabled metatranscriptomic shotgun sequencing approach with paired DNA and RNA samples collected from the cecal content of the mice used in the previous analyses. Metagenomic reads from mock-infected cecal communities were assembled into contigs and putative genes were identified resulting in 234,124 (streptomycin), 83,096 (cefoperazone), and 35,977 (clindamycin) potential genes in each metagenome. Streptomycin treatment resulted in a significantly more diverse community (Fig. S1a) than other groups, so a larger detectable metagenome was expected. Putative genes were then annotated according to KEGG and the subset of genes the were successfully annotated with function were utilized for the next analysis. DNA and cDNA reads from both infected and uninfected conditions were then mapped to the gene catalog that corresponded to their antibiotic treatment group of origin. The resulting abundances were normalized to both sequencing read length and target gene length to yield a per base mapping abundance. Finally, after equal subsampling, metatranscriptomic read abundances for each gene were divided by their corresponding metagenomic coverage in order to normalize for overrepresented genes and species. Therefore, final expression values represent the level of expression upregulation for each gene outside of those from which transcript is abundant but is only expressed at low levels from genes which are highly abundant.

Utilizing the fully normalized metatranscriptomes, we first focused on differences in gene expression in broadly defined pathways and gene categories for each antibiotic treatment with and without *C. difficile*-infection. Based on the metabolomic results, we hypothesized that pathways with the greatest differences would include those involved in the metabolism of carbohydrates and amino acids. We then calculated the difference in cDNA abundance for each pathway between infected and uninfected conditions, represented as delta-cDNA abundance. To highlight the largest dfferences, we limited the analysis to the top 5 KEGG pathways with the most change between mock and *C. difficile*-infected conditions within each treatment (Fig. 5a). In streptomycin and clindamycin treatments, greater expression of KEGG pathways was observed in the *C. difficile*-infected metatranscriptomes. Both groups displayed large changes in amino sugar metabolism and ABC transporters, however other distinct carbon metabolism pathways were upregulated in each. Glycolysis and oxidative phosphorylation were overrepresented in streptomycin treated mice while starch/sucrose metabolism and PTS systems were more abundant associated with clindamycin treated mice. Together these shifts suggest that communities differentially adapt carbon metabolism pathways in response to colonization of *C. difficile*. Conversely, the largest differences seen in cefoperazone treated mice were over-expressed in the absence of *C. difficile* infection. These pathways included three separate pathways for the replication or manipulation of genetic material (RNA Processing, tRNA Sythesis, & Homologous Recombination). Instead, many genes involved in anaerobic glucose metabolism and select ABC-transporters were upregulated (Table S3). These results indicate that the cecal microbiota of infected mice shifts its metabolism toward catabolizing simple carbohydrates. We then moved on to perform a more fine-scale analysis of changes at the gene level, by selecting the genes in each antibiotic treatment group with the largest disparity in normalized cDNA abundance between mock and *C. difficile*-infected groups (Fig. 5B-5D). In agreement with pathway-level differences in expression, the majority of genes with the greatest difference between mock and infected mice belonged to pathways highlighted in Fig. 5A with three additional pathways relating to amino acid metabolism (I: Glutathione metabolism, J: Valine/Leucine/Isoleucine metabolism, & K: Glycine/Serine/Threonine metabolism). Numerous genes for transport of simple carbohydrates and glycolysis were also differentially overrepresented under infected conditions across treatment groups when compared to mock infection of untreated, resistant communities (Fig S6). These data expand on our interpretation of pathway-level analysis and support our hypothesis that *C. difficile* colonization leads to changes in community-level expression of genes for nutrient acquisition and catabolism.

***C. difficile* colonization corresponds with large-scale changes in expression of genes from specific bacterial taxa** Because not all bacterial taxa share identical metabolic capabilities, we hypothesized that specific subsets of bacteria were differentially affected by the presence or metabolic activity of *C. difficile*. We sought to delineate the transcriptomic contributions of separate bacterial taxa within each metatranscriptomic dataset. To accomplish this we utilized the genus level taxonomic information associated with each KEGG annotation to identify which group likely contributed a given gene to the metagenome. Many genes in the KEGG database are annotated as hypothetical or uncharacterized but still possess a taxonomic annotation. This resulted in substantially more genes from the total being conserved for analysis in each group. With these data, we narrowed the focus onto transcription for genera that represented >0.01% of genes receiving taxonomic annotations in any of the metagenomic assemblies. We then directly compared the normalized cDNA abundances for each gene between infected and uninfected states for each antibiotic treatment (Fig. 3a-3c). Coordinates were determined by the relative expression of each gene in mock and *C. difficile* infection for x and y axes respectively. This causes genes with equal transcription in both conditions being compared to be strongly correlated and positioned proximal to the central diagonal line. As such, we applied linear correlation and a squared residual cutoff to define those genes that are most strongly upregulated in either condition, and finally calculated the mean distance of outliers in each group from the center line (represented in arbitrary units or AU). This resulted in 2473 outliers at an average distance of 2.545 AU associated with streptomycin, 2930 outliers at an average distance 3.854 AU with cefoperazone, and only 727 outliers at an average distance of 2.414 AU in clindamycin treatment. Overall, the clindamycin treatment was associated with the fewest gene outliers of expression that were a shorter distance from even expression in uninfected and infection conditions than either of the other antibiotic groups.

Incorporation of the genes-level taxonomic information for each transcript revealed that outlier genes came were contributed by underrepresented genera. First, in streptomycin-treated mice, the most prominent differences were in 937 genes belonging to *Lactobacillus* that were upregulated with *C. difficile* infection (Fig. 6a). Next, in cefoperazone treatment 2290 genes belonging to *Bacteroides* were upregulated in mock infected mice (Fig. 6b). A consistent trend in streptomycin and cefoperazone treatments was an overrepresentation of highly expressed genes from genera belonging to Bacteroidetes during mock infection. The metatransciptomes within both of these treatment conditions poorly correlated between mock and infected conditions, indicating a high degree of change induced by *C. difficile* colonization (*r* = 0.0334 & *r* = 0.031). Finally, in clindamycin treated mice the largest difference in transcription was for 510 *Lactobacillus* genes during *C. difficile* infected mice (Fig. 6c). Infected and uninfected metatranscriptomes associated with this antibiotic correlated the more strongly than either other treatment (*r* = 0.862), further supporting that *C. difficile* colonization had a low impact on transcription of the cecal microbiota. TThese data support that *C. difficile* may differentially modify the transcriptional activity of separate microbial taxa based on the context of the community in which it is colonizing. This could have implications in altering the ecosystem of the gut to promote persistence and ultimately negatively affect the ability of the community to clear infection.

### Discussion

Our results demonstrate that distinct intestinal ecosystems are impacted differently by *C. difficile* colonization and that these changes to community metabolism could have implications for the ability of the pathogen to persist in those environments. Furthermore, discordant modifications to OTU abundances, transcriptional activity of the microbiota, and cecal metabolome shortly after infection suggest that *C. difficile* manipulates the niche landscape of the intestinal tract in order to better maintain long-term colonization. This hypothesis is best supported by the disparity of community-level phenotypes between clindamycin and cefoperazone/streptomycin treatment models. Only in the clindamyin treatment model were animals able to go on to clear detectable *C. difficile* colonization within 18 hours. Unlike the other conditions, clindamycin-treated communities were significantly altered in community structure following *C. difficile* colonization, but not at the metabolomic level (Fig. S1f & 4a). This disconnect between community structure differences and overall metabolic output was explained by the low levels of change in the metatranscriptomes of these communities when compared to the other treatment groups in response to infection (Fig. 4d & 5c). Collectively these results advance the idea that in order for *C. difficile* to maintain colonization for longer periods of time, it must partition desired niche spaces in the context of a given microbiome, and that the activities of certain subsets may be more readily reshaped than others. Instances of active nutrient niche restructuring in the gut have been documented previously for prominent symbiotic bacterial species in gnotobiotic mice [33], but never before in a model of infection with a normal community of microbes. Interestingly, taxonomic groups most highly represented as outliers in the normalized metatranscriptomes of each antibiotic treatments were non-dominant species of each respective cecal community by 16S rRNA gene sequencing (on average <5% of community; Fig. 2a & 6). These data give the impression that *C. difficile* may "attack the loser", meaning those populations more targeted by the antibiotic treatment and in the midst of recovery, in order to have the highest probability of success in the gut environment it is currently colonizing. Previous studies have found that rare taxonomic groups, even those at a low abundance as a result of a spontaneous perturbation, may have disproportion effects on the metabolic environment of the community at large [34]. For example, this strategy has been observed in temperate lakes where conditionally rare microbes were found to be far more metabolically active than highly abundant taxa [35], and this concept would likely apply to bacterial groups recovering population density following cessation of antibiotic treatment. As such, *C. difficile* may preferentially seek to compete with these organisms to ultimately affect greater change to the entire ecosystem and open a long-lasting nutrient niche. While this hypothesis requires further exploration to adequately support, it provides an ecological framework for future research questions concerning the interactions of *C. difficile* with susceptible communities in the gut.

This study is one of the first *in vivo* observations of a medically relevant bacterial pathogen altering metabolic activity of a host-associated community to potentially promote colonization. Another group had previously identified potential metabolite markers of *C. difficile* infection in patient feces [36], but were not able to make connections to changes to community metabolism that were afforded to us by paired untargeted metabolomic analyasis and metatranscriptomic sequencing. In a recent study, one group found that a tick-vectored bacterial pathogen alters the ability of the resident microbiota of the tick by interrupting proper biofilm formation and allowing lasting colonization [37]. In both cases the pathogen modifies aspects of the microbiota it is colonizing, however in the case of *C. difficile* the interaction appears to more centered on access to nutrients than a persistent spatial niche. While we acknowledge that this study may not elucidate the specific mechanism by which this interaction occurs, the combined systems analysis strengthens each individual level of observation and only when employed together does a clearer definition of *C. difficile*-related microbial ecology in the gut emerge. This research lays the groundwork for a more rationale consideration of the metabolic functionalities of bacterial taxa to consider when attempting to rebuild *C. difficile* colonization resistance across differentially perturbed gut environments.

Several groups have attempted to identify single bacterial species or limited strain consortia that are able to replicate this effect, but each has been met with incomplete restoration of colonization resistance or function through yet unexplored means [30,38–40]. The effect we observed of *C. difficile* colonization on community metabolic activity could also be linked to pathogen strain and may offer explanation to the propensity of some strains to persist over others where toxin activity could play a role [41]. Moreover, the current work contributes to the existing concept that the healthy gut microbiota maintains colonization resistance to *C. difficile* by outcompeting the pathogen for preferred nutrient niche space. Ultimately, our results suggest that each susceptible and subsequently infected microbiome may be unique and require specific microbes or functionalities to restore colonization resistance to *C. difficile* in that specific context. Conversely, colonization resistance against *C. difficile* may be the result of contributions by distinct subcommunities of bacteria across each unique resistant gut community. As the microbiome is so intimately connected to colonization resistance against the bacterium, it has become imperative to understand what factors allow some gut environments to be persistently colonized while others are not. This research lays the groundwork for future studies to assess context dependent restoration of *C. difficile* colonization resistance and what factors are able to interfere with the ability of *C. difficile* to modify gut ecology in order to promote clearance.

### Materials and Methods

**Animal care and antibiotic administration.** For a more detailed description of the procedure, refer to Jenior et al. (2017) [19]. In short, even number of male and female conventionally-reared six-to-eight week-old C57BL/6 mice in each experimental group were administered one of three antibiotics; cefoperazone, streptomycin, or clindamycin (As described in Table S1) before oral *C. difficile* infection. All animal protocols were approved by the University Committee on Use and Care of Animals at the University of Michigan and carried out in accordance with the approved guidelines.

***C. difficile* infection and necropsy.** For a more detailed description of the procedure, refer to [19]. On the day of challenge, 1×103 *C. difficile* spores were administered to mice via oral gavage in phosphate-buffered saline (PBS) vehicle. Mock-infected animals were given an oral gavage of 100 μl PBS at the same time as those mice administered *C. difficile* spores. 18 hours following infection, mice were euthanized by CO2 asphyxiation and necropsied to obtain the cecal contents. Aliquots were immediately flash frozen for later DNA extraction and toxin titer analysis. A third aliquot was transferred to an anaerobic chamber for quantification of *C. difficile* abundance. The remaining content in the ceca was mixed in a stainless steel mortar housed in a dry ice and ethanol bath. Cecal contents from all mice within each treatment group were pooled into the mortar prior to grinding to a fine powder. The ground content was then stored at -80° C for subsequent RNA extraction. For 10-day colonization studies, fresh stool was collected from infected mice each day beginning on the day of infection. Mice were monitored for overt signs of disease and were euthanized after the final stool collection.

***C. difficile* cultivation and quantification.** For a more detailed description of the procedure, refer to [19]. Briefly, cecal samples were weighed and serially diluted under anaerobic conditions with anaerobic PBS. Differential plating was performed to quantify both *C. difficile* spores and vegetative cells by plating diluted samples on CCFAE plates (fructose agar plus cycloserine, cefoxitin, and erythromycin) at 37° C for 24 hours under anaerobic conditions [42]. In parallel, undiluted samples were heated at 60° C for 30 minutes to eliminate vegetative cells and leave only spores [29]. These samples were serially diluted under anaerobic conditions in anaerobic PBS and plated on CCFAE with taurocholate at 37° C for 24 hours. Plating was simultaneously done for heated samples on CCFAE to ensure all vegetative cells had been eliminated. CFU quantification for 10-day colonization experiments was performed from stoll using TCCFAE to measure total *C. difficile* load in these animals over time.

***C. difficile* toxin titer assay.** To quantify the titer of toxin in the cecum a Vero cell rounding assay was performed as described elsewhere [19,43]. Briefly, filtered-sterilized cecal content was serially diluted in PBS and added to Vero cells in a 96-well plate. Plates were viewed after 24 hour incubation for cell rounding.

**DNA/RNA extraction and sequencing library preparation.** DNA for downstream shotgun metagenomic and 16S rRNA gene sequencing was extracted from approximately 50 mg of cecal content from each mouse using the PowerSoil-htp 96 Well Soil DNA isolation kit (MO BIO Laboratories) and an epMotion 5075 automated pipetting system (Eppendorf). The V4 region of the bacterial 16S rRNA gene was amplified using custom barcoded primers [44]. Equal molar rations of raw isolated DNA within each treatment group were then pooled and ~2.5 ng of material was used to generate shotgun libraries with a modified 10-cycle Nextera XT genomic library construction protocol (Illumina). This was done to mimic the pooling strategy necessary for metatranscriptomic library preparation. Final libraries were pooled at equal molar ratios and stored at -20° C. For RNA extraction, a more detailed description of the procedure can be found in [19]. Briefly, immediately before RNA extraction, 3 ml of lysis buffer (2% SDS, 16 mM EDTA and 200 mM NaCl) contained in a 50 ml polypropylene conical tube was heated for 5 minutes in a boiling water bath [45]. The hot lysis buffer was added to the frozen and ground cecal content. The mixture was boiled with periodic vortexing for another 5 minutes. After boiling, an equal volume of 37° C acid phenol/chloroform was added to the cecal content lysate and incubated at 37° C for 10 minutes with periodic vortexing. The mixture was the centrifuged at 2,500 x g at 4° C for 15 minutes. The aqueous phase was then transferred to a sterile tube and an equal volume of acid phenol/chloroform was added. This mixture was vortexed and centrifuged at 2,500 x g at 4° for 5 minutes. The process was repeated until aqueous phase was clear. The last extraction was performed with chloroform/isoamyl alcohol to remove acid phenol. An equal volume of isopropanol was added and the extracted nucleic acid was incubated overnight at -20° C. The following day the sample was centrifuged at 12000 x g at 4° C for 45 minutes. The pellet was washed with 0° C 100% ethanol and resuspended in 200 μl of RNase-free water. Following the manufacturer's protocol, samples were then treated with 2 μl of Turbo DNase for 30 minutes at 37° C. RNA samples were retrieved using the Zymo Quick-RNA MiniPrep according the manufacturer's protocol. The Ribo-Zero Gold, immediately before RNA extraction, 3 ml of lysis buffer (2% SDS, 16 mM EDTA and 200 mM NaCl) contained in a 50 ml polypropylene conical tube was heated for 5 minRNA Removal Kit Epidemiology was then used to deplete prokaryotic and eukaryotic rRNA from the samples according the manufacturer's protocol. Stranded RNA-Seq libraries were made constructed with the TruSeq Total RNA Library Preparation Kit v2, both using the manufacturer's protocol. Average length of amplicon libraries for both DNA and cDNA sequencing was performed using an Agilent BioAnalyzer with High Sensativity DNA Analysis kits. Completed libraries were pooled in equal molar ratios within their respective groups and stored at -20° C until time of sequencing.

**High-throughput sequencing and raw read curation.** Sequencing of 16S rRNA gene amplicon libraries was performed using an Illumina MiSeq sequencer as described previously [44]. The 16S rRNA gene sequences were curated using the mothur software package (v1.36) as described in [19]. Shotgun metagenomic sequencing was performed in 2 phases. Libraries from mock-infected communities, that were also to be utilized for contig assembly, were sequenced using an Illumina HiSeq 2500 on 2x250 paired-end settings and was repeated across 2 lanes to normalize for inter-run variation. *C. difficile*-infected metagenomic libraries were sequenced with an Illumina NextSeq 300 with 2x150 settings across 2 runs to also normalize for inter-run variation. These efforts resulted in an average of 280 million paired raw reads per sample. Metatranscriptomic sequencing was performed on an Illumina HiSeq 2500 with 2x50 settings and was repeated across 4 lanes for normalization and to obtain necessary coverage [46]. This gave an average of 380 million raw cDNA per library. Both metagenomic and metatranscriptomic sequencing was performed at the University of Michigan Sequencing Core. Raw sequencing read curation for both metagenomic and metatranscriptomic datasets was performed in a two step process. Residual 5’ and 3’ Illumina adapter sequences were trimmed using CutAdapt [47] on a per library basis. Reads were quality trimmed using Sickle [48] with a quality cutoff of Q30. This resulted in approximately 270 million reads per library (both paired and orphaned) for both metagenomic and metatranscriptomic sequencing. Actual read abundances for individual metagenomic and metatranscriptomic sequencing efforts can be found in Table S2.

**Metagenomic contig assembly and gene annotation.** Metagenomic contigs were assembled using Megahit [49] with the following settings; minimum kmer size of 87, maximum kmer size of 127, and a kmer step size of 10. Progigal was utilized to to identify putative gene sequences, and were screen for a minimum length of 250 nucleotides. These sequences were translated to amino acids and peptides were annotated against the KEGG protein database [50] using Diamond implementation of BLASTp [51]. Peptide-level gene annotations were assigned to the corresponding nucleotide sequence, and genes failing to find a match in KEGG were preserved as unannotated genes. Final nucleotide fasta files with KEGG annotations were then utilized in the construction of Bowtie2 [52] mapping databases from downstream analyses.

**DNA/cDNA read mapping and normalization.** Mapping was accomplished using Bowtie2 [52] and the default stringent settings. Optical and PCR duplicates were then removed using Picard MarkDuplicates (<http://roadinstitute.github.io/picard/>). The remaining mappings were converted to idxstats format using Samtools [53] and the read counts per gene were tabulated. Discordant pair mappings were discarded and counts were then normalized to read length and gene length to give a per base report of gene coverage. Unless indicated otherwise, each collection of reads was then 1000-fold iteratively subsampled to 90% of the lowest sequence total within each analysis, and a median expression value for each gene was calculated.

**Quantification of *in vivo* metabolite relative concentrations.** For a more detailed description of the procedure, refer to Jenior et al. (2017) [19]. Metabolomic analysis performed by Metabolon (Durham, NC), a brief description of their methods is as follows. All methods utilized a Waters ACQUITY ultra-performance liquid chromatography (UPLC) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer at 35,000 mass resolution. Samples were dried then reconstituted in solvents compatible to each of the four methods. The first, in acidic positive conditions using a C18 column (Waters UPLC BEH C18-2.1x100 mm, 1.7 µm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). The second method was identical to the first but was chromatographically optimized for more hydrophobic compounds. The third approach utilized a basic negative ion optimized conditions using a separate dedicated C18 column. Basic extracts were gradient eluted from the column using methanol and water, however with 6.5mM Ammonium Bicarbonate at pH 8. Samples were then analyzed via negative ionization following elution from a hydrophilic interaction chromatography column (Waters UPLC BEH Amide 2.1x150 mm, 1.7 µm) using a gradient consisting of water and acetonitrile with 10mM Ammonium Formate, pH 10.8. The MS analysis alternated between MS and data-dependent MS n scans using dynamic exclusion. The scan range varied slighted between methods but covered 70-1000 m/z. Library matches for each compound were checked for each sample and corrected if necessary. Peaks were quantified using area under the curve.

**Statistical methods.** All statistical analyses were performed using R (v.3.2.0). Significant differences between community structure of treatment groups from 16S rRNA gene sequencing were determined with AMOVA in the mothur software package. Significant differences of Inv. Simpson diversity, cfu, toxin titer, and metabolite concentrations were determined by Wilcoxon signed-rank test with Benjamini-Hochberg correction. Undetectable points used half the limit of detection for cfu and toxin statistical calculations. LEfSe analysis with OTU data was performed with a cutoff that each OTU must appear in all samples from their respective groups to be considered true signal (n=9). Random forest was performed using the implementation in R [54], with the informative threshold of MDAs greater than the abolute value of the lowest MDA defined by [55]. Distances of outlier points from center line during metatranscriptomic comparisons was accomplished using 2-dimensional linear geometry.

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### Figure Legends

**Figure 1 | Experimental models of *C. difficile* infection and distinct virulence patterns.** **(a)** Experimental timelines of antibiotic treatment and infection mouse models. **(b)** *C. difficile* 630 CFU in stool of infected mice following each antibiotic treatment regime over 10 days of infection. Median and interquartile range are shown for each time point. **(c)** Quantification of *C. difficile* cfu and toxin titer in cecal content across antibiotic treatment models ater 18 hours of infection. Black lines indicate median values. Gray asterisks indicate significant difference from no antibiotic controls by Wilcoxon rank-sum test with the Benjamini-Hochberg correction (all *p* < 0.001).

**Figure 2 | Impact of *C. difficile* colonization on other bacterial populations abundances in the gut microbiota.** **(a)** Relative abundance of family-level taxonomic classification for OTUs in each treatment group. **(b-d)** Discriminating OTUs with LEfSe analysis between Mock and *C. difficile*-infected communities within each treatment group, *p*-values from LEfSe are shown on the left with taxonomic information (all *p* < 0.05). Relative abundance of the respective OTUs from each mouse along with medians are shown for each treatment group.

**Figure 3 | Metabolite markers of *C. difficile* infection susceptibility.** Results from UPLC/MS metabolomic analysis of cecal content from mice also used in previous analyses. Only mock-infected metabolomic results were included this analysis to identify markers of susceptibility. **(a-b)** NMDS ordinations of Bray-Curtis distances comparing the cecal metabolome dissimilarity of mice receiving no treatment or one of the three distinct classes of antibiotics. (a) Metabolomes of resistant mice are significantly different from antibiotic treated animals (*p* < 0.001). (b) Antibiotic treated metabolomes and also significantly distinct from one another (*p* < 0.001). Significant differences for NMDS analyses were calculated with AMOVA. **(c-d)** Scaled intensities of metabolites with highest mean decrease in accuracy (MDA) from random forest feature selection discriminating groups from (a-b). MDA is labeled in brackets beside each metabolite name and out-of-bag error from internal cross-validation is labeling along the bottom axis. Asterisks along the right axis indicate significant difference by Wilcoxon rank-sum test. (c) Relative concentrations of metabolites that distinguish cecal content from mice resistant to *C. difficile* colonization from susceptible animals. (d) Metabolites concentrations for those molecules that are able to differentiate antibiotic treatment groups. Multiple comparisons were accounted for using the Benjamini-Hochberg correction (\*\*\* < 0.001, \*\* <= 0.01, \* <= 0.05).

**Figure 4 | *C. difficile* infection has differential effects on the cecal metabolome.** **(a-c)** NMDS ordinations of Bray-Curtis distances comparing infection groups within each antibiotic treatment; (a) Streptomycin, (b) Cefoperazone, and (c) Clindamycin. Significant differences were determined by AMOVA. **(d-f)** Relative concentrations of metabolites with highest Mean Decrease Accuracy (MDA) from Random Forest feature selection to discriminate between Mock and *C. difficile*-infected conditions within each antibiotic treatment. Respective MDA is labeled in brackets next to each metabolite name and out of bag error from internal cross-validation of each model is labeled under the plotting area. (d) Streptomycin treatment, (e) Cefoperazone treatment, and (f) Clindamycin treatment. Significant differences are labeled along the right axis and were calculated with Wilcoxon rank-sum test with Benjamini-Hochberg correction (\*\*\* < 0.001, \*\* <= 0.01, \* <= 0.05).

**Figure 5 | *C. difficile* infection alters community-level select pathway and gene expression of the gut microbiota across perturbed communities** Metatranscriptomic (cDNA) read abundances associated with each gene were normalized to their associated metaganomic (DNA) coverage, resulting in values that reflect upregulation. **(a)** Differences in read abundance for top 5 KEGG pathways with the largest amount of change between Mock and *C. difficile*-infected states within each antibiotic treatment. Values above the center line were expressed more during *C. difficile* infection, and those below the line were expressed more in Mock-infected animals. Dotted lines indicate average expression of pathways associated with each condition. **(b-d)** cDNA read abundances of the top 10 genes with the largest differences in expression within each indicated antibiotic treatment group. Shown are the expression levels for the genes displayed on the left during Mock (white) or *C. difficile* (black) infection. Gene names and member pathways indicated by letter codes along the left axis (pathway legend below).

**Figure 6 | *C. difficile* colonization alters gene expression of taxonomic groups differentially between antibiotic treatments.** Each point represents a unique gene from the respective metagenomic assembly. Coordinates were determined by the log2-transformed expression level of each gene between *C. difficile*-infected and mock-infected conditions. Metatranscriptomic read abundances were normalized to their associated metagenomic coverage. Colored indicate genus of origin, and gray areas denote genes with consistent expression between conditions and outliers to this region were determined by least squares regression analysis with a minimum residual value of 2. Antibiotic treatments; **(a)** Streptomycin-treated, **(b)** Cefoperazone-treated, and **(c)** Clindamycin-treated.

**Supplementary Figure 1 | Impact of antibiotic treatment and *C. difficile* infection on cecal community structure** **(a)** Inverse-Simpson diversity of cecal communities from all treatment groups. Gray stars indicate significant difference from no antibiotic controls (all *p* < 0.001). Black stars denote within treatment group significant difference between mock and *C. difficile*-infected communities. Differences were calculated using Wilcoxon rank-sum test with Benjamini-Hochberg correction. NMDS ordinations of Bray-Curtis distances comparing the groups labeled in the bottom left of each plotting area. **(b)** Antibiotic-treated compared to Untreated controls and **(c)** comparison between only antibiotic treatment groups. **(d-f)** Within antibiotic treatment comparisons for the effect of *C. difficile* colonization on community structure. Significant differences and correlation coefficients for ordination analyses were found using ANOSIM.

**Supplementary Figure 2 | Relative concentrations of select metabolite groups in each group** Metabolites included in this analysis were chosen based on their previously published links to *C. difficile* physiology or susceptibility to infection. Groups are as follows; **(a)** Bile acids, **(b)** Carbohydrates, and **(c)** Amino Acids. Significant differences were determined by Wilcoxon rank-sum test with Benjamini-Hochberg correction.

**Supplementary Figure 3 | Effect of infection on the cecal metabolome across treatment groups** Pooled analysis of antibiotic treated animals only. **(a)** NMDS ordination of Bray-Curtis distances differentiating mock and *C. difficile*-infected metabolomes (*p* = 0.075). **(b)** Random forest classification results for metabolites that effectively distinguish infected and uninfected conditions. Shown are relative concentrations of the top 10 metabolites with the highest mean decrease in accuracy from internal cross-validation. Shown in the top right corner of each panel are the metabolite names and mean decrease accuracy of each. Significant differences in concentration between mock and *C. difficile*-infected groups were determined by Wilcoxon rank-sum test with Benjamini-Hochberg correction.

**Supplementary Figure 4 | Heatmap comparing Resistant and Susceptible mice for all measured metabolites** Relative concentrations of metabolites in each animal across all expremerimental groups. Sample names are listed along the right side, and *C. difficile* susceptibility is along the left. Hierarchical clustering was perormed for each KEGG compound category included in the untargeted metabolomic analysis separately and are listed in order under the figure panel.

**Supplementary Figure 5 | Gene-level expression for each community compared to the level expressed in resistant mice for each gene** Murine cecal metatransctipromic sequencing results. Both reads and genes atributed to *C. difficile* were removed prior to analysis to focus on the changes in the community caused by infection. Shown are genes in each groups with the largest disparity from a metatranscriptome from a community resistant to *C. difficile* colonization. Panels are displayed as follows; Streptomycin treatment: **(a)** mock infection, **(b)** *C. difficile* infection. Cefoperazone treatment: **(c)** mock infection, **(d)** *C. difficile* infection. Clindamycin treatment: **(e)** mock infection, **(f)** *C. difficile* infection.

**Supplementary Table 1 | Chosen antibiotic treatment regimes** Antibiotic classes, mechanisms, and dosage information for each treatment.

**Supplementary Table 2 | High-throughput sequencing read counts and metagenomic assembly quality** Raw and curated read abundances for both metagenomic and metatranscriptomic sequencing efforts. Raw read curation steps are outlined in Materials & Methods. Metagenomic contig summary statistics reflect the quality of assembly for each group.

**Supplementary Table 3 | Normalized cDNA abundances for each antibiotic treated group** Final cDNA read abundances for each gene from the corresponding metagenomic assembly. Normalization reflects standardizing cDNA read abundances to both read length and target gene length as well as metagenomic coverage for each gene (also normalized).

**Supplementary Table 4 | Median scaled intensity of metabolites for each antibiotic treatment and infection group**

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