The Effect of Gut Microbiota Variation on *Clostridioides difficile* Infection

by

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“Injustice anywhere is a threat to justice everywhere.”

— Martin Luther King Jr., Letter from the Birmingham Jail

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Dedication

For Camilla, my family, you are capable of anything.

Stay curious and inquisitive.

For the lives that contributed to this body of work.

I am grateful for their contribution to the advancement of scientific knowledge. I acknowledge the whole person, not a sample, data point, or person with CDI, but an individual with personalities, memories, relationships and loved ones.

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Abstract

The bacteria, viruses, fungi, archaea, and protozoa, collectively known as microbiota, present throughout our intestine prevent the pathogenic bacteria *Clostridioides difficile* from infecting. This protection known as colonization resistance is lost when the gut microbiota is perturbed by antibiotics. This disruption opens a niche for *C. difficile* to colonize. *C. difficile* is removed with antibiotic treatment but that also leaves niches open for *C. difficile* to reinfect. There have been efforts to develop bacterial consortia to eliminate *C. difficile* and resolve the open niches. However, a few recent studies have revealed differences in *C. difficile* colonization associated with different perturbations. I hypothesized that, due to the variation in the gut microbiota and susceptibility to *C. difficile* infection (CDI), the gut microbiota has perturbation-specific effects on CDI. To test this hypothesis, I challenged mice with diverse gut microbiota, via either human fecal inoculation of germ-free mice or antibiotic treatments of conventional mice, to test whether microbiota variation affected disease severity, clearance of *C. difficile* colonization, or restoration of colonization resistance. In Chapter II, I varied the gut microbiota by colonizing germ-free mice with human fecal communities prior to *C. difficile* challenge. We then observed the effect of the gut microbiota variation on the resultant disease. These data showed that variation in the bacterial community associated with variation in moribundity and cecal tissue histopathology. In Chapter III, I varied the gut microbiota by treating to conventional mice from the same colony, containing a homogenous gut communities, with either clindamycin, cefoperazone, or streptomycin. I varied the antibiotic dose to permit *C. difficile* colonization and subsequent natural clearance to test whether the communities had similar changes in the gut

bacterial community associated elimination of *C. difficile*. This set of experiments showed that the bacterial community undergoes antibiotic-specific changes concurrent with the elimination of *C. difficile*. In Chapter IV, I created variation in the gut microbiota with the three antibiotics previously used then tested whether a fecal community transplant (FCT) can restore colonization resistance. Mice treated with clindamycin became colonized and then cleared *C. difficile,* regardless if they received PBS or a FCT. Cefoperazone-treated mice were able to clear *C. difficile* colonization only when they received a FCT. Streptomycin-treated mice were able to resist colonization with a diluted FCT. These experiments showed the effectiveness of a simplified community, such as a diluted FCT, was specific to the antibiotic perturbation. My results show that the niche *C. difficile* occupies affects disease manifestation, the community changes associated with eliminating *C. difficile* colonization, and the bacteria necessary to fill the available niches to prevent colonization. These studies show that to prevent CDI we need to better understand the range in available niches in susceptible communities that *C. difficile* can occupy and the requirements to fill the available niches.

Chapter I

Introduction

1.1 Gut Microbiota Provides Colonization Resistance

The bacteria, viruses, fungi, archaea, and protozoa that colonize the human intestinal tract are known as the gut microbiota. Early microbiology was focused on the negative interactions between microbes and human health. However, the pathogenic microbes make up only a small portion of the microbes we come into contact with. The approximately 10^{11} bacterial cells/ml of the gut microbiome in the colon provide many critical benefits to the human host (1). Some of the benefits include development of the intestinal villi and gut-associated lymphoid tissue, reduced intestinal epithelial cell permeability, and vascularization of the gut (2). There are also functions of the gut microbiota that in response the host reciprocates benefits to the microbiota. The intestinal mucus layer, consisting of an inner and an outer layer, promotes transport through the gastrointestinal (GI) tract and protects against bacterial invasion (3). It also functions as a habitat and nutrient source for specific bacteria. The bacteria and their byproducts also affect the thickness and composition of the mucus layer, as germ-free mice have a thinner mucus layer and increased disease activity (4). This reciprocal relationship between the gut microbiota and the mucus layer occurs with the immune system as well. The gut bacteria promote the secretion of IgA into the intestinal lumen. There the IgA binds microbes and prevents them from translocating and infecting the host (5). In addition to nutrients gained from the mucus layer, the

gut microbiota can obtain other nutrients passing through the GI tract which are inaccessible to the host. The expanded metabolic capabilities of the gut bacteria enable them to consume the inaccessible polysaccharides and supply the host with essential nutrients, short-chain fatty acids (SCFAs), and vitamins (6). The gut microbiota provide many different benefits to the host, and many of them contribute to preventing pathogenic bacteria from infecting the gut.

The protection against infection by an unperturbed indigenous gut microbiota is known as colonization resistance. The gut bacteria have many mechanisms, in addition to the ones already described, to prevent microbes from infecting the gut. Bacteria produce antimicrobial products. They can ferment indigestible carbohydrates into SCFAs, such as butyrate, which can affect intracellular pH and metabolism, impairing growth (7). Bile acid metabolism also contributes to colonization resistance. Primary bile acids are released into the intestine to aid digestion of lipids. The gut microbiota can deconguate or dehydroxylate the primary bile acids into secondary bile acids, which can be bactericidal (8, 9). Bacteria can also produce bacteriocins, which are small toxic peptides that inhibit growth of other species (10). Another mechanism of colonization resistance is nutrient competition. Gut bacteria consume key nutrients required by pathogenic bacteria, impairing growth (11, 12). Lastly, bacteriophages of the gut microbiota can exclude specific species of bacteria from the gut. All of these biotic and abiotic factors together produce an environment lacking a niche for *C. difficile* growth and persistence.

1.2 Clostridioides difficile Infection

C. difficile, a Gram-positive, anaerobic, spore-forming bacterium, causes nearly half a million infections per year in the United States (13). *C. difficile* infection (CDI) is the most common hospital-acquired infection. Patients can experience varied disease, from naturally resolving diarrhea to pseudomembranous colitis and even death (14). Pathogenesis is primarily

driven by the glycotransferase activities of toxins TcdA and TcdB. These toxins disrupt actin regulation and intestinal epithelial cell tight junctions (15). Some strains of *C. difficile*, such as the epidemic NAP1/027/BI strain, produce an additional toxin, binary toxin. However, individual *C. difficile* strain isolates can vary in the genome, growth rate, sporulation, germination, and toxin production (16). The primary treatment for CDI is antibiotics which is also the primary risk factor of CDI. Vancomycin, fidaxomicin, or metronidazole are recommended to treat CDI, dependent on cost, availability, disease severity, and recurrence (18). These antibiotic treatments, as well as any other antibiotic treatment, increases the risk of CDI, and 15% to 30% of patients will become reinfected (19). A fecal microbiota transplant (FMT) may be necessary to eliminate CDI for patients with multiple recurrences or infections recalcitrant to antibiotics. While we have improved our capabilities of defining the composition of the gut microbiota, we are still missing information about its association with long-term disease and unknown pathogenic microbes. As such, FMT carries potential unknown long-term risks as well as short-term risks, potentially fatal (20). The relationship between CDI, risks, and treatments that modify the gut microbiota reveals the critical role it plays in CDI.

1.3 Interaction of Microbiota and *C. difficile*

When the gut microbiota becomes perturbed with antibiotics, *C. difficile* is able to colonize. Antibiotics are necessary and sufficient in animal models of CDI to overcome colonization resistance (21). *C. difficile* spores require primary bile acids in conjunction with either amino acids or divalent cations to germinate in the intestine (22). The gut microbiota converts available primary bile acids into secondary bile acids, preventing germination. However, when a perturbance eliminates bacteria that can deconguate or dehydroxylate bile acids, primary bile acids are allowed to accumulate and trigger *C. difficile* spores to germinate

into metabolically active cells (23). Once active, *C. difficile* needs to access a niche which will permit its growth and persistence. A balance of bile acids is necessary since deoxycholate, a secondary bile acid, can either inhibit growth or promote biofilm formation depending on its concentration. Nutrients must be sufficient and accessible for *C. difficile* to colonize. *C. difficile* is a generalist and can adapt to different nutrient niches, typically consuming amino acids and carbohydrates (24, 25). Also it can access nutrients via cross-feeding, such as the sialic acid released from mucosal glycoconjugates by *Bacteroides thetaiotaomicron* (26). Once established in the gut, *C. difficile* modifies the intestinal environment to eliminate other microbes and increases available niches. It has been shown to induce indole production by *Escherichia coli* which can inhibit the growth of many anaerobic gut bacteria (27). *C. difficile* can also directly inhibit Gram-negative bacteria of *Bacteroidadeae* and *Enterobacteriaceae* via fermenting tyrosine to *para-cresol* (28). While colonized, *C. difficile* secretes toxins TcdA and TcdB in response to resources becoming limited. Deoxycholate, proline, cysteine, butanol, and glucose have the ability to suppress toxin expression, whereas, butyrate can increase expression (9, 29–31). From germination through to toxin production, the gut microbiota and the available ecological niche affects the activity of *C. difficile*.

Specific bacterial members of the gut microbiota have been associated with inhibiting *C. difficile*. Murine experiments and human studies help us to better understand which community members are involved in inhibiting *C. difficile* and preventing CDI. Each host and their gut microbiota can have a different response to each antibiotic (32, 33). This variation can allow us to identify the conserved community members associated with CDI. A murine model using a five-antibiotic cocktail plus a clindamycin intraperitoneal injection revealed a loss of *Lachnospiraceae* and an increased population of *Enterobacteriaceae* associated with CDI (34).

Subsequently, mice monocolonized with a *Lachnospiraceae* isolate had reduced *C. difficile* colonization and disease compared to germ-free and *E. coli* monocolonized mice (35). Buffie *et al.* modeled gut microbiota changes in mice given either clindamycin, ampicilin, or enrofloxacin and challenged with *C. difficile* in addition to the microbiota of people undergoing allogeneic hematopoeietic stem cell transplantation receiving antibiotics which became infected with *C. difficile* (36). They identified *Clostridium scindens*, a bile acid 7-dehydroxylating and antibiotic-producing intestinal bacterium, which has since been shown to reduce *C. difficile* colonization (36–38). Although, *C. scindens* has also been detected in patients with CDI and may even assist *C. difficile* via biofilm formation (39, 40). This single bacterium cannot prevent CDI on its own. The only single-species shown to be effective at preventing CDI is *C. difficile* itself. When a non-toxigenic strain of *C. difficile* is precolonized, it prevents a lethal infection by a toxigenic strain via eliminating the co-germinant glycine (12). However, this protection is dependent on the non-toxigenic strain being significantly more abundant when the toxigenic strain infects and may not be sufficient once the toxigenic strain has already germinated. Therefore, individual species of bacteria may be able to inhibit *C. difficile,* but, aside from *C. difficile,* none have been able to prevent or eliminate CDI.

Therefore, another approach to inhibit CDI has been to identify a consortium of bacteria. In a human study comparing people with either non-CDI diarrhea, CDI, or no diarrhea, bacteria related to *Bacteroides*, *Lachnospiraceae*, *Porphyromonadaceae*, and *Ruminococcaceae* were least abundant in people with CDI and most abundant in people without diarrhea (41). When comparing the effect of FMT on CDI, *Bacteroidiaceae*, *Lachnospiraceae* and *Ruminococcaceae* were less abundant and *Enterococcaceae*, *Streptococcaceae* and *Veillonellaceae* were more abundant in the samples prior to FMT, relative to donor and post-FMT samples (42). Mouse

models have been used to find simplified communities to assist in recovering the lost bacterial populations and filling available niches of susceptible gut communities. In a recent top-down approach, Auchtung *et al.* began with human fecal communities, reduced the communities through dilution, and then screened them for the ability to inhibit *C. difficile* colonization in a humanized mouse model (43). This led to the identification of a simplified community that was able to maintain inhibition of *C. difficile*. For a bottom-up approach, Lawley *et al.* developed a consortium of six bacteria, *Anaerostipes*, *Bacteroidetes*, *Enterococcus*, *Enterorhabdus*, *Lactobacillus*, and *Staphylococcus*, cultured from feces that could restore colonization resistance to a mouse model of persistent CDI (44). While these consortia are promising to restore colonization resistance, it is unclear if they are applicable beyond the model in which they were developed in.

Variation in the gut microbiota may reduce the utility of these bacterial consortia. Often we have found bacteria that inhibit *C. difficile* using an individual mouse model with a homogeneous microbiome or have identified common bacteria across heterogeneous communities. However, this generalization could mask or ignore specificity in the available niches of different community susceptibilities. Ghimire *et al.* isolated 66 speices from the gut microbiota which inhibited *C. difficile* growth in co-culture (45). When culturing multiple inhibitory isolates with *C. difficile*, the level of inhibition changed and some cases had a positive effect on *C. difficile* growth. We can create different communities by treating mice with different types of antibiotics and *C. difficile* can colonize each to similar levels (33). Similar to the features identified in the human studies (41, 42), colonization was positively associated with *Escherichia* and *Streptococcus* populations and negatively associated with *Lachnospiraceae* and *Porphyromonadaceae* populations but were dependent on the other community members and

their abundances. When investigating three of these antibiotics further, *C. difficile* colonized different niches and had antibiotic-specific transcript expression for diverse nutrients (25). To investigate if this variation in the gut microbiota can affect *C. difficile* colonization, Tomkovich *et al.* challenged mice from different vendors and mouse colonies with *C. difficile* (46). These mice were all colonized one day post infection but eliminated the colonization at different rates based on the source of the mice. Therefore, the variation in the gut microbiota and available niches of a susceptible gut microbiota affects *C. difficile* colonization but it is unclear what the effect is on CDI disease and resolution of the infection.

1.4 Direction of Thesis Work

The gut microbiota protects the host from invading pathogens until it becomes perturbed. When antibiotics eliminate the colonization resistance, *C. difficile* is able to colonize exposed niches and infect the host. In order to resolve the infection, the gut microbiota needs to be restored and eliminate the available niches. We have established many of the mechanisms involved in pathogenesis and colonization resistance of CDI. But we do not have a clear understanding of how these mechanisms interact and vary within the gut microbiota to prevent CDI. Often missing from CDI experiments and studies is accounting for the gut microbiota variation.

In Chapter II, we tested whether variation in the gut microbiota affects CDI disease severity. We colonized germ-free mice with human fecal communities to generate a diverse set of gut microbiota. Once the community colonized the gut, the mice were challenged with *C. difficile*. Interestingly, CDI resulted in a range of disease severities and grouped by the fecal community they received. These data show that the composition of the gut microbiota can potentiate the disease caused by *C. difficile*.

In Chapter III, we tested whether variation in the gut microbiota affects community changes associated with CDI elimination. We used three different antibiotics and a titration of dosages to make mice susceptible to *C. difficile*. The range in antibiotic dosages allowed us to observe cases which *C. difficile* could colonize (> 10^6 CFU) and then spontaneously reduce *C. difficile* below detection. We associated changes in gut bacterial groups with the clearance of *C. difficile* for each antibiotic treatment. These data show that gut microbiota has interactions associated with eliminating *C. difficile* infection specific to the antibiotic that induced susceptibility.

In Chapter IV, we tested whether variation in the gut microbiota affects the requirements to restore colonization resistance. We treated mice with one of three antibiotics and then inoculated them with either a fecal community or PBS. Then we challenged the mice with *C. difficile*. Each antibiotic treatment resulted in a different response to the fecal community transplant. Only one antibiotic-treatment, streptomycin, had colonization resistance restored with the fecal community as well as a diluted fecal community. These data show that the effectiveness of a fecal community transplant to enable the gut microbiota to inhibit *C. difficile* colonization was dependent on the antibiotic treatment.

Variation in the gut microbiota affects CDI. *C. difficile* colonizes different niches of differently perturbed gut communities. In order to treat and prevent CDI, we need a better understanding of the range of niches in susceptible communities that *C. difficile* can colonize and the necessary bacteria to fill them. If we treat the susceptiblity common across all communities, *C. difficile* will have the potential to colonize another available niche specific to the infected community, prolonging the infection. Once we know the niches *C. difficile* can colonize, we can identify the bacteria to eliminate them. As we improve our understanding of the characteristics of

susceptible communities, it may be possible to identify people who are susceptible to CDI and supplement their gut microbiota to fill the available niches and restore its colonization resistance.

Chapter II

The Gut Bacterial Community Potentiates *Clostridioides difficile* Infection Severity.

2.1 Notes

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2.2 Abstract

The severity of *Clostridioides difficile* infections (CDI) has increased over the last few decades. Patient age, white blood cell count, creatinine levels as well as *C. difficile* ribotype and toxin genes have been associated with disease severity. However, it is unclear whether there is an

association between members of the gut microbiota and disease severity. The gut microbiota is known to interact with *C. difficile* during infection. Perturbations to the gut microbiota are necessary for *C. difficile* to colonize the gut. The gut microbiota can inhibit *C. difficile* colonization through bile acid metabolism, nutrient consumption and bacteriocin production. Here we sought to investigate whether members of the gut bacterial communities can also contribute to disease severity. We derived diverse gut communities by colonizing germ-free mice with different human fecal communities. The mice were then infected with a single *C. difficile* ribotype 027 clinical isolate which resulted in moribundity and histopathologic differences. The variation in severity was associated with the human fecal community that the mice received. Generally, bacterial populations with pathogenic potential, such as *Escherichia*, *Helicobacter*, and *Klebsiella*, were associated with more severe outcomes. Bacterial groups associated with fiber degradation, bile acid metabolism and lantibiotic production, such as *Anaerostipes* and *Coprobacillus*, were associated with less severe outcomes. These data indicate that, in addition to differences in hosts and strains of *C. difficile*, populations of gut bacteria can influence CDI disease severity.

2.3 Importance

Clostridioides difficile colonization can be asymptomatic or develop into an infection, ranging in severity from mild diarrhea to toxic megacolon, sepsis, and death. Models that predict severity and guide treatment decisions are based on clinical factors and *C. difficile* characteristics. Although the gut microbiome plays a role in protecting against CDI, its effect on CDI disease severity is unclear and has not been incorporated into disease severity models. We demonstrated that variation in the microbiome of mice colonized with human feces yielded a range of disease outcomes. These results revealed groups of bacteria associated with both severe

and mild *C. difficile* infection outcomes. Gut bacterial community data from patients with CDI could improve our ability to identify patients at risk of developing more severe disease and improve interventions which target *C. difficile* and the gut bacteria to reduce host damage.

2.4 Introduction

Clostridioides difficile infections (CDI) have increased in incidence and severity since *C. difficile* was first identified as the cause of antibiotic-associated pseudomembranous colitis (47). CDI disease severity can range from mild diarrhea to toxic megacolon and death. The Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA) guidelines define severe CDI in terms of a white blood cell count greater than 15,000 cells/mm^3 and/or a serum creatinine greater than 1.5 mg/dL. Patients who develop shock or hypotension, ileus, or toxic megacolon are considered to have fulminant CDI (18). Since these measures are CDI outcomes, they have limited ability to predict risk of severe CDI when the infection is first detected. Schemes have been developed to score a patient's risk for severe CDI outcomes based on clinical factors but have not been robust for broad application (48). Thus, we have limited ability to prevent patients from developing severe CDI.

Missing from CDI severity prediction models are the effects of the indigenous gut bacteria. *C. difficile* interacts with the gut community in many ways. The indigenous bacteria of a healthy intestinal community provide a protective barrier preventing *C. difficile* from infecting the gut. A range of mechanisms can disrupt this barrier, including antibiotics, medications, or dietary changes, and lead to increased susceptibility to CDI (15, 49, 50). Once *C. difficile* overcomes the protective barrier and colonizes the intestine, the indigenous bacteria can either promote or inhibit *C. difficile* through producing molecules or modifying the environment (23,

25). Bile acids metabolized by the gut bacteria can inhibit *C. difficile* growth and affect toxin production (9, 51). Bacteria in the gut also can compete more directly with *C. difficile* through antibiotic production or nutrient consumption (12, 38, 52). While the relationship between the gut bacteria and *C. difficile* has been established, the effect the gut bacteria can have on CDI disease severity is unclear.

Recent studies have demonstrated that mice with diverse microbial communities when challenged with a high-toxigenic *C. difficile* strain had varied disease severity (53), and when challenged with a low-toxigenic strain specific members of the gut microbial community associated with variation in colonization (46). Here, we sought to further elucidate the relationship between members of the gut bacterial community and CDI disease severity when challenged with a high-toxigenic strain, *C. difficile* ribotype 027 (RT027). We hypothesized that since specific groups of gut bacteria affect the metabolism of *C. difficile* and its infection dynamics, we can also identify groups of bacteria that affect the disease severity of the infection. To test this hypothesis, we colonized germ-free C57BL/6 mice with human fecal samples to create varied gut communities. We then challenged the mice with *C. difficile* RT027 and followed the mice for the development of severe outcomes of moribundity and histopathologic cecal tissue damage. Since the murine host and *C. difficile* isolate were the same and only the gut community varied, the variation in disease severity we observed was attributable to the gut microbiome.

2.5 Results

***C. difficile* is able to infect germ-free mice colonized with human fecal microbial communities without antibiotics.** To produce gut microbiomes with greater variation than those

found in conventional mouse colonies, we colonized germ-free mice with bacteria from human feces (54). We inoculated germ-free C57BL/6 mice with homogenized feces from each of 15 human fecal samples via oral gavage. These human fecal samples were selected because they represented diverse community structures based on community clustering (41). The gut communities were allowed to equilibrate for two weeks post-inoculation (55). We then surveyed the bacterial members of the gut communities by 16S rRNA gene sequencing of murine fecal pellets (Figure 2.1A). The bacterial communities from each mouse grouped more closely to those communities from mice that received the same human fecal donor community than to the mice who received a different human fecal donor community (Figure 2.1B). The communities were primarily composed of populations of *Clostridia*, *Bacteroidia*, *Erysipelotrichia*, *Bacilli*, and *Gammaproteobacteria*. However, the gut bacterial communities of each donor group of mice harbored unique relative abundance distributions of the shared bacterial classes.

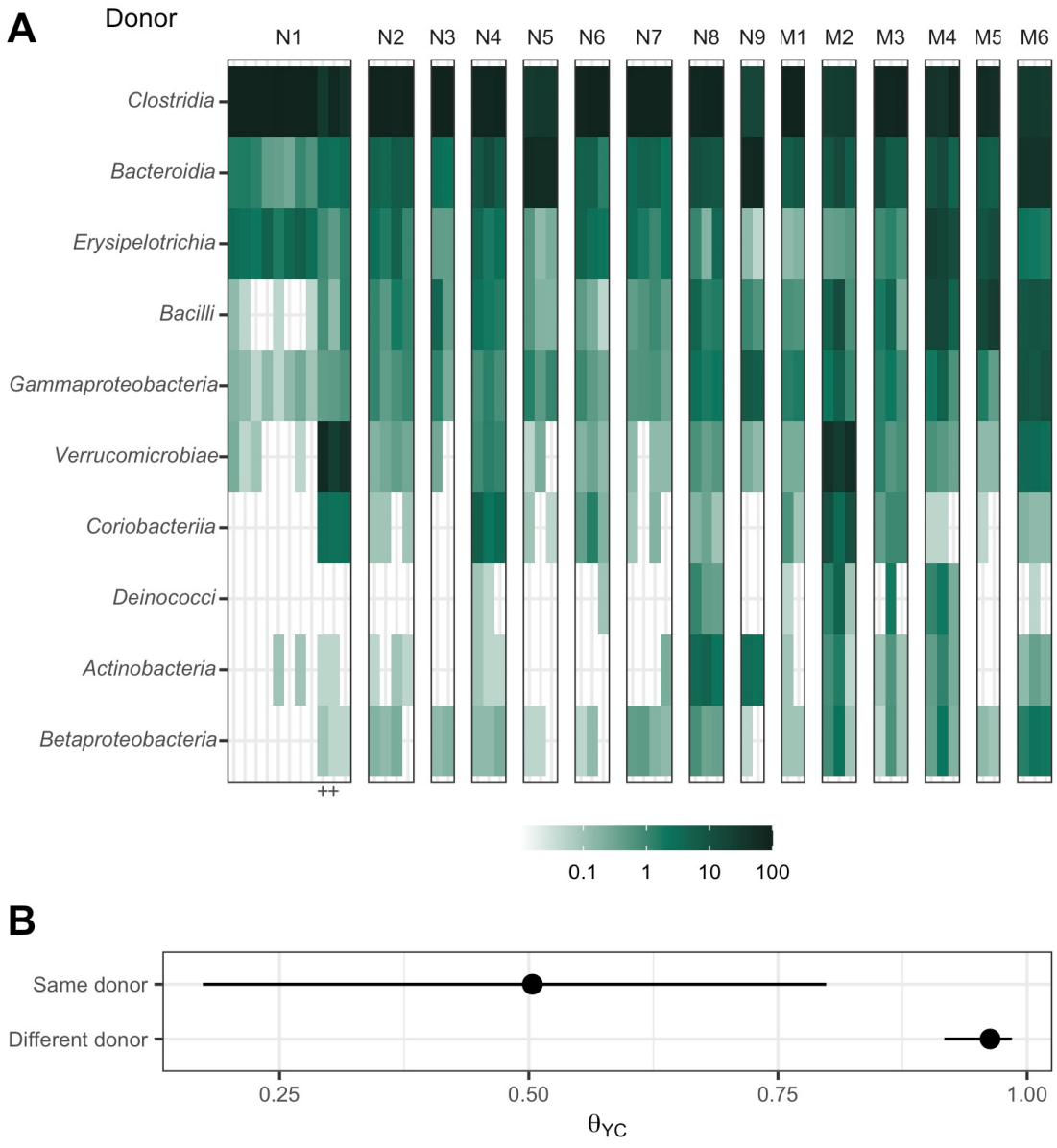


Figure 2.1. Human fecal microbial communities established diverse gut bacterial communities in germ-free mice.

(A) Relative abundances of the 10 most abundant bacterial classes observed in the feces of previously germ-free C57Bl/6 mice 14 days post-colonization with human fecal samples (i.e., day 0 relative to *C. difficile* challenge). Each column of abundances represents an individual mouse. Mice that received the same donor feces are grouped together and labeled above with a letter (N for non-moribund mice and M for moribund mice) and number (ordered by mean histopathologic score of the donor group). + indicates the mice which did not have detectable *C. difficile* CFU (Figure 2.2). (B) Median (points) and interquartile range (lines) of β-diversity (θ_{YC}) between an individual mouse and either all others which were inoculated with feces from the same donor or from a different donor. The β-diversity among the same donor comparison

group was significantly less than the β-diversity of the different donor group ($P < 0.05$, calculated by Wilcoxon rank sum test).

Next, we tested this set of mice with their human-derived gut microbial communities for susceptibility to *C. difficile* infection. A typical mouse model of CDI requires pre-treatment of conventional mice with antibiotics, such as clindamycin, to become susceptible to *C. difficile* colonization (21, 33). However, we wanted to avoid modifying the gut communities with an antibiotic to maintain their unique microbial compositions and ecological relationships. Since some of these communities came from people at increased risk of CDI, such as recent hospitalization or antibiotic use (41), we tested whether *C. difficile* was able to infect these mice without an antibiotic perturbation. We hypothesized that *C. difficile* would be able to colonize the mice who received their gut communities from a donor with a perturbed community. Mice were challenged with 10^3 *C. difficile* RT027 clinical isolate spores. The mice were followed for 10 days post-challenge, and their stool was collected and plated for *C. difficile* colony forming units (CFU) to determine the extent of the infection. Surprisingly, communities from all donors were able to be colonized (Figure 2.2). Two mice were able to resist *C. difficile* colonization, both received their community donor N1, which may be attributed to experimental variation since this group also had more mice. By colonizing germ-free mice with different human fecal communities, we were able to generate diverse gut communities in mice, which were susceptible to *C. difficile* infection without further modification of the gut community.

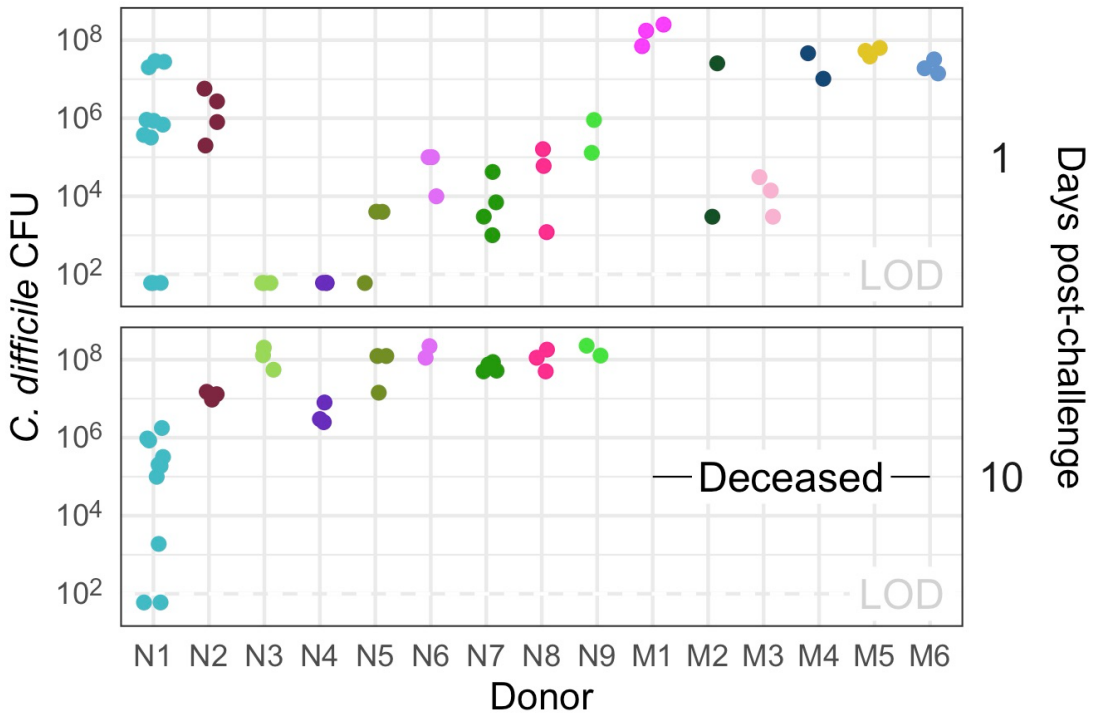


Figure 2.2. All donor groups resulted in *C. difficile* infection but with different outcomes.

C. difficile CFU per gram of stool was measured the day after challenge with 10^3 *C. difficile* RT027 clinical isolate 431 spores and at the end of the experiment, 10 days post-challenge. Each point represents an individual mouse. Mice are grouped by donor and labeled by the donor letter (N for non-moribund mice and M for moribund mice) and number (ordered by mean histopathologic score of the donor group). Points are colored by donor group. Mice from donor groups N1 through N6 succumbed to the infection prior to day 10 and were not plated on day 10 post-challenge. LOD = Limit of detection.

Infection severity varies by initial community. After we challenged the mice with *C. difficile*, we investigated the outcome from the infection and its relationship to the initial community. We followed the mice for 10 days post-challenge for colonization density, toxin production, and mortality. Seven mice, from Donors N1, N3, N4, and N5, were not colonized at detectable levels on the day after *C. difficile* challenge but were infected ($>10^6$) by the end of the experiment. All mice that received their community from Donor M1 through M6 succumbed to the infection and became moribund within 3 days post-challenge. The remaining mice, except the uninfected Donor N1 mice, maintained *C. difficile* infection through the end of the experiment (Figure 2.2). At 10 days post-challenge, or earlier for the moribund mice, mice were euthanised and fecal material was assayed for toxin activity and cecal tissue was collected and scored for histopathologic signs of disease (Figure 2.3).

Overall, there was greater toxin activity detected in the stool of the moribund mice ($P = 0.003$). However, when looking at each group of mice, we observed a range in toxin activity for both the moribund and non-moribund mice (Figure 2.3A). Non-moribund mice from Donors N2 and N5 through N9 had comparable toxin activity as the moribund mice. Additionally, not all moribund mice had toxin activity detected in their stool.

Next, we examined the cecal tissue for histopathologic damage. Moribund mice had high levels of epithelial damage, tissue edema, and inflammation (Figure 2.4) similar to previously reported histopathologic findings for *C. difficile* RT027 (56). As observed with toxin activity, the moribund mice had higher histopathologic scores than the non-moribund mice ($P < 0.001$). However, unlike the toxin activity, all moribund mice had consistently high histopathologic summary scores (Figure 2.3B). The non-moribund mice, Donor groups N1 through N9, had a range in tissue damage from none detected to similar levels as the moribund mice, which

grouped by community donor. Together, the toxin activity, histopathologic score, and moribundity showed variation across the donor groups but were largely consistent within each donor group.

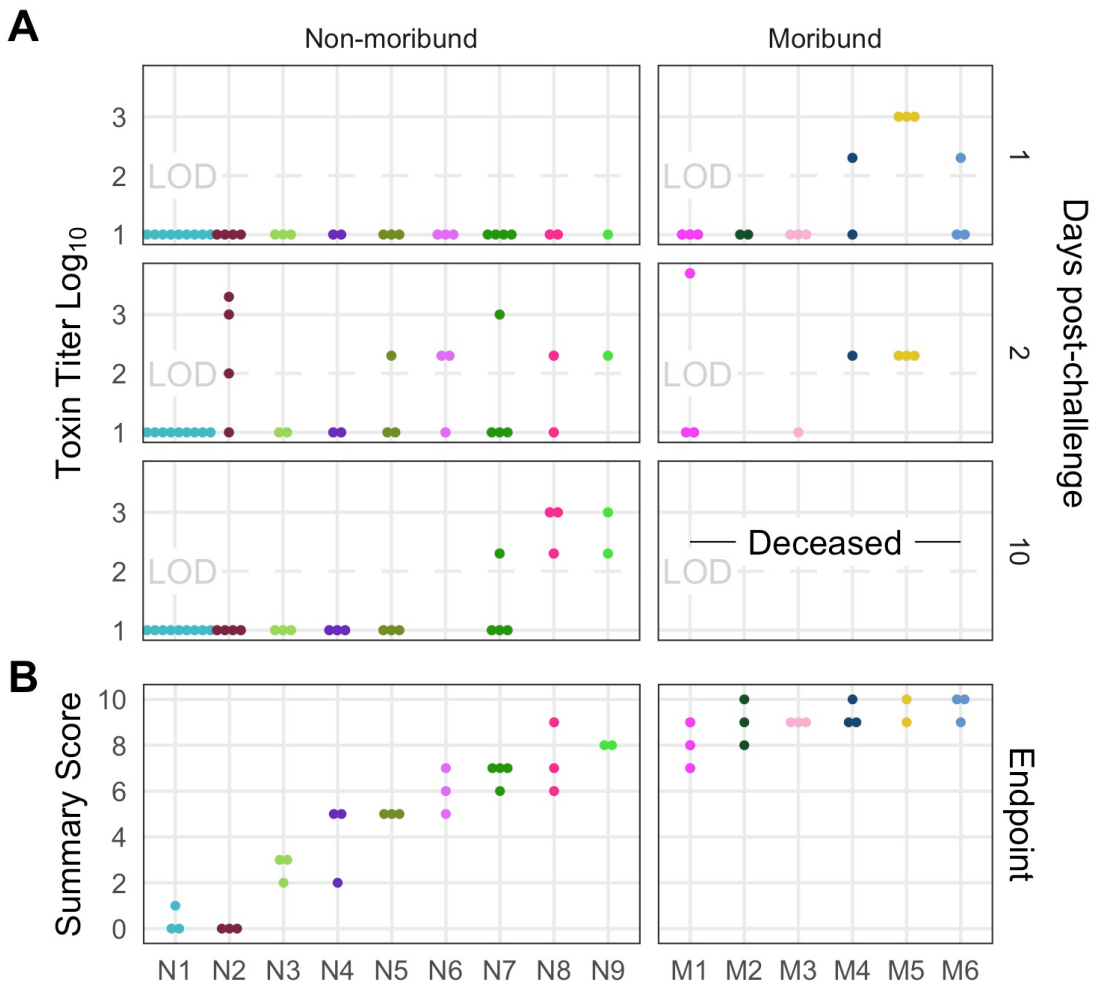


Figure 2.3. Histopathologic score and toxin activity varied across donor groups.

(A) Fecal toxin activity was detected in some mice post *C. difficile* challenge in both moribund and non-moribund mice. (B) Cecum scored for histopathologic damage from mice at the end of the experiment. Samples were collected for histopathologic scoring on day 10 post-challenge for non-moribund mice or the day the mouse succumbed to the infection for the moribund group (day 2 or 3 post-challenge). Each point represents an individual mouse. Mice are grouped by donor and labeled by the donor letter (N for non-moribund mice and M for moribund mice) and number (ordered by mean histopathologic score of the donor group). Points are colored by donor group. Mice in group N1 that have a summary score of 0 are the mice which did not have detectable *C. difficile* CFU (Figure 2.2). Missing points are from mice that had insufficient fecal sample collected for assaying toxin or cecum for histopathologic scoring. LOD = Limit of detection.

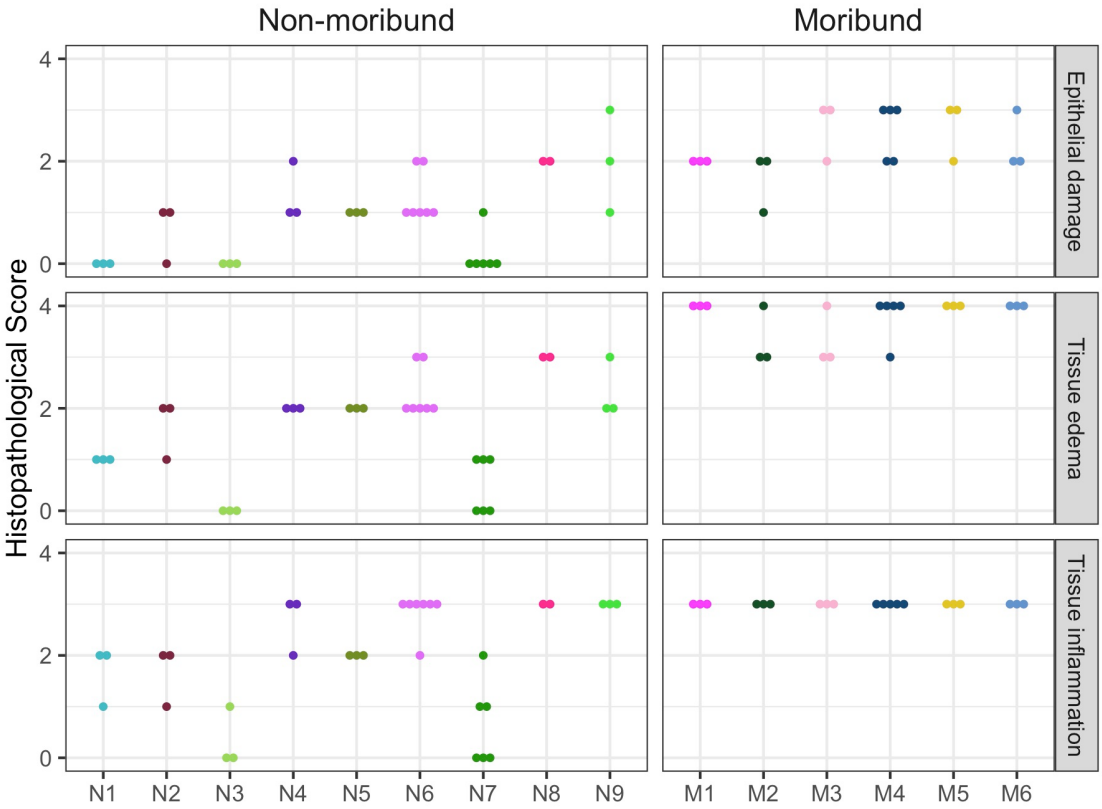


Figure 2.4. Histopathologic score of tissue damage at the endpoint of the infection.

Tissue collected at the endpoint, either day 10 post-challenge (Non-moribund) or day mice succumbed to infection (Moribund), were scored from histopathologic damage. Each point represents an individual mouse. Mice (points) are grouped and colored by their human fecal community donor. Missing points are from mice that had insufficient sample for histopathologic scoring.

Microbial community members explain variation in CDI severity. We next interrogated the bacterial communities at the time of *C. difficile* challenge (day 0) for their relationship to infection outcomes using linear discriminant analysis (LDA) effect size (LEfSe) analysis to identify individual bacterial populations that could explain the variation in disease severity. We split the mice into groups by severity level based on their moribundity and histopathologic score. We dichotomized the histopathologic scores into high and low groups by splitting on the median score of 5. This analysis revealed 20 genera that were significantly different by the disease severity (Figure 2.5A). Bacterial genera *Turicibacter*, *Streptococcus*, *Staphylococcus*, *Pseudomonas*, *Phocaeicola*, *Parabacteroides*, *Bacteroides*, and *Escherichia/Shigella* were detected at higher relative abundances in the mice that became moribund. Populations of *Anaerotignum*, *Coprobacillus*, *Enterocloster*, and *Murimonas* were more abundant in the non-moribund mice that would develop only low intestinal injury. To understand the role of toxin activity in disease severity, we applied LEfSe to identify the genera most likely to explain the differences between the presence and absence of detected toxin activity (Figure 2.5B). Many genera that associated with the presence of toxin were also associated with moribundity, such as populations of *Escherichia/Shigella* and *Bacteroides*. Likewise, there were genera such as *Anaerotignum*, *Enterocloster*, and *Murimonas* that were associated with no detected toxin that also exhibited greater relative abundance in communities from non-moribund mice with a low histopathologic score. Lastly, we tested for correlations between the endpoint relative abundances of bacterial operational taxonomic units (OTUs) and the histopathologic summary score (Figure 2.5C). The endpoint relative abundance of *Bacteroides* was positively correlated with histopathologic score, as its day 0 relative abundance did with disease severity (Figure 2.5A). Populations of *Klebsiella* and *Prevotellaceae* were positively correlated with the

histopathologic score and were increased in the group of mice with detectable toxin. This analysis identified bacterial genera that were associated with the variation in moribundity, histopathologic score, and toxin.

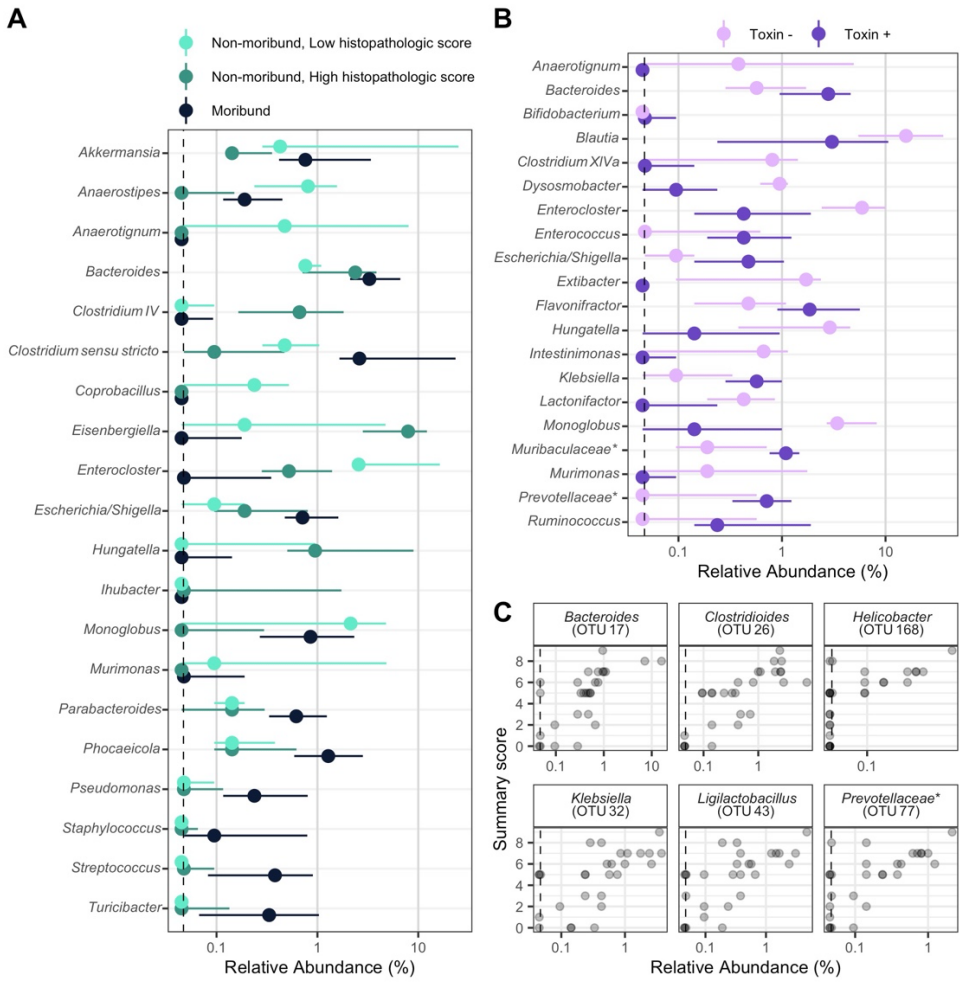


Figure 2.5. Individual fecal bacterial community members of the murine gut associated with *C .difficile* infection outcomes.

(A and B) Relative abundance of genera at the time of *C. difficile* challenge (Day 0) that varied significantly by the moribundity and histopathologic summary score or detected toxin by LEfSe analysis. Median (points) and interquartile range (lines) are plotted. Genera are ordered alphabetically to ease comparisons across analyses. (A) Relative abundances were compared across infection outcome of moribund (colored black) or non-moribund with either a high histopathologic score (score greater than the median score of 5, colored green) or a low histopathologic summary score (score less than the median score of 5, colored light green). (B) Relative abundances were compared between mice which toxin activity was detected (Toxin +, colored dark purple) and which no toxin activity was detected (Toxin -, colored light purple). (C) Endpoint bacterial OTUs correlated with histopathologic summary score. Each individual mouse is plotted (transparent gray point). Spearman's correlations were statistically significant after Benjamini-Hochberg correction for multiple comparisons. All bacterial groups are ordered alphabetically. * indicates that the bacterial group was unclassified at lower taxonomic classification ranks.

We next determined whether, collectively, bacterial community membership and relative abundance could be predictive of the CDI disease outcome. We trained random forest models with bacterial community relative abundance data from the day of colonization at each taxonomic rank to predict toxin, moribundity, and day 10 post-challenge histopathologic summary score. For predicting if detectable toxin would be produced, microbial populations aggregated by phylum rank classification performed similarly as models using lower taxonomic ranks (AUROC = 0.83, Figure 2.6). *C. difficile* was more likely to produce detectable toxin when the community infected had less abundant populations of *Verrucomicrobia* and *Campilobacterota* and had more abundant populations of *Proteobacteria* (Figure 2.7A). Next, we assessed the ability of the community to predict moribundity. Bacteria grouped by class rank classification was sufficient to predict which mice would succumb to the infection before the end of the experiment (AUROC = 0.91, Figure 2.6). The features with the greatest effect showed that communities with greater populations of bacteria belonging to *Bacilli* and *Firmicutes* and reduced populations of *Erysipelotrichia* were more likely to result in moribundity (Figure 2.7B). Only one other class of bacteria was decreased in moribund mice, a group of unclassified *Clostridia*. Lastly, the relative abundances of genera were able to predict a high or low histopathologic score (histopathologic scores were dichotomized as in previous analysis, AUROC = 0.99, Figure 2.6). No genera had a significantly greater effect on the model performance than any others, indicating the model was reliant on many genera for the correct prediction. The model used some of the genera identified in the LEfSe analysis, such as *Coprobacillus*, *Anaerostipes*, and *Hungatella*. Communities with greater abundances of *Hungatella*, *Eggerthella*, *Bifidobacterium*, *Duncaniella* and *Neisseria* were more likely to have high histopathologic scores. These models have shown that the relative abundance of bacterial

populations and their relationship to each other could be used to predict the variation in moribundity, histopathologic score, and detectable toxin of CDI.

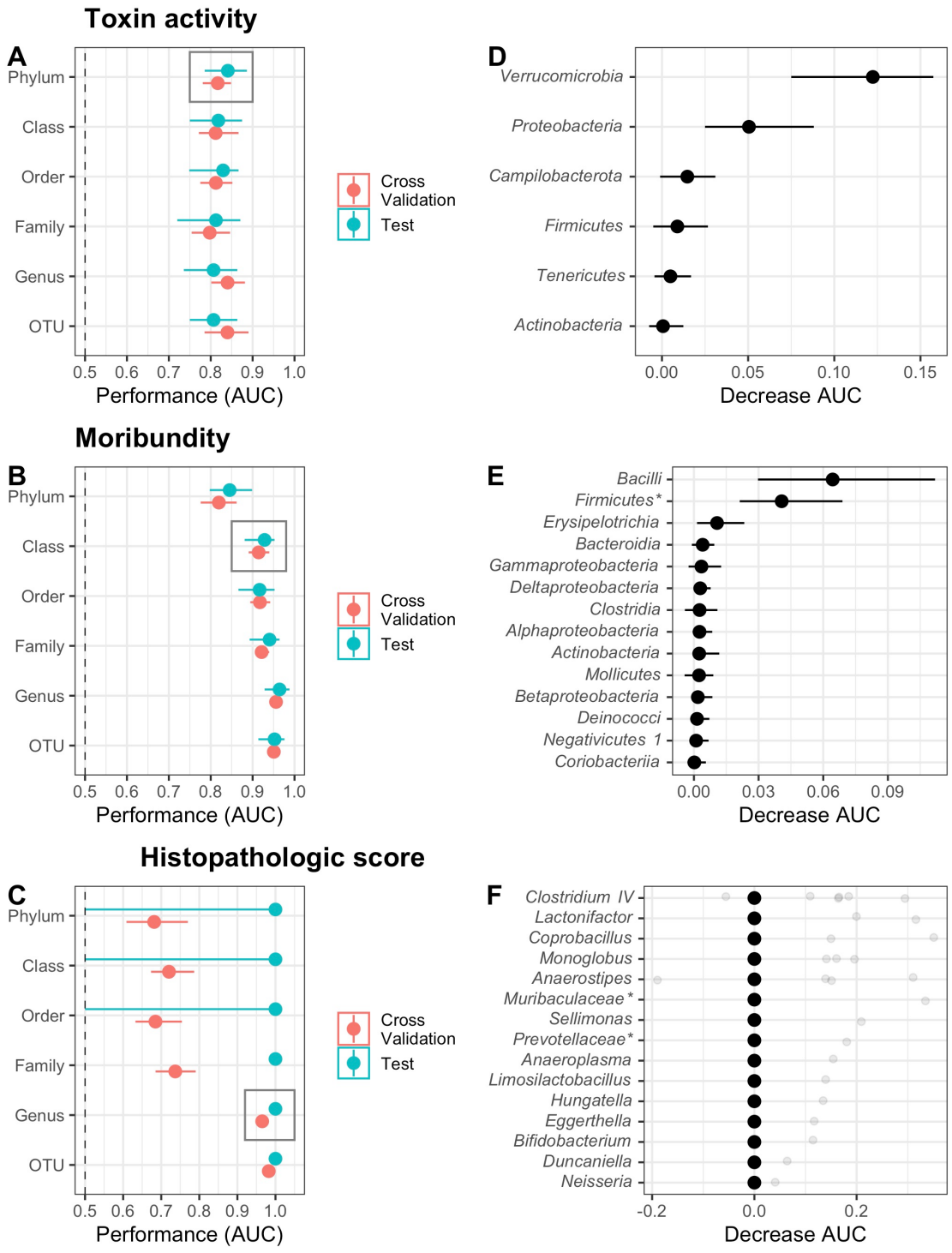


Figure 2.6. Random forest models predicted outcomes of the *C. difficile* challenge.

(A-C) Taxonomic classification rank model performance. Relative abundance at the time of *C. difficile* challenge (Day 0) of the bacterial community members grouped by different classification rank were modeled with random forest to predict the infection outcome. The

models used the highest taxonomic classification rank performed as well as the lower ranks. Black rectangle highlights classification rank used to model each outcome. (D-F) Model feature importance. Bacterial groups are ordered by their decrease in area under receiver-operator curve (AUC) when its relative abundances was permuted. Individual relative abundances were added to F since differences in AUC were outside the interquartile range. * indicates bacterial group was unclassified at lower taxonomic classification ranks. For all plots, median (solid points) and interquartile range (lines) are plotted. (A) Toxin production modeled which mice would have toxin detected during the experiment. (B) Moribundity modeled which mice would succumb to the infection prior to day 10 post-challenge. (C) Histopathologic score modeled which mice would have a high (score greater than the median score of 5) or low (score less than the median score of 5) histopathologic summary score. (D) Bacterial phyla which affected the performance of predicting detectable toxin activity when permuted. (E) Bacterial classes which affected the performance of predicting moribundity when permuted. (D) Bacterial genera which affected the performance of predicting histopathologic score when permuted.

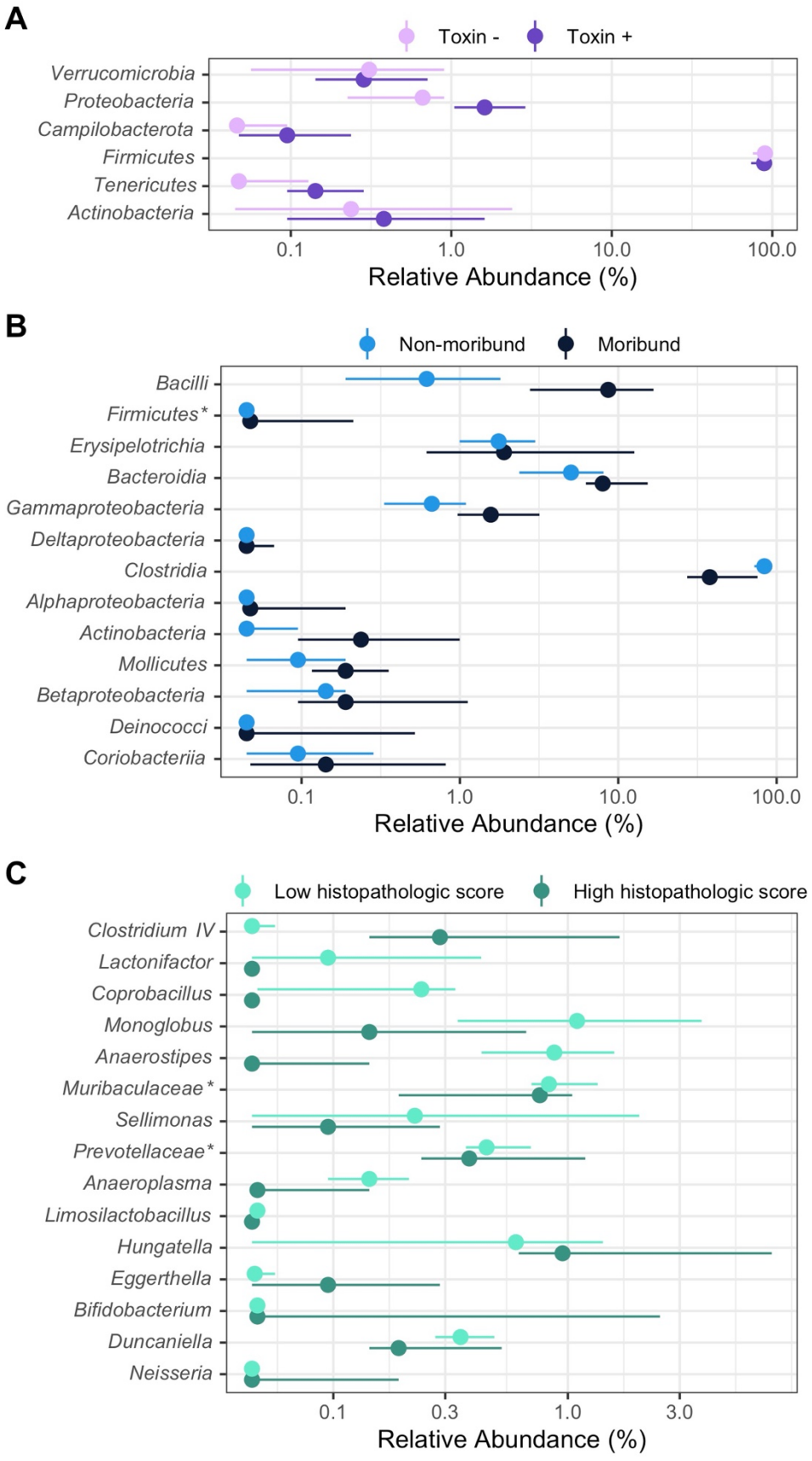

A
Toxin -
Toxin +
Verrucomicrobia
Proteobacteria
Campilobacterota
Firmicutes
Tenericutes
Actinobacteria
0.1
1.0
10.0
100.0
Relative Abundance (%)
B
Non-moribund
Moribund
Bacilli
Firmicutes*
Erysipelotrichia
Bacteroidia
Gammaproteobacteria
Deltaproteobacteria
Clostridia
Alphaproteobacteria
Actinobacteria
Mollicutes
Betaproteobacteria
Deinococci
Coriobacteriia
0.1
1.0
10.0
100.0
Relative Abundance (%)
C
Low histopathologic score
High histopathologic score
Clostridium IV
Lactonifactor
Coprobacillus
Monoglobus
Anaerostipes
Muribaculaceae*
Sellimonas
Prevotellaceae*
Anaeroplasma
Limosilactobacillus
Hungatella
Eggerthella
Bifidobacterium
Duncaniella
Neisseria
0.1
0.3
1.0
3.0
Relative Abundance (%)

Figure 2.7. Fecal bacterial community members of the murine gut at the time of *C. difficile* infection predicted outcomes of the infection.

On the day of infection (Day 0), bacterial community members grouped by different classification rank were modeled with random forest to predict the infection outcome. The models used the highest taxonomic classification rank that performed as well as the lower ranks. Median (solid points) and interquartile range (lines) of the group relative abundance are plotted. Bacterial groups are ordered by their importance to the model; taxonomic group at the top of the plot had the greatest decrease in performance when its relative abundances were permuted. * indicates that the bacterial group was unclassified at lower taxonomic classification ranks. (A) Bacterial members grouped by phyla predicted which mice would have toxin activity detected at any point throughout the infection (Toxin +, dark purple). (B) Bacterial members grouped by class predicted which mice would become moribund (dark blue). (C) Bacterial members grouped by genera predicted if the mice would have a high (score greater than the median score of 5, colored dark green) or low (score less than the median score of 5, colored light green) histopathologic summary score.

2.6 Discussion

Challenging mice colonized with different human fecal communities with *C. difficile* RT027 demonstrated that variation in members of the gut microbiome affects *C. difficile* infection disease severity. Our analysis revealed an association between the relative abundance of bacterial community members and disease severity. Previous studies investigating the severity of CDI disease involving the microbiome have had limited ability to interrogate this relationship between the microbiome and disease severity. Studies that have used clinical data have limited ability to control variation in the host, microbiome or *C. difficile* ribotype (57). Murine experiments typically use a single mouse colony and different *C. difficile* ribotypes to create severity differences (58). Recently, our group has begun uncovering the effect microbiome variation has on *C. difficile* infection. We showed the variation in the bacterial communities between mice from different mouse colonies resulted in different clearance rates of *C. difficile* (46). We also showed varied ability of mice to spontaneously eliminate *C. difficile* infection when they were treated with different antibiotics prior to *C. difficile* challenge (59). Overall, the results presented here have demonstrated that the gut bacterial community contributed to the severity of *C. difficile* infection.

C. difficile can lead to asymptomatic colonization or infections with severity ranging from mild diarrhea to death. Physicians use classification tools to identify patients most at risk of developing a severe infection using white blood cell counts, serum albumin level, or serum creatinine level (18, 60, 61). Those levels are driven by the activities in the intestine (62). Research into the drivers of this variation have revealed factors that make *C. difficile* more virulent. Strains are categorized for their virulence by the presence and production of the toxins TcdA, TcdB, and binary toxin and the prevalence in outbreaks, such as ribotypes 027 and 078

(21, 63–66). However, other studies have shown that disease is not necessarily linked with toxin production (67) or the strain (68). Furthermore, there is variation in the genome, growth rate, sporulation, germination, and toxin production in different isolates of a strain (16, 17, 69, 70). This variation may help explain why severe CDI prediction tools often miss identifying many patients with CDI that will develop severe disease (48, 58, 71, 72). Therefore, it is necessary to gain a full understanding of all factors contributing to disease variation to improve our ability to predict severity.

The state of the gut bacterial community determines the ability of *C. difficile* to colonize and persist in the intestine. *C. difficile* is unable to colonize an unperturbed healthy murine gut community and is only able to become established after a perturbation (33). Once colonized, the different communities lead to different metabolic responses and dynamics of the *C. difficile* population (25, 59, 73). Gut bacteria metabolize primary bile acids into secondary bile acids (74, 75). The concentration of these bile acids affects germination, growth, toxin production and biofilm formation (9, 39, 51, 76). Members of the bacterial community also affect other metabolites *C. difficile* utilizes. *Bacteroides thetaiotaomicron* produce sialidases which release sialic acid from the mucosa for *C. difficile* to utilize (26, 77). The nutrient environment affects toxin production (78). Thus, many of the actions of the gut bacteria modulate *C. difficile* in ways that could affect the infection and resultant disease.

A myriad of studies have explored the relationship between the microbiome and CDI disease. Studies examining difference in disease often use different *C. difficile* strains or ribotypes in mice with similar microbiota as a proxy for variation in disease, such as strain 630 for non-severe and RT027 for severe (21, 44, 63, 64). Studies have also demonstrated variation in infection through tapering antibiotic dosage (33, 34, 59) or by reducing the amount of *C.*

difficile cells or spores used for the challenge (21, 34). These studies often either lack variation in the initial microbiome or have variation in the *C. difficile* infection itself, confounding any association between variation in severity and the microbiome. Recent studies have shown variation in the initial microbiome, via different murine colonies or colonizing germ-free mice with human feces, that were challenged with *C. difficile* resulted in varied outcomes of the infection (46, 53).

Our data have demonstrated gut bacterial relative abundances associate with variation in toxin production, histopathologic scoring of the cecal tissue and mortality. This analysis revealed populations of *Akkermansia*, *Anaerostipes*, *Coprobacillus*, *Enterocloster*, *Lactonifactor*, and *Monoglobus* were more abundant in the microbiome of non-moribund mice which had low histopathologic scores and no detected toxin. The protective role of these genera are supported by previous studies. *Coprobacillus*, *Lactonifactor*, and *Monoglobus* have been shown to be involved in dietary fiber fermentation and associated with healthy communities (79–82). *Anaerostipes* and *Coprobacillus*, which produce short chain fatty acids, have been associated with healthy communities (83–85). Furthermore, *Coprobacillus*, which was abundant in mice with low histopathologic scores but rare in all other mice, has been shown to contain a putative type I lantibiotic gene cluster and inhibit *C. difficile* colonization (86–88). *Akkermansia* and *Enterocloster* were also identified as more abundant in mice which had a low histopathologic scores but have contradictory supporting evidence in the current literature. In our data, *Akkermansia* was most abundant in the non-moribund mice with low histopathologic scores but there were some moribund mice which had increased populations of *Akkermansia*. This could be attributed to either a more protective mucus layer was present inhibiting colonization (88, 89) or mucus consumption by *Akkermansia* could have been crossfeeding *C. difficile* or exposing a

niche for *C. difficile* (90–92). Similarly, *Enterocloster* was more abundant and associated with low histopathologic scores. It has been associated with healthy populations and has been used to mono-colonize germ-free mice to reduce the ability of *C. difficile* to colonize (35, 93). However, *Enterocloster* has also been involved in infections, such as bacteremia (94, 95). These data have exemplified populations of bacteria that have the potential to be either protective or harmful. Thus, the disease outcome is not likely based on the abundance of individual populations of bacteria, rather it is the result of the interactions of the community.

The groups of bacteria that were associated with either a higher histopathologic score or moribundity are members of the indigenous gut community that also have been associated with disease, often referred to as opportunistic pathogens. Many of the populations with pathogenic potential that associated with worse outcomes are also facultative anaerobes. *Enterococcus*, *Klebsiella*, *Shigella/Escherichia*, *Staphylococcus*, and *Streptococcus* have been shown to expand after antibiotic use (41, 96, 97) and are commonly detected in CDI cases (98–101). In addition to these populations, *Eggerthella*, *Prevotellaceae* and *Helicobacter*, which associated with worse outcomes, have also been associated with intestinal inflammation (102–104). Recently, *Helicobacter hepaticus* was shown to be sufficient to cause susceptibility to CDI in IL-10 deficient C57BL/6 mice (105). In our experiments, when *Helicobacter* was present, the infection resulted in a high histopathologic score (Figure 2.5C). While we did not use IL-10 deficient mice, it is possible the bacterial community or host response are similarly modified by *Helicobacter*, allowing *C. difficile* infection and host damage. Aside from *Helicobacter*, these groups of bacteria that associated with more severe outcomes did not have a conserved association between their relative abundance and the disease severity across all mice.

Since we observed groups of bacteria that were associated with less severe disease it may be appropriate to apply the damage-response framework for microbial pathogenesis to CDI (106, 107). This framework posits that disease is not driven by a single entity, rather it is an emergent property of the responses of the host immune system, infecting microbe, *C. difficile*, and the indigenous microbes at the site of infection. In the first set of experiments, we used the same host background, C57BL/6 mice, the same infecting microbe, *C. difficile* RT027 clinical isolate 431, with different gut bacterial communities. The bacterial groups in those communities were often present in both moribund and non-moribund and across the range of histopathologic scores. Thus, it was not merely the presence of the bacteria but their activity in response to the other microbes and host which affect the extent of the host damage. Additionally, while each mouse and *C. difficile* population had the same genetic background, they too were reacting to the specific microbial community. Disease severity is driven by the cumulative effect of the host immune response and the activity of *C. difficile* and the gut bacteria. *C. difficile* drives host damage through the production of toxin. The gut microbiota can modulate host damage through the balance of metabolic and competitive interactions with *C. difficile*, such as bacteriocin production or mucin degradation, and interactions with the host, such as host mucus glycosylation or intestinal IL-33 expression (53, 108). For example, low levels of mucin degradation can provide nutrients to other community members producing a diverse non-damaging community (109). However, if mucin degradation becomes too great it reduces the protective function of the mucin layer and exposes the epithelial cells. This over-harvesting can contribute to the host damage due to other members producing toxin. Thus, the resultant intestinal damage is the balance of all activities in the gut environment. Host damage is the emergent property of numerous damage-response curves, such as one for host immune response,

one for *C. difficile* activity and another for microbiome community activity, each of which are a composite curve of the individual activities from each group, such as antibody production, neutrophil infiltration, toxin production, sporulation, fiber and mucin degradation. Therefore, while we have identified populations of interest, it may be necessary to target multiple types of bacteria to reduce the community interactions contributing to host damage.

Here we have shown several bacterial groups and their relative abundances associated with variation in CDI disease severity. Further understanding how the microbiome affects severity in patients could reduce the amount of adverse CDI outcomes. When a patient is diagnosed with CDI, the gut community composition, in addition to the traditionally obtained clinical information, may improve our severity prediction and guide prophylactic treatment. Treating the microbiome at the time of diagnosis, in addition to *C. difficile*, may prevent the infection from becoming more severe.

2.7 Methods

Animal care. 6- to 13-week old male and female germ-free C57BL/6 were obtained from a single breeding colony in the University of Michigan Germ-free Mouse Core. Mice (N1 n=11, N2 n=7, N3 n=3, N4 n=3, N5 n=3, N6 n=3, N7 n=7, N8 n=3, N9 n=2, M1 n=3, M2 n=3, M3 n=3, M4 n=3, M5 n=7, M6 n=3) were housed in cages of 2-4 mice per cage and maintained in germ-free isolators at the University of Michigan germ-free facility. All mouse experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan.

***C. difficile* experiments.** Human fecal samples were obtained as part of Schubert *et al.* and selected based on community clusters (41) to result in diverse community structures. Feces

were homogenized by mixing 200 mg of sample with 5 ml of PBS. Mice were inoculated with 100 μl of the fecal homogenate via oral gavage. Two weeks after the fecal community inoculation, mice were challenged with *C. difficile*. *C. difficile* clinical isolate 431 came from Carlson *et al.* which had previously been isolated and characterized (16, 69) and has recently been further characterized (70). Spores concentration were determined both before and after challenge (110). 10^3 *C. difficile* spores were given to each mouse via oral gavage.

Sample collection. Fecal samples were collected on the day of *C. difficile* challenge and the following 10 days. Each day, a fecal sample was collected and a portion was weighed for plating (approximately 30 mg) and the remaining sample was frozen at -20°C. Anaerobically, the weighed fecal samples were serially diluted in PBS, plated on TCCFA plates, and incubated at 37°C for 24 hours. The plates were then counted for the number of colony forming units (CFU) (111).

DNA sequencing. From the frozen fecal samples, total bacterial DNA was extracted using MOBIO PowerSoil-htp 96-well soil DNA isolation kit. We amplified the 16S rRNA gene V4 region and sequenced the resulting amplicons using an Illumina MiSeq as described previously (112).

Sequence curation. Sequences were processed with mothur(v.1.44.3) as previously described (112, 113). In short, we used a 3% dissimilarity cutoff to group sequences into operational taxonomic units (OTUs). We used a naive Bayesian classifier with the Ribosomal Database Project training set (version 18) to assign taxonomic classifications to each OTU (114). We sequenced a mock community of a known community composition and 16s rRNA gene

sequences. We processed this mock community with our samples to calculate the error rate for our sequence curation, which was an error rate of 0.19%.

Toxin cytotoxicity assay. To prepare the sample for the activity assay, fecal material was diluted 1:10 weight per volume using sterile PBS and then filter sterilized through a 0.22-μm filter. Toxin activity was assessed using a Vero cell rounding-based cytotoxicity assay as described previously (64). The cytotoxicity titer was determined for each sample as the last dilution, which resulted in at least 80% cell rounding. Toxin titers are reported as the log10 of the reciprocal of the cytotoxicity titer.

Histopathology evaluation. Mouse cecal tissue was placed in histopathology cassettes and fixed in 10% formalin, then stored in 70% ethanol. McClinchey Histology Labs, Inc. (Stockbridge, MI) embedded the samples in paraffin, sectioned, and created the hematoxylin and eosin-stained slides. The slides were scored using previously described criteria by a board-certified veterinary pathologist who was blinded to the experimental groups (64). Slides were scored as 0-4 for parameters of epithelial damage, tissue edema, and inflammation and a summary score of 0-12 was generated by summing the three individual parameter scores.

Statistical analysis and modeling. To compare community structures, we calculated Yue and Clayton dissimilarity matrices (θ_{YC}) in mothur (115). We rarefied samples to 2,107 sequences per sample to limit uneven sampling biases. We tested for differences in individual taxonomic groups that would explain the outcome differences with LEfSe (116) in mothur. Remaining statistical analysis and data visualization was performed in R (v4.0.5) with the tidyverse package (v1.3.1). We tested for significant differences in β-diversity (θ_{YC}) using the Wilcoxon rank sum test. We used Spearman's correlation to identify which OTUs that had a

correlation between their relative abundance and the histopathologic summary score. *P* values were then corrected for multiple comparisons with a Benjamini and Hochberg adjustment for a type I error rate of 0.05 (117). We built random forest models using the mikropml package (118) with relative abundance summed by taxonomic ranks from day 0 samples using mtry values of 1 through 10, 15, 20, 25, 40, 50, 100. The split for training and testing varied by model to avoid overfitting the data. To determine the optimal split, we tested splits (50%, 60%, 70%, 80%, 90% data used for training) to find the greatest portion of data that could be used to train the model while still maintaining the same performance for the training model as the model with the held-out test data. The toxin and moribundity models were trained with 60% of the data. The histopathologic score model was trained with 80% of the data. Lastly, we did not compare murine communities to donor community or clinical data because germ-free mice colonized with non-murine fecal communities have been shown to more closely resemble the murine communities than the donor species community (119). Furthermore, it is not our intention to make any inferences regarding human associated bacteria and their relationship with human CDI outcome.

Chapter III

Clearance of *Clostridioides difficile* Colonization is Associated with Antibiotic-Specific Bacterial Changes

3.1 Notes

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Conceptualization: N.A.L., A.M.S., P.D.S.; Data curation: NAL; Formal analysis: NAL; Investigation: N.A.L., A.M.S., H.S., P.D.S.; Methodology: N.A.L., A.M.S., H.S., P.D.S.; Resources: N.A.L., A.M.S., P.D.S.; Software: N.A.L.; Visualization: N.A.L., P.D.S.; Writing - original draft: N.A.L.; Writing - review & editing: N.A.L., A.M.S., H.S., P.D.S.; Funding acquisition: P.D.S.; Project administration: P.D.S.; Supervision: P.D.S.

3.2 Abstract

The gut bacterial community prevents many pathogens from colonizing the intestine. Previous studies have associated specific bacteria with clearing *Clostridioides difficile* colonization across different community perturbations. However, those bacteria alone have been

unable to clear *C. difficile* colonization. To elucidate the changes necessary to clear colonization, we compared differences in bacterial abundance between communities able and unable to clear *C. difficile* colonization. We treated mice with titrated doses of antibiotics prior to *C. difficile* challenge which resulted in no colonization, colonization and clearance, or persistent colonization. Previously, we observed that clindamycin-treated mice were susceptible to colonization but spontaneously cleared *C. difficile*. Therefore, we investigated whether other antibiotics would show the same result. We found reduced doses of cefoperazone and streptomycin permitted colonization and clearance of *C. difficile*. Mice that cleared colonization had antibiotic-specific community changes and predicted interactions with *C. difficile*. Clindamycin treatment led to a bloom in populations related to *Enterobacteriaceae*. Clearance of *C. difficile* was concurrent with the reduction of those blooming populations and the restoration of community members related to the *Porphyromonadaceae* and *Bacteroides*. Cefoperazone created a susceptible community characterized by a drastic reduction in the community diversity, interactions, and a sustained increase in abundance of many facultative anaerobes. Lastly, clearance in streptomycin-treated mice was associated with the recovery of multiple members of the *Porphyromonadaceae*, with little overlap in the specific *Porphyromonadaceae* observed in the clindamycin treatment. Further elucidation of how *C. difficile* colonization is cleared from different gut bacterial communities will improve *C. difficile* infection treatments.

3.3 Importance

The community of microorganisms, known as the microbiota, in our intestines prevents pathogens, such as *C. difficile*, from colonizing and causing infection. However, antibiotics can disturb the gut microbiota, which allows *C. difficile* to colonize. *C. difficile* infections (CDI) are primarily treated with antibiotics, which frequently leads to recurrent infections because the

microbiota have not yet returned to a resistant state. The recurrent infection cycle often ends when the fecal microbiota from a presumed resistant person are transplanted into the susceptible person. Although this treatment is highly effective, we do not understand the mechanism. We hope to improve the treatment of CDI through elucidating how the bacterial community eliminates CDI. We found *C. difficile* colonized susceptible mice but was spontaneously eliminated in an antibiotic-treatment specific manner. These data indicate each community had different requirements for clearing colonization. Understanding how different communities clear colonization will reveal targets to improve CDI treatments.

3.4 Introduction

A complex consortium of bacteria and microbes that inhabits our gut, known as the microbiota, prevent pathogens from colonizing and causing disease. This protection, known as colonization resistance, is mediated through many mechanisms such as activating host immune responses, competing for nutrients, producing antimicrobials, and contributing to the maintenance of the mucosal barrier (14). However, perturbations to the intestinal community or these functions opens the possibility that a pathogen can colonize (120). For example, the use of antibiotics perturb the gut microbiota and can lead to *Clostridioides difficile* infection (CDI).

CDI is especially problematic due to its burden on the healthcare system (13, 121). *C. difficile* can cause severe disease, such as toxic megacolon, diarrhea, and death (122). CDI is primarily treated with antibiotics (123). CDIs recalcitrant to antibiotics are eliminated by restoring the community with a fecal microbiota transplant (FMT), returning the perturbed community to a healthier protective state (124, 125). However, FMTs are not always effective

against CDI and have the risk of transferring a secondary infection (20, 126). Therefore, we need to better understand how the microbiota clears the infection to develop more effective treatments.

Previous research has shown that the microbiota affects *C. difficile* colonization. Mouse models have identified potential mechanisms of colonization resistance such as bile salt metabolism and nutrient competition (24, 25, 36, 127). However, studies that have restored those functions were unable to restore complete resistance (44, 128). This could be attributed to the complexity of the community and the mechanisms of colonization resistance (43, 45). We previously showed that when *C. difficile* colonizes different antibiotic-treated murine communities it modifies its metabolism to fit each specific environment (25, 33, 73). Therefore, we have investigated the bacterial community dynamics concurrent with clearance of *C. difficile* below the limit of detection across uniquely perturbed communities.

Jenior et al. (73) observed that clindamycin-treated mice spontaneously cleared *C. difficile* colonization whereas mice treated with cefoperazone and streptomycin did not. Here, we continued to explore the different effects these three antibiotics have on *C. difficile* colonization. The purpose of this study was to elucidate the gut bacterial community changes concurrent with clearance of *C. difficile* colonization. We hypothesized that each colonized community had perturbation-specific susceptibilities and requires specific changes to clear the pathogen. To induce a less severe perturbation, we reduced the doses of cefoperazone and streptomycin. This resulted in communities that were initially colonized to a high level ($>10^6$ CFU/g feces) and then spontaneously cleared *C. difficile*. We found each antibiotic resulted in unique changes in the microbiota that were associated with the persistence or clearance of *C. difficile*. These data further support the hypothesis that *C. difficile* can exploit numerous niches in perturbed communities.

3.5 Results

Reduced doses of cefoperazone and streptomycin allowed communities to spontaneously clear *C. difficile* colonization. To understand the dynamics of colonization and clearance of *C. difficile*, we first identified conditions which would allow colonization and clearance. Beginning with clindamycin, mice were treated with an intraperitoneal injection of clindamycin (10 mg/kg) one day prior to challenge with *C. difficile*. All mice (N = 11) were colonized to a high level (median CFU = 3.07×10^7) the next day and cleared the colonization within 10 days; 6 mice cleared *C. difficile* within 6 days (Figure 3.1A). Previous *C. difficile* infection models using cefoperazone and streptomycin have not demonstrated clearance. So we next explored whether cefoperazone and streptomycin could permit colonization and subsequent clearance with lower doses. We began with replicating the previously established *C. difficile* infection models using these antibiotics (73). We treated mice with cefoperazone or streptomycin in their drinking water for 5 days (0.5 mg/mL and 5 mg/mL, respectively) and then challenged them with *C. difficile*. For both antibiotics, *C. difficile* colonization was maintained for the duration of the experiment as previously demonstrated (Figure 3.1B-C) (73). Then we repeated the *C. difficile* challenge with reduced doses of the antibiotics (cefoperazone - 0.3 and 0.1 mg/mL; streptomycin - 0.5 and 0.1 mg/mL). For both antibiotic treatments, the lowest dose resulted in either no colonization (N = 8) or a transient, low level colonization (N = 8, median length = 1 day, median CFU/g = 2.8×10^3) (Figure 3.1B-C). The intermediate dose of both antibiotics resulted in a high level colonization (median CFU/g = 3.5×10^6) and half (N = 8 of 16) of the mice clearing the colonization within 10 days. Based on our previous research, which showed each of these antibiotics uniquely changed the microbiota, we hypothesized that the microbiota varied across these antibiotic treatments that resulted in colonization clearance. To

focus on the changes related to clearance and not antibiotic dosage, the remaining analysis aggregated mice which had *C. difficile* present in their stool post-challenge by whether *C. difficile* was detected (i.e. colonized) or not (i.e. cleared) at the end of the experiment.

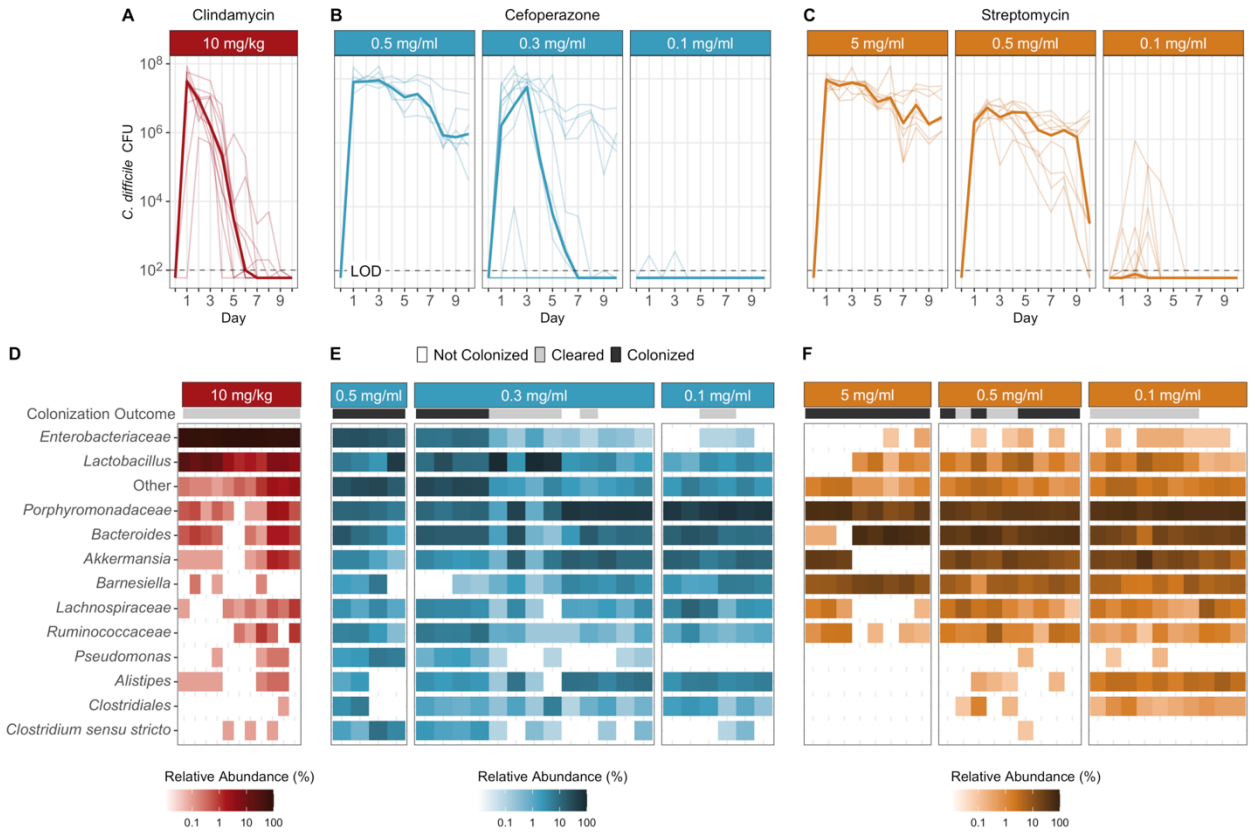


Figure 3.1. Reduced antibiotic doses permitted murine communities to be colonized and spontaneously clear that *C. difficile* colonization.

(A-C) Daily CFU of *C. difficile* in fecal samples of mice treated with clindamycin, cefoperazone, or streptomycin from time of challenge (Day 0) with 10^3 *C. difficile* strain 630Δerm spores through 10 days post infection (dpi). The bold line is the median CFU of the group and the transparent lines are the individual mice. (D-F) Relative abundance of twelve most abundant taxonomic groups, labeled with the lowest level of classification, at the time of *C. difficile* challenge, all other taxanomic groups are combined into Other. Each column is an individual mouse. (clindamycin - 10 mg/kg N =11; cefoperazone - 0.5 mg/mL N = 6, 0.3 mg/mL N = 13, 0.1 mg/mL N = 6; streptomycin - 5.0 mg/mL N = 8, 0.5 mg/mL N = 9, 0.1 mg/mL N = 11) LOD = Limit of detection.

Clearance of *C. difficile* was associated with antibiotic-specific changes to the microbiota. Beginning with the clindamycin-treated mice, we analyzed their fecal 16S rRNA gene sequences to identify the community features related to *C. difficile* colonization and clearance. First, we compared the most abundant bacterial genera of the communities at the time of *C. difficile* challenge. The clindamycin-treated mice became dominated by relatives of *Enterobacteriaceae* with a concurrent reduction in the other abundant genera, except for populations of *Lactobacillus* (Figure 3.1D, 3.2). These community changes permitted *C. difficile* to colonize all of these mice, but all of the mice were also able to clear the colonization. We next investigated how the microbiota diversity related to *C. difficile* clearance. Clindamycin treatment decreased the α-diversity ($P < 0.05$) and similarity to the pre-clindamycin community at the time of *C. difficile* challenge ($P < 0.05$) (Figure 3.3A). But it was not necessary to restore the community similarity to its initial state to clear *C. difficile*. Therefore we investigated the temporal differences in the abundance of the operational taxonomic units (OTUs) between the initial untreated community and post-clindamycin treatment at the time of challenge and between the time of challenge and the end of the experiment. Clindamycin treatment resulted in large decreases in 21 OTUs and a bloom of relatives of *Enterobacteriaceae* (Figure 3.4A). With the elimination of *C. difficile*, we observed a drastic reduction of the relatives of *Enterobacteriaceae* and recovery of 10 populations related to *Porphyromonadaceae*, *Bacteroides*, *Akkermansia*, *Lactobacillus*, *Bifidobacterium*, *Lachnospiraceae*, and *Clostridiales* (Figure 3.4A). Thus, clindamycin reduced most of the natural community allowing *C. difficile* to colonize. The recovery of only a portion of the community was associated with eliminating the *C.difficile* population.

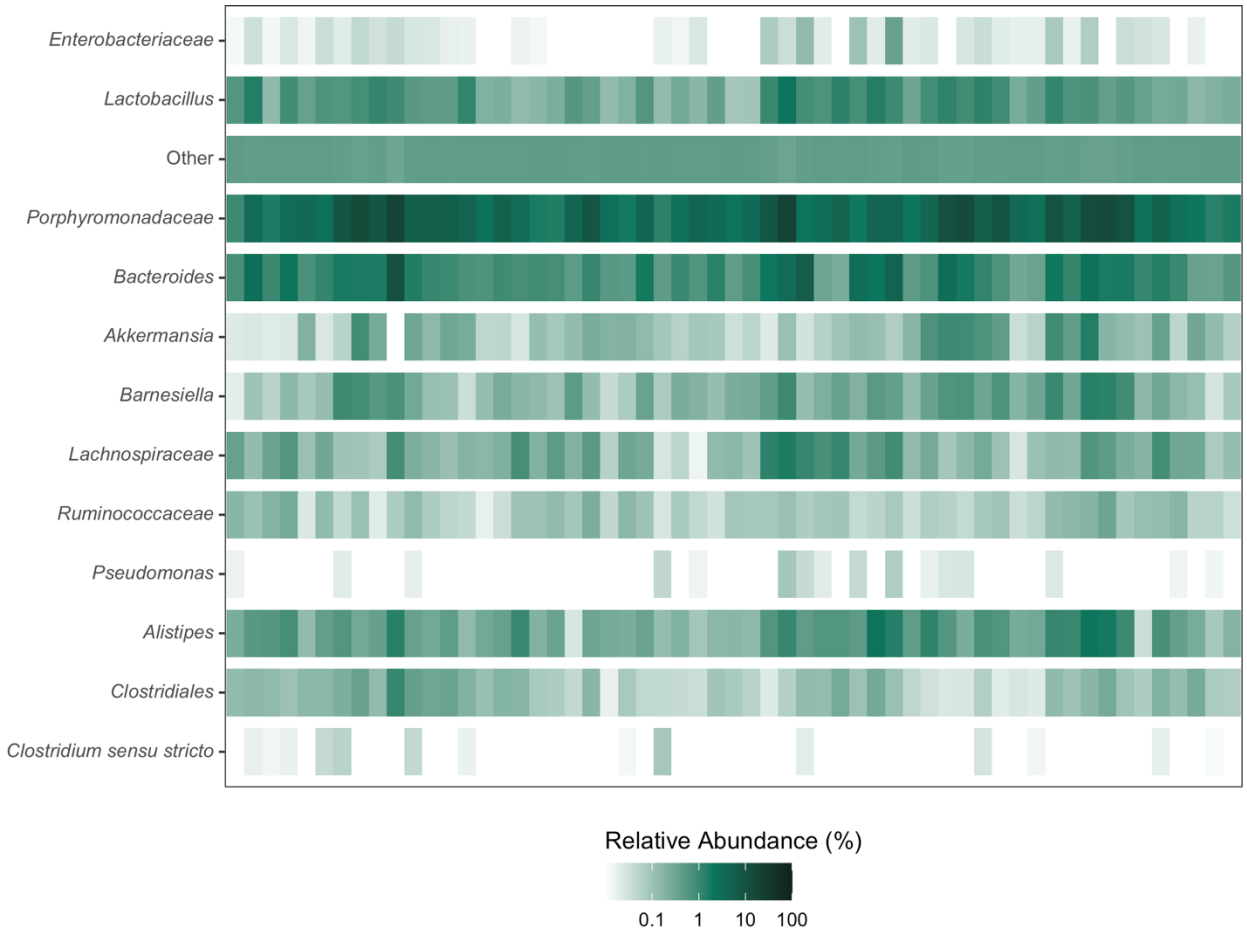


Figure 3.2. Initial microbiota relative abundance of mice prior to antibiotic treatment.

Initial community shows the most abundant taxa. The plot shows the relative abundance at the beginning of the experiment prior to antibiotic treatment of twelve most abundant taxonomic groups, labeled with the lowest level of classification. All other taxonomic groups are combined into Other. Each column is an individual mouse fecal community. Color intensity is $\log_{10}$-transformed mean percent relative abundance.

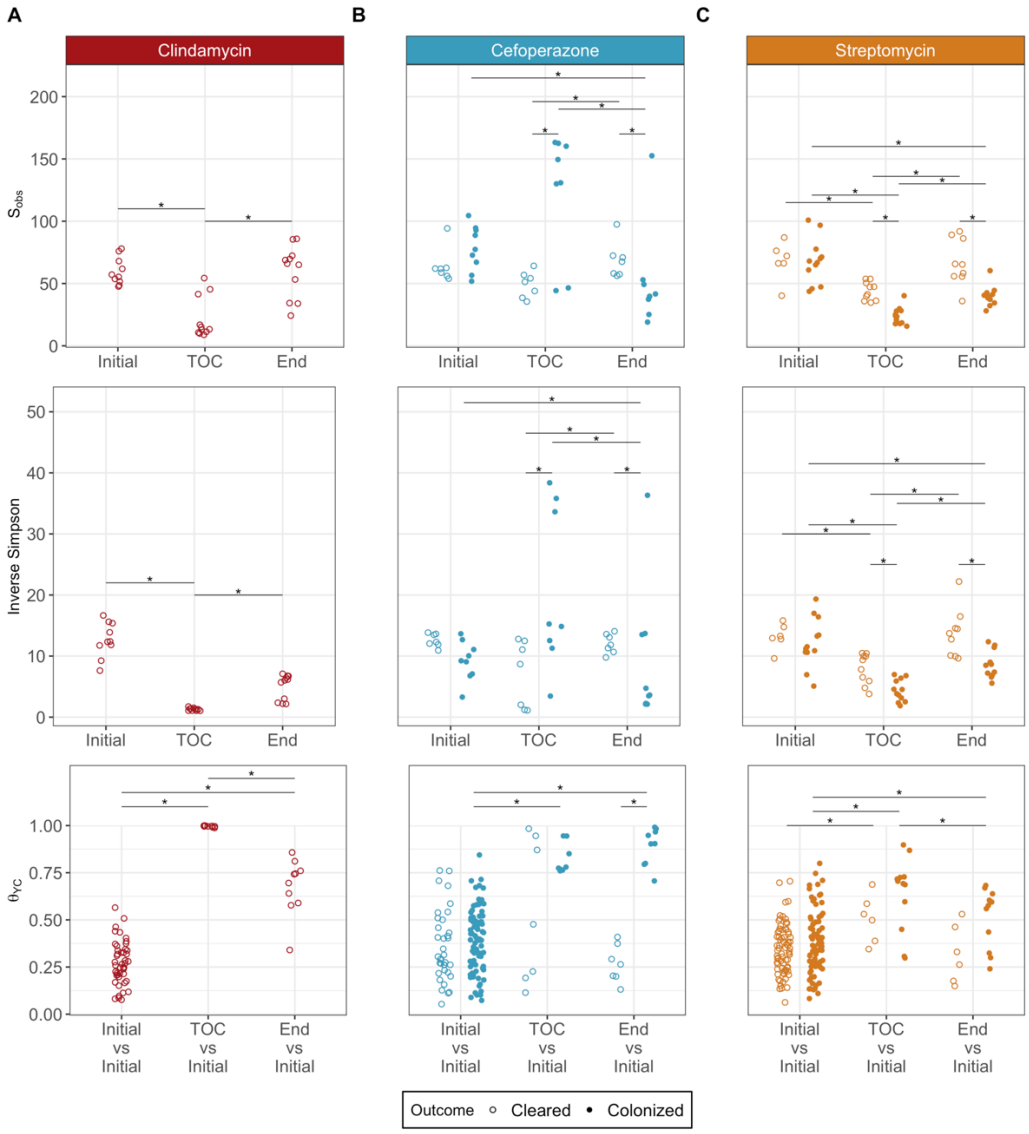


Figure 3.3. Microbiota community diversity showed antibiotic-specific trends associated with *C. difficile* colonization clearance.

For communities colonized with *C. difficile* from mice treated with clindamycin (A), cefoperazone (B), and streptomycin (C), microbiota α-diversity (S_{obs} and Inverse Simpson) and β-diversity (θ_{YC}) were compared at the initial pre-antibiotic treatment state, time of *C. difficile* challenge (TOC), and end of the experiment. β-diversity (θ_{YC}) was compared between the initial pre-antibiotic treatment to all other initial pre-antibiotic treatment communities treated with the same antibiotic, the initial community to the same community at the time of *C. difficile* challenge, and the initial community to the same community at end of the experiment. (clindamycin - cleared N = 11; cefoperazone - cleared N = 7, colonized N = 9; streptomycin - cleared N = 9, colonized N = 11). * indicates statistical significance of $P < 0.05$, calculated by Wilcoxon rank sum test with Benjamini-Hochberg correction.

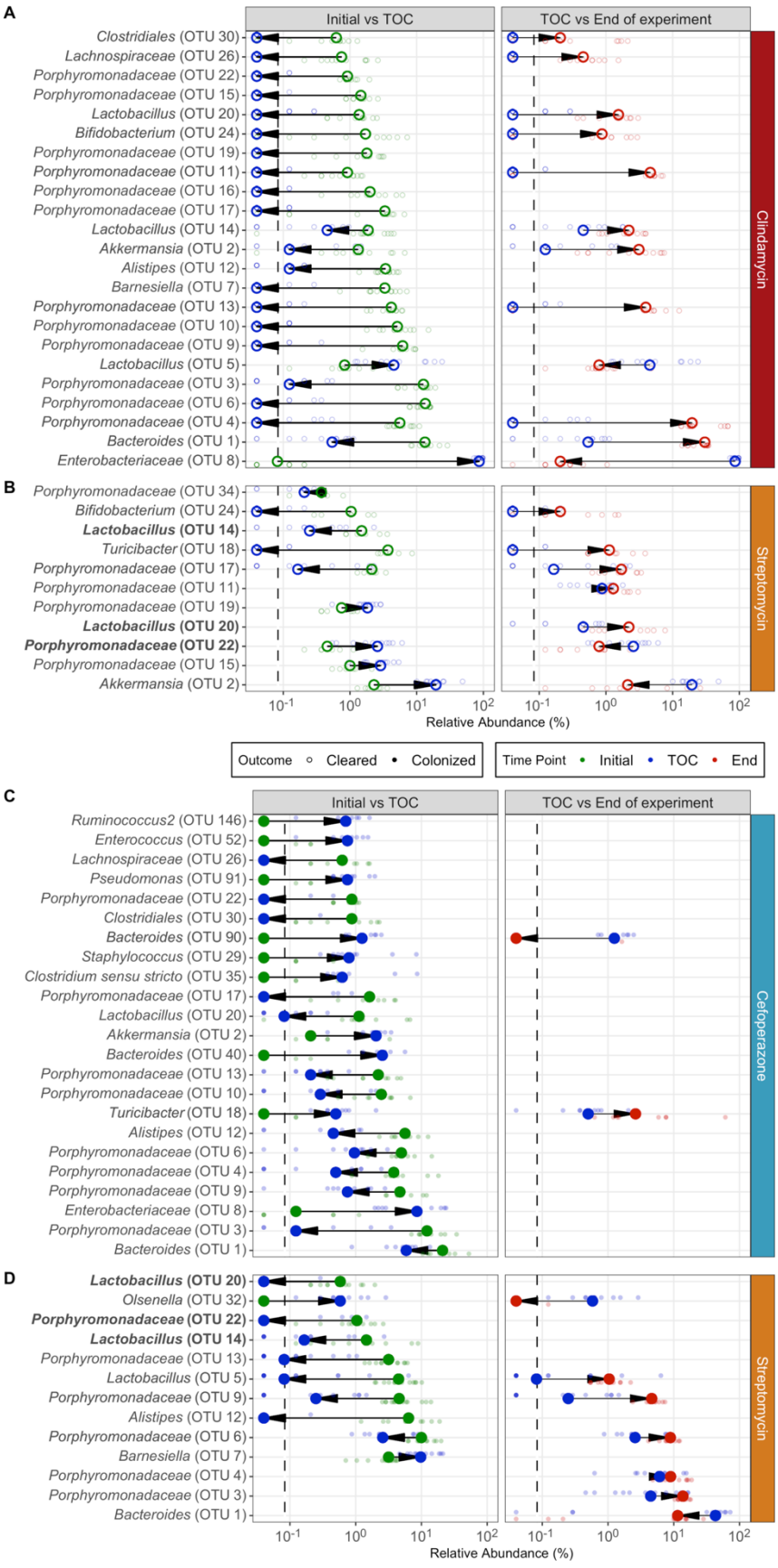

A
Initial vs TOC
TOC vs End of experiment
Clostridiales (OTU 30)
Lachnospiraceae (OTU 26)
Porphyromonadaceae (OTU 22)
Porphyromonadaceae (OTU 15)
Lactobacillus (OTU 20)
Bifidobacterium (OTU 24)
Porphyromonadaceae (OTU 19)
Porphyromonadaceae (OTU 11)
Porphyromonadaceae (OTU 16)
Porphyromonadaceae (OTU 17)
Lactobacillus (OTU 14)
Akkermansia (OTU 2)
Alistipes (OTU 12)
Barnesiella (OTU 7)
Porphyromonadaceae (OTU 13)
Porphyromonadaceae (OTU 10)
Porphyromonadaceae (OTU 9)
Lactobacillus (OTU 5)
Porphyromonadaceae (OTU 3)
Porphyromonadaceae (OTU 6)
Porphyromonadaceae (OTU 4)
Bacteroides (OTU 1)
Enterobacteriaceae (OTU 8)
Clindamycin
B
Porphyromonadaceae (OTU 34)
Bifidobacterium (OTU 24)
Lactobacillus (OTU 14)
Turicibacter (OTU 18)
Porphyromonadaceae (OTU 17)
Porphyromonadaceae (OTU 11)
Porphyromonadaceae (OTU 19)
Lactobacillus (OTU 20)
Porphyromonadaceae (OTU 22)
Porphyromonadaceae (OTU 15)
Akkermansia (OTU 2)
Streptomycin
Relative Abundance (%)
Outcome Cleared Colonized
Time Point Initial TOC End
C
Initial vs TOC
TOC vs End of experiment
Ruminococcus2 (OTU 146)
Enterococcus (OTU 52)
Lachnospiraceae (OTU 26)
Pseudomonas (OTU 91)
Porphyromonadaceae (OTU 22)
Clostridiales (OTU 30)
Bacteroides (OTU 90)
Staphylococcus (OTU 29)
Clostridium sensu stricto (OTU 35)
Porphyromonadaceae (OTU 17)
Lactobacillus (OTU 20)
Akkermansia (OTU 2)
Bacteroides (OTU 40)
Porphyromonadaceae (OTU 13)
Porphyromonadaceae (OTU 10)
Turicibacter (OTU 18)
Alistipes (OTU 12)
Porphyromonadaceae (OTU 6)
Porphyromonadaceae (OTU 4)
Porphyromonadaceae (OTU 9)
Enterobacteriaceae (OTU 8)
Porphyromonadaceae (OTU 3)
Bacteroides (OTU 1)
Cefoperazone
D
Lactobacillus (OTU 20)
Olsenella (OTU 32)
Porphyromonadaceae (OTU 22)
Lactobacillus (OTU 14)
Porphyromonadaceae (OTU 13)
Lactobacillus (OTU 5)
Porphyromonadaceae (OTU 9)
Alistipes (OTU 12)
Porphyromonadaceae (OTU 6)
Barnesiella (OTU 7)
Porphyromonadaceae (OTU 4)
Porphyromonadaceae (OTU 3)
Bacteroides (OTU 1)
Streptomycin
Relative Abundance (%)

Figure 3.4. Each antibiotic had specific sets of temporal changes in OTU abundance associated with *C. difficile* colonization and clearance.

For clindamycin (A), cefoperazone (C), and streptomycin (B, D), the difference in the relative abundance of OTUs that were significantly different between time points within each *C. difficile* colonization outcome for each antibiotic treatment were identified. Dark larger points in foreground are median relative abundance and transparent smaller points in background are relative abundance of individual mice. Lines connect points within each comparison to show difference in medians. Arrows point in the direction of the temporal change of the relative abundance. Only OTUs at time points with statistically significant differences, $P < 0.05$, were plotted (calculated by Wilcoxon rank sum test with Benjamini-Hochberg correction). Bold OTUs were shared across outcomes. Limit of detection (LOD).

We applied the same analysis to the cefoperazone-treated mice to understand what community features were relevant to clearing *C. difficile*. Increasing the dose of cefoperazone shifted the dominant community members from relatives of the *Porphyromonadaceae*, *Bacteroides* and *Akkermansia* to relatives of the *Lactobacillus* and *Enterobacteriaceae* at the time of challenge (Figure 3.1E, 3.2). We saw a similar increase in relatives of *Enterobacteriaceae* with clindamycin. However, the cefoperazone-treated mice that had larger increases in *Enterobacteriaceae* were unable to clear *C. difficile*. We next investigated the differences between the cefoperazone-treated mice that cleared *C. difficile* to those that did not. For the communities that cleared *C. difficile*, diversity was maintained throughout the experiment (Figure 3.3B). A subset of mice treated with cefoperazone that remained colonized experienced an increase in α-diversity, possibly driven by the decrease in highly abundant populations and increase in low abundant populations (Figure 3.1E, 3.5). These persistently colonized communities also had a large shift away from the initial community structure caused by the antibiotic treatment ($P < 0.05$), which remained through the end of the experiment ($P < 0.05$) (Figure 3.3B). The α-diversity of mice treated with cefoperazone did not vary significantly by dosage (Figure 3.6). These data suggested that it was necessary for cefoperazone-treated mice to become more similar to the initial pre-antibiotic community structure to clear *C. difficile*.

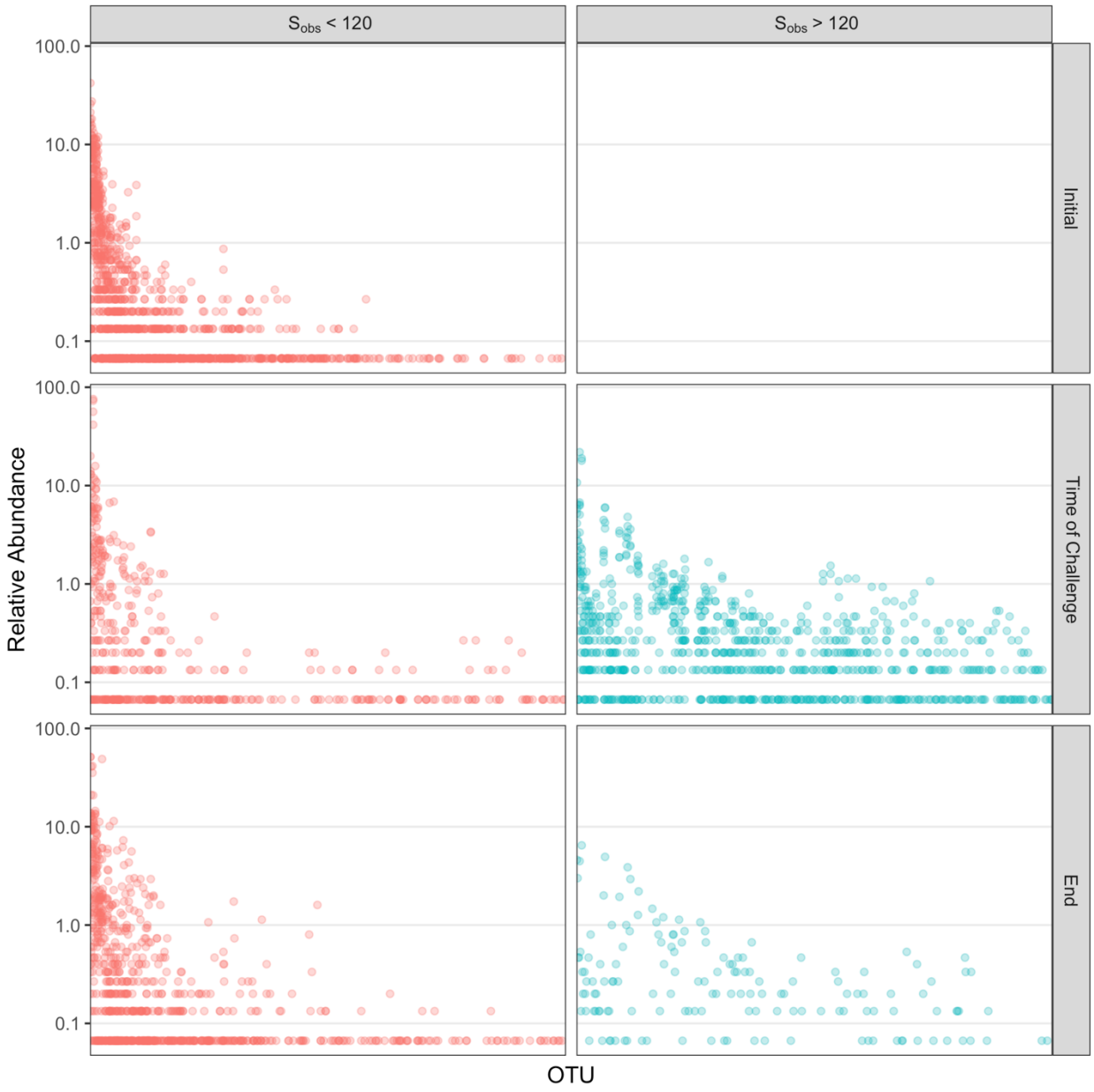


Figure 3.5. Cefoperazone-treated mice with increased S_{obs} have increased abundance initially low abundant OTUs.

Relative abundance of each OTU plotted for mice treated with cefoperazone. OTUs arranged numerically along the x-axis. Each point is the relative abundance of a single OTU of an individual mouse. Split at S_{obs} = 120 to separate the communities that increased in α-diversity above the α-diversity in the untreated initial communities (Figure 3.3). S_{obs} < 120 - Initial N = 16, Time of Challenge N = 9, End N = 15; S_{obs} > 120 - Initial N = 0, Time of Challenge N = 6, End N = 1.

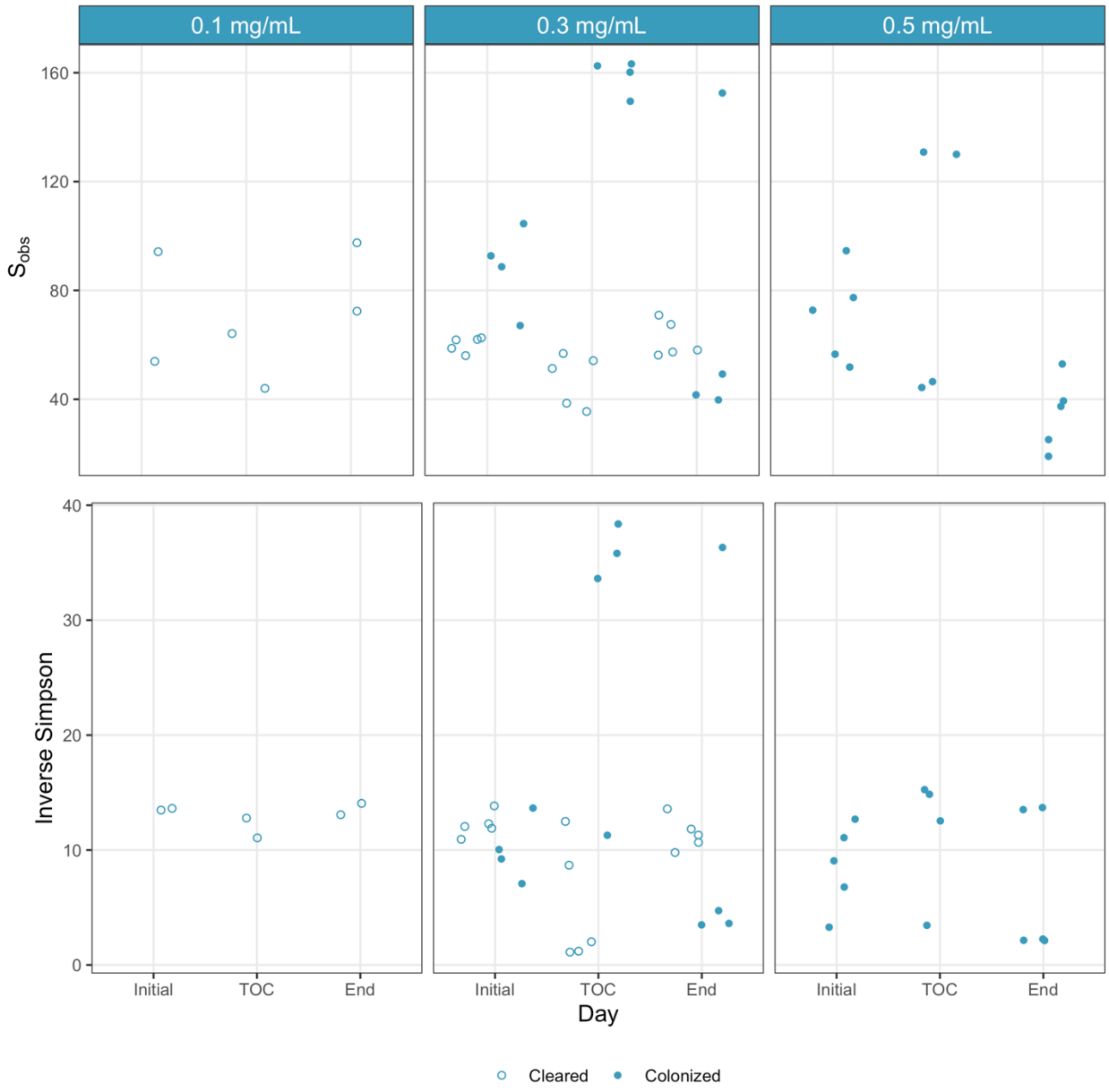


Figure 3.6. α-diversity of communities from cefoperazone-treated mice that remained colonized with *C. difficile* was not different by antibiotic dosage.

S_{obs} and inverse simpson were plotted by the time point, *C. difficile* colonization outcome, and cefoperazone dosage and tested by Wilcoxon rank sum test with Benjamini-Hochberg correction for differences. The group with the largest difference, at the time of challenge for mice that remained colonized, was not significant ($P = 0.1142857$). Mice that remained colonized are represented with filled points and those that cleared are unfilled. Points are shaped by cefoperazone dosage - circle 0.1 mg/mL, triangle 0.3 mg/mL, 0.5 mg/mL.

We next investigated the changes in OTU abundances between the communities that cleared *C. difficile* and those that did not to elucidate the community members involved in clearance. Communities that remained colonized were significantly enriched in facultative anaerobic populations including *Enterococcus*, *Pseudomonas*, *Staphylococcus*, and *Enterobacteriaceae* at the time of challenge. Communities that cleared *C. difficile* had significant enrichment in 10 different OTUs related to the *Porphyromonadaceae* at the end of the experiment (Figure 3.7A). We were also interested in the temporal changes within each community so we investigated which OTUs changed due to antibiotic treatment or during the *C. difficile* colonization. The majority of significant temporal differences in OTUs for cefoperazone-treated mice occurred in persistently colonized communities. Persistently colonized communities had a persistent loss of numerous relatives of the *Porphyromonadaceae* and increases in the relative abundance of facultative anaerobes (Figure 3.4C, 3.8). Overall, persistent *C. difficile* colonization in cefoperazone-treated mice was associated with a shift in the microbiota to a new community structure which was unable to recover from the antibiotic perturbation, whereas clearance occurred when the community was capable of returning to its original structure.

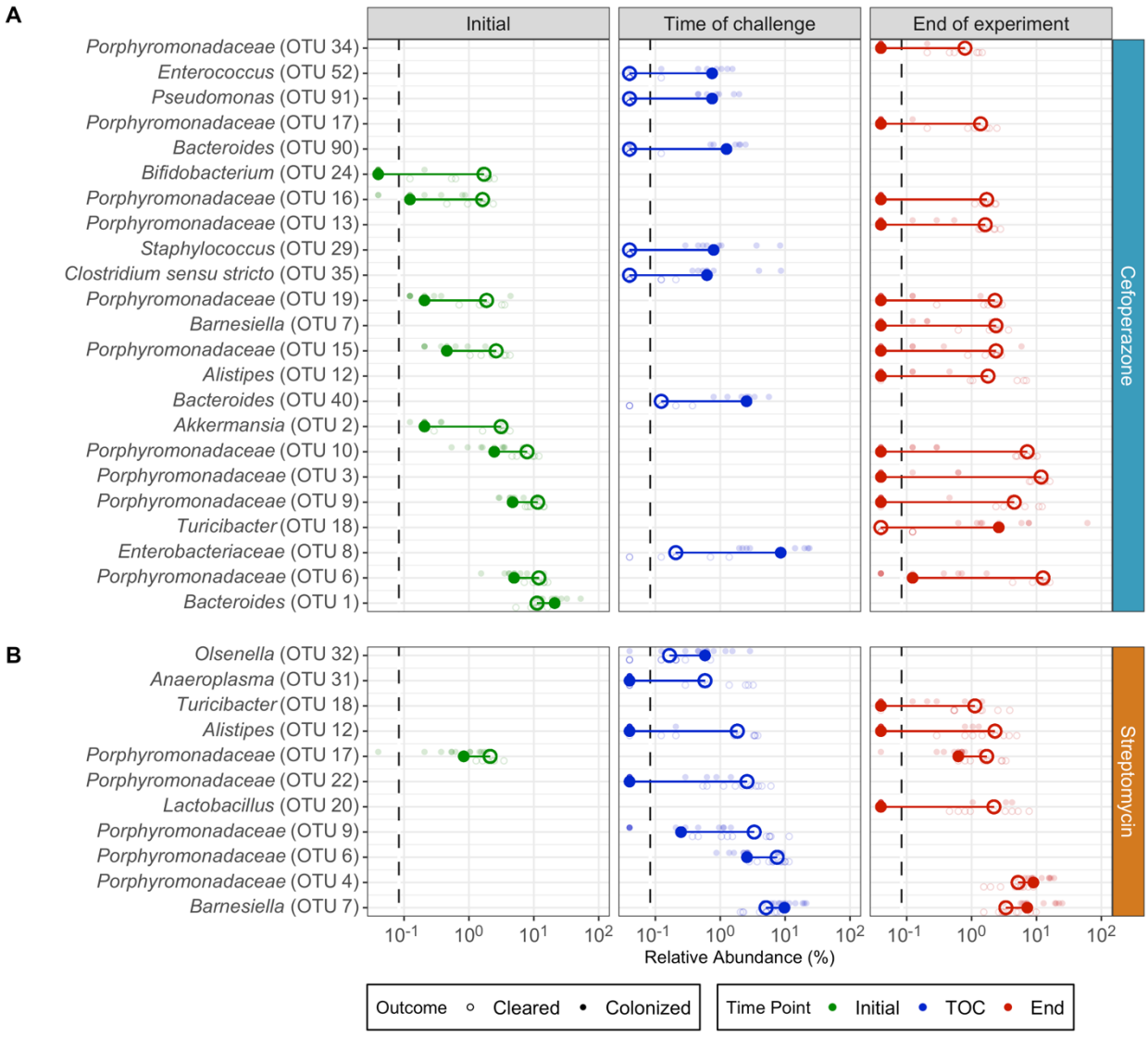


Figure 3.7. OTU abundance differences between communities that cleared *C. difficile* colonization and remained colonized are unique to each treatment.

For cefoperazone (A) and streptomycin (B), the difference in the relative abundance of OTUs that were significantly different between communities that eliminated *C. difficile* colonization and those that remained colonized within each antibiotic treatment for each time point were identified. Dark larger points in foreground are median relative abundance and transparent smaller points in background are relative abundance of individual mice. Lines connect points within each comparison to show difference in medians. Only OTUs at time points with statistically significant differences, $P < 0.05$, were plotted (calculated by Wilcoxon rank sum test with Benjamini-Hochberg correction). Limit of detection (LOD).

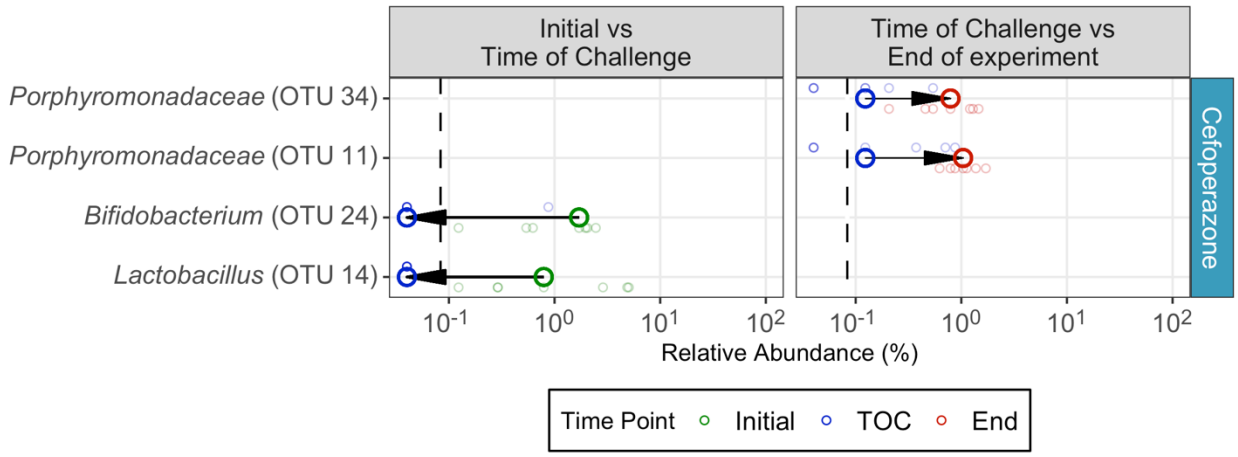


Figure 3.8. Temporally differing OTU for cefoperazone-treated mice that cleared *C. difficile* colonization.

Bold points are median relative abundance and transparent points are relative abundance of individual mice. Lines connect points within each comparison to show difference in medians. Arrows point in the direction of the temporal change of the relative abundance. Only OTUs at time points with statistically significant differences, $P < 0.05$, were plotted (calculated by Wilcoxon rank sum test with Benjamini-Hochberg correction). Limit of detection (LOD).

Finally, we identified the differences in *C. difficile* colonization for streptomycin-treated mice. Increasing the dose of streptomycin maintained the abundance of relatives of the *Porphyromonadaceae* and *Bacteroides*, but reduced most of the other genera including populations of the *Lactobacillus*, *Lachnospiraceae*, *Ruminococcaceae*, *Alistipes*, and *Clostridiales* (Figure 3.1F). Both communities that cleared and those that remained colonized had similar changes in diversity. Streptomycin-treated mice became mildly dissimilar ($P < 0.05$) and less diverse ($P < 0.05$) with streptomycin treatment but by the end of the experiment returned to resemble the pre-antibiotic community ($P < 0.05$) (Figure 3.3C). Those communities that remained colonized had slightly lower alpha-diversity than those that cleared *C. difficile*. ($P < 0.05$). Persistently colonized mice had reduced relative abundance of relatives of *Alistipes*, *Anaeroplasma*, and *Porphyromonadaceae* at time of challenge compared to the mice that cleared *C. difficile* (Figure 3.7B). At the end of the experiment the mice that were still colonized had lower abundances of *Turicibacter*, *Alistipes*, and *Lactobacillus*. Since most of the differences were reduced relative abundances in the colonized mice, we were interested to explore what temporal changes occurred between pre-antibiotic treatment, the time of challenge, and the end of the experiment for the communities that cleared *C. difficile*. The temporal changes in streptomycin-treated mice were more subtle than those observed with the other antibiotic treatments. At the time of challenge, the communities that remained colonized had reductions in 4 OTUs related to the *Porphyromonadaceae*. Those that cleared *C. difficile* also had changes in OTUs related to the *Porphyromonadaceae*, however, 2 populations decreased and 2 increased in abundance (Figure 3.4B, D). At the end of the experiment, all communities experienced recovery of the abundance of many of the populations changed by the streptomycin treatment, but the communities that remained colonized did not recover 5 of the OTUs of *Alistipes*, *Lactobacillus*,

and *Porphyromonadaceae* that were reduced by streptomycin. The differences between the streptomycin-treated mice that remained colonized and those that had been cleared of *C. difficile* were not as distinct as those observed with the cefoperazone treatment. The differences between colonized and cleared streptomycin-treated mice were minimal, which suggested the few differences may be responsible for the clearance. Overall, these data revealed that while there were commonly affected families across the antibiotic treatments, such as the *Porphyromonadaceae*, *C. difficile* clearance was associated with community and OTU differences specific to each antibiotic.

Distinct features of the bacterial community at the time of infection predicted end point colonization. To determine whether the community composition at the time of *C. difficile* challenge could predict *C. difficile* clearance, we built a machine learning model using L2 logistic regression. We modeled all treatments together to prevent overfitting of the data and allow the model to reveal which OTUs were able to correctly predict clearance in the context of the other OTU relative abundances. We evaluated the predictive performance of the model using the area under the receiver operating characteristic curve (AUROC), where a value of 0.5 indicated the model was random and 1.0 indicated the model always correctly predicts the outcome. Our model resulted in a AUROC of 0.986 [IQR 0.970-1.000], which suggested that the model was able to use the relative abundance of OTUs at the time of challenge to accurately predict colonization clearance (Figure 3.9). To assess the important features, we randomly permuted each OTU feature by removing it from the training set to determine its effect on the prediction (Figure 3.10A). The most important feature was an OTU related to the *Enterobacteriaceae*, whose abundance predicted clearance. This result appears to have been strongly driven by the clindamycin data (Figure 3.10B, C). The remaining OTUs did not have a

large effect on the model performance, which suggested that the model decision was spread across many features. These results revealed the model used the relative abundance data of the community members and the relationship between those abundances to correctly classify clearance. There were many OTUs with treatment and outcome specific abundance patterns that did not agree with the odds ratio of the OTU used by the model. For example, *Enterobacteriaceae* abundance influenced the model to predict clearance (Figure 3.10B), however in experiments that used cefoperazone, the communities that remained colonized had higher abundances of *Enterobacteriaceae* than the communities that cleared colonization (Figure 3.10C). The model arrived at the correct prediction through the collective influence of other OTUs. Therefore, the model used different combinations of multiple OTUs and their relative abundances across treatments to predict *C. difficile* clearance. These data can offer a basis for hypotheses regarding the distinct combinations of bacteria that promote *C. difficile* clearance.

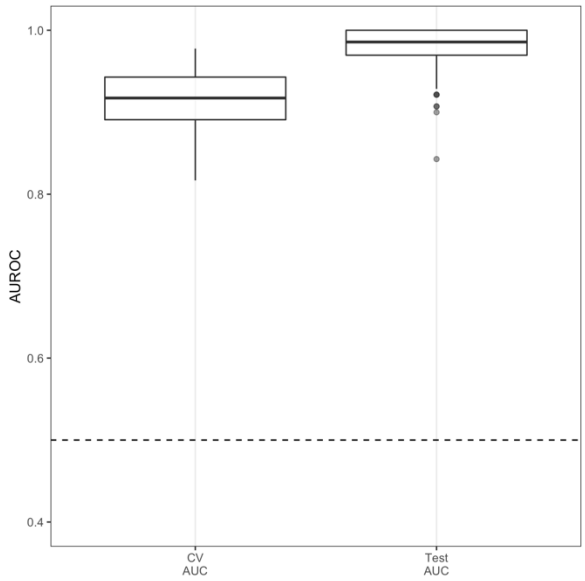


Figure 3.9. Bacterial community at the time of infection can classify endpoint colonization.

Classification performance of L2 logistic regression. Area under the receiver-operator curve for classifying if the community will remain colonized based on the OTUs present at the time of *C. difficile* infection (Day 0). Cross-validation of model performed on half of the data to tune model (CV AUC) and then tuned model was tested on the held-out data (Test AUC).

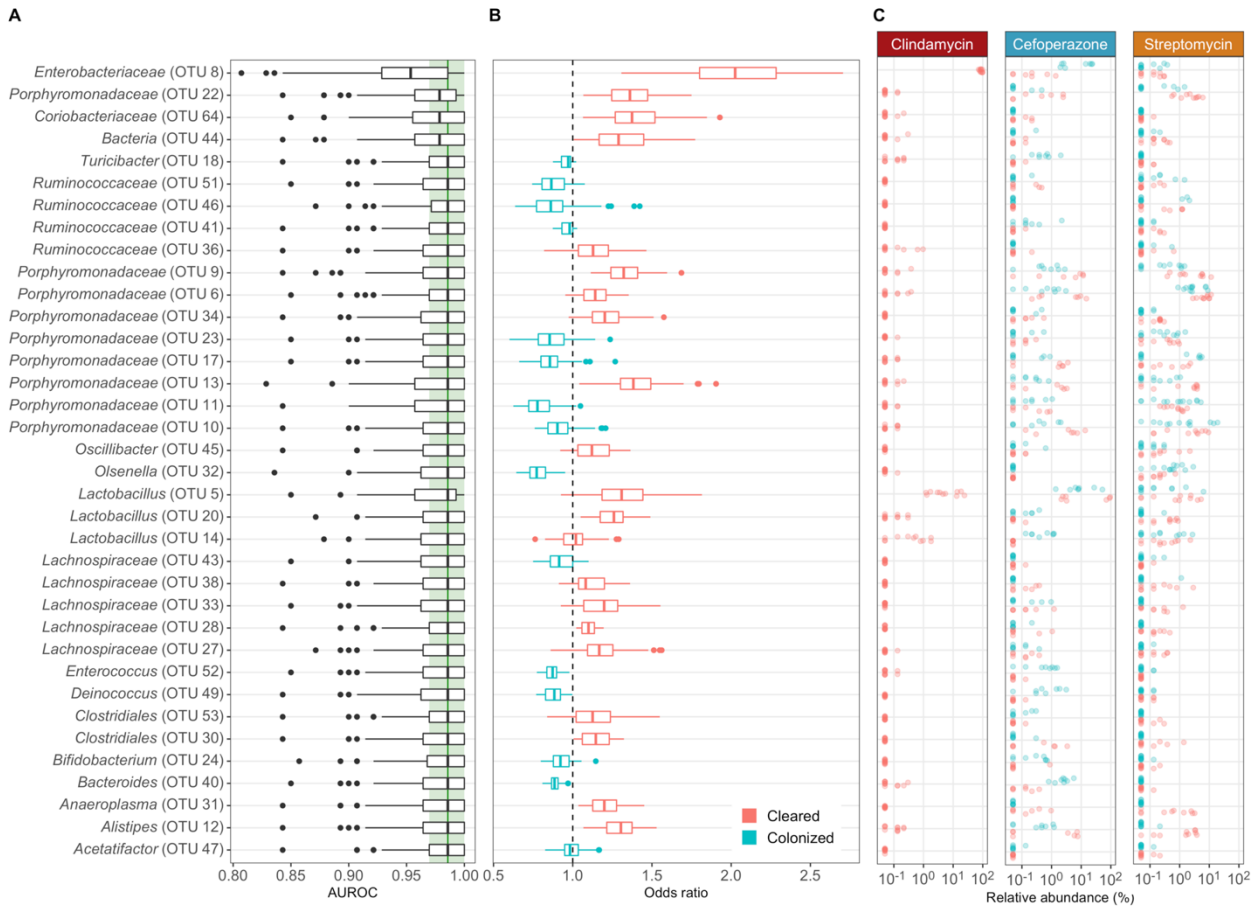


Figure 3.10. Distinct features of the bacterial community at the time of infection can classify end point colonization.

(A) L2 logistic regression model features' importance determined by the decrease in model performance when randomizing an individual feature. All OTUs affecting performance shown. Light green band in the background shows the interquartile range and the dark green line shows the median AUROC of the final model with all features included. (B) Distribution of odds ratio used in L2 logistic regression model. Values above 1 indicate abundance predicted the community cleared colonization (red) and values below 1 indicate abundance predicted *C. difficile* remained colonized (blue). Feature label and boxplot are colored to match the median odds ratio. (C) Relative abundance difference in features used by L2 logistic regression model displayed by antibiotic treatment.

Conditional independence networks revealed treatment-specific relationships between the community members and *C. difficile* during colonization clearance. Finally, we explored the relationship between temporal changes in the community and *C. difficile* by building a conditional independence network for each treatment using SPIEC-EASI (sparse inverse covariance estimation for ecological association inference) (129). First, we focused on the first-order associations of *C. difficile* (Figure 3.11A). In clindamycin-treated mice, *C. difficile* had positive associations with relatives of *Enterobacteriaceae*, *Pseudomonas*, and *Olsenella* and negative associations with relatives of the *Lachnospiraceae* and *Clostridium* XIVa. *C. difficile* had limited associations in cefoperazone-treated mice; the primary association was positive with relatives of *Enterobacteriaceae*. In streptomycin-treated mice, *C. difficile* had negative associations with relatives of the *Porphyromonadaceae* and positive associations with populations of the *Ruminococcaceae*, *Bacteroidetes*, *Clostridium* IV and *Olsenella*. Next, we quantified the degree centrality, the number of associations between each OTU for the whole network of each antibiotic and outcome, and betweenness centrality, the number of associations connecting two OTUs that pass through an OTU (Figure 3.11B). This analysis revealed cefoperazone treatment resulted in networks primarily composed of singular associations with much lower degree centrality ($P < 0.05$) and betweenness centrality ($P < 0.05$) than the other antibiotic treatments. Communities that were treated with cefoperazone that resulted in cleared or persistent colonization had 10 to 100-fold lower betweenness centrality values than communities treated with clindamycin or streptomycin. Collectively, these networks suggest *C. difficile* colonization was affected by unique sets of OTUs in mice treated with clindamycin and streptomycin, but cefoperazone treatment eliminated bacteria critical to maintaining community interactions and had few populations that associated with *C. difficile*.

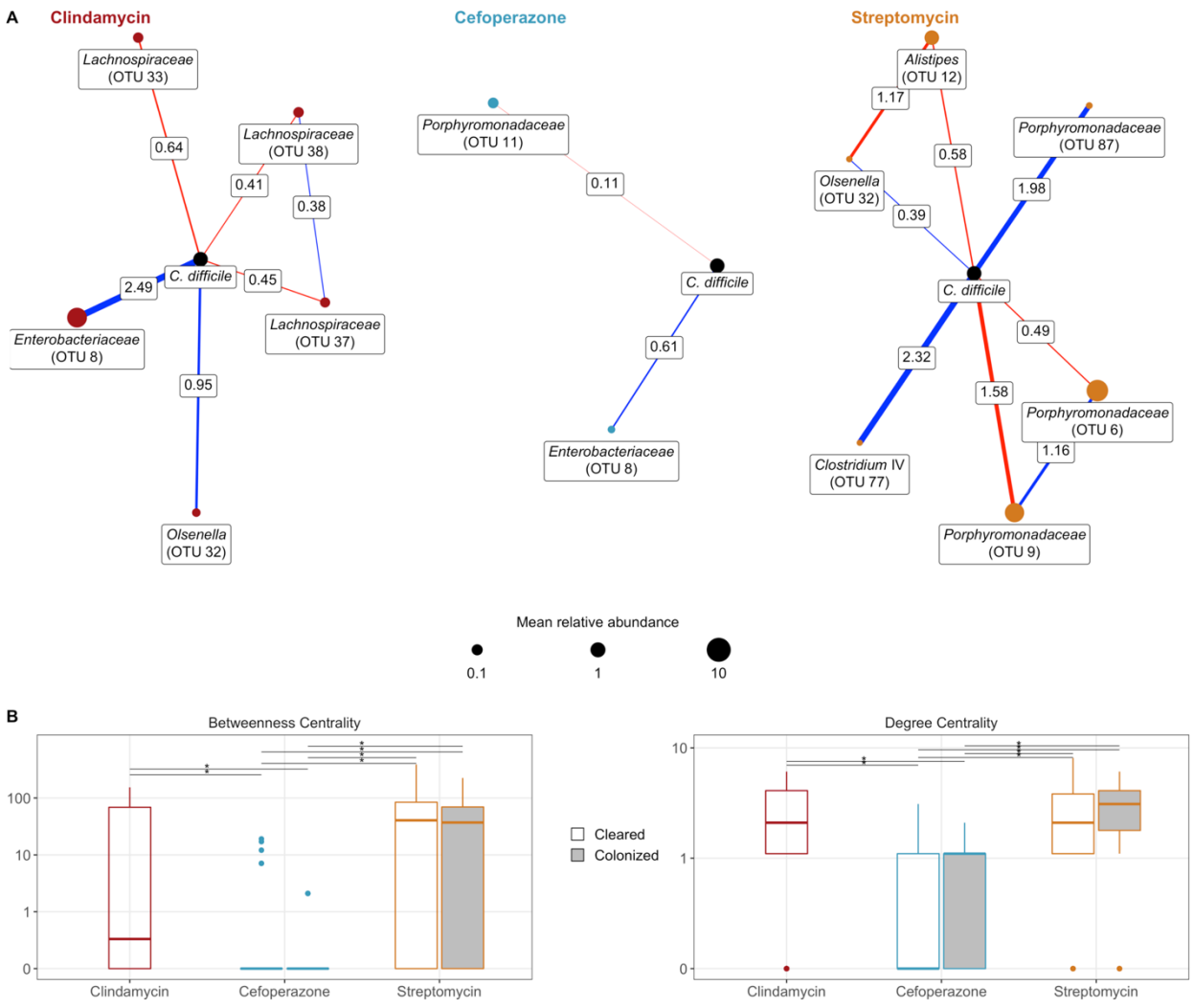


Figure 3.11. Conditional independence networks reveal treatment-specific relationships between the community and *C. difficile* during colonization clearance.

(A) SPIEC-EASI (sparse inverse covariance estimation for ecological association inference) networks showing conditionally independent first-order relationships between *C. difficile* and the community as *C. difficile* was cleared from the gut environment. Nodes are sized by median relative abundance of the OTU. A red colored edge indicates a negative interaction and blue indicates a positive interaction, while edge thickness indicates the interaction strength. (B) Network centrality measured with betweenness, i.e. how many paths between two OTUs pass through an individual, and degree, i.e. how many connections an OTU had. * indicates statistical significance of $P < 0.05$, calculated by Wilcoxon rank sum test with Benjamini-Hochberg correction.

3.6 Discussion

We have shown that different antibiotic treatments resulted in specific changes to the microbiota that were associated with *C. difficile* clearance. Clindamycin-treated mice became susceptible with a dominant bloom in populations related to *Enterobacteriaceae*. Clearance was associated with the resolution of the bloom and recovery of bacteria that were reduced by the antibiotic treatment. Cefoperazone-treated mice became susceptible with the expansion of numerous facultative anaerobes. Communities with a sustained presence of these facultative anaerobes were unable to recover from the initial antibiotic perturbation or clear the colonization, whereas the communities that returned to their initial community were able to clear *C. difficile* colonization. Streptomycin-treated mice became susceptible with fewer and smaller changes than the other treatments. The communities that cleared colonization had slightly higher α-diversity than those that remained colonized. Additionally, all communities in mice treated with streptomycin had similar numbers of OTUs changing through the experiment but the specific OTUs were different for each outcome. These observations support our hypothesis that each colonized community has antibiotic-specific changes that create unique conditions for *C. difficile* colonization and requires specific changes within each community to clear *C. difficile*.

Previous studies have identified microbiota associated with reduced *C. difficile* colonization in either a set of closely related murine communities or collectively across many different susceptible communities (34, 36, 44). Bacteria from these studies have since been tested in *C. difficile* infection models. These experiments either showed decreased colonization not elimination of *C. difficile* (35, 36) or only demonstrated elimination in the model it was developed (44). Rather than looking for similarities across all susceptible communities, we explored the changes that were associated with *C. difficile* clearance for each antibiotic. Even

though these mice all came from the same breeding colony with similar initial microbiomes, *C. difficile* clearance was associated with antibiotic-specific changes in community diversity, OTU abundances, and associations between OTUs. Our data suggest that the set of bacteria necessary to restore colonization resistance following one antibiotic perturbation may not be effective for all antibiotic perturbations. We have developed this modeling framework starting from a single mouse community. It should also be relevant when considering interpersonal variation among humans (46).

Recent studies have begun to uncover how communities affect *C. difficile* colonization (33, 43, 45, 46, 73). We attempted to understand the general trends in each antibiotic treatment that lead to clearance of *C. difficile*. We categorized the general changes and microbial relationships of these experiments into three models. First, a model of temporary opportunity characterized by the transient dominance of a facultative anaerobe which permits *C. difficile* colonization but *C. difficile* is not able to persist, as with clindamycin treatment. We hypothesize this susceptibility is due to a transient repression of community members and interventions which further perturb the community may worsen the infection. Time alone may be sufficient for the community to clear colonization (34, 44, 130) but treating the community with an antibiotic or the bowel preparation for an FMT (131, 132) may prolong susceptibility by eliminating protective functions or opening new niches. Second, a model of an extensive opportunity characterized by a significant perturbation leading to a persistent increase in facultative anaerobes and exposing multiple niches, as with cefoperazone treatment. These communities appear to have been severely depleted of multiple critical community members and are likely lacking numerous protective functions (73). We hypothesize multiple niches are made available for *C. difficile* to colonize through reduced populations of bacteria that produce inhibitory

molecules or compete for either nutrients or space, increasing available resources. In this scenario, community restoration will require transplantation with microbes that provide adequate diversity and abundance to outcompete and occupy all the exposed niches. If this diversity is not provided through a single FMT, multiple FMTs (133, 134) or transplant of an enriched fecal community (97) may be necessary to recover the microbiota enough to outcompete *C. difficile* for the nutrient niches and replace the missing protective functions. Third, a model of a specific opportunity characterized by a perturbation that only affects a select portion of the microbiota, leading to small changes in relative abundance and a slight decrease in diversity, opening a limited niche for *C. difficile* to colonize, as with streptomycin treatment. We hypothesize that a few specific bacterial species with key inhibitory functions would be necessary to recolonize the exposed niche and eliminate *C. difficile* colonization (45, 127). A fecal microbiota transplant may contain the bacterial diversity needed to fill the open niche and help supplant *C. difficile* from the exposed niche of the colonized community. Analyzing each of these colonization models individually allowed us to understand how each may clear *C. difficile* colonization.

Future investigations can further identify the exposed niches of susceptible communities and the requirements to clear *C. difficile* colonization. One common theme for susceptibility across treatments was the increased abundance of facultative anaerobes. These blooms of facultative anaerobes could be attributed to the loss of the indigenous obligate anaerobes with antibiotic treatment (135, 136). However, it is unclear what prevents the succession from the facultative anaerobes back to the obligate anaerobes in cefoperazone-treated mice. Future studies should investigate the relationship between facultative anaerobe blooms and susceptibility to colonization as well as interventions to recover the obligate anaerobes. Another aspect to consider in future experiments is *C. difficile* strain specificity. Other strains may fill different

niches and fill other community interactions (9, 16, 64). For example, more virulent strains, like *C. difficile* VPI 10463, may have a greater effect on the gut environment since it produces more toxin and drives a stronger immune response (44, 64, 67). Those differences could lead to greater increases in inflammatory conditions and further increase populations that thrive in these conditions, such as *Enterobacteriaceae*, and change the requirements to clear *C. difficile* (135, 137, 138). Finally, we have shown that the functions found in communities at peak colonization were antibiotic-specific (73). We found that the bacterial population changes associated with *C. difficile* clearance were antibiotic-specific. It is unknown how the community functions contributing to *C. difficile* clearance compare across antibiotics. It is possible while we observed different changes in the bacteria populations but the functions eliminating *C. difficile* were conserved. Additionally, it is unclear how specific these functions are to the OTUs we observed. It is possible that phylogentically diverse OTUs have similar functional potiential as well as phylogentically similar OTUs having specific functions. Examining the changes in transcription and metabolites during clearance will help define the activities necessary to clear *C. difficile* and if they are specific to the perturbation. This information will build upon the community differences presented in this study and move us closer to elucidating how the microbiota clears *C. difficile* colonization and developing targeted therapeutics.

We have shown that mice became susceptible to *C. difficile* colonization after three different antibiotic treatments and then differed in their ability to clear the colonization. These experiments have shown that each antibiotic treatment resulted in different community changes leading to *C. difficile* clearance. These differences suggest that a single mechanism of infection and one treatment for all *C. difficile* infections may not be appropriate. While our current use of FMT to eliminate CDI is highly effective, it does not work in all patients and has even resulted in

adverse consequences (20, 124–126). The findings in this study may help explain why FMTs may be ineffective. Although an FMT transplants a whole community, it may not be sufficient to replace the missing community members or functions to clear *C. difficile*. Alternatively, the FMT procedure itself may disrupt the natural recovery of the community. The knowledge of how a community clears *C. difficile* colonization will advance our ability to develop targeted therapies to manage CDI.

3.7 Methods

Animal care. 5- to 8-week-old male and female C57BL/6 mice were obtained from a single breeding colony. Mice were housed in cages of 2-5 mice maintained in specific-pathogen-free (SPF) conditions at the University of Michigan animal facility. Each experimental treatment used 6-11 mice and was repeated 2-4 times. All mouse protocols and experiments were approved by the University Committee on Use and Care of Animals at the University of Michigan and completed in agreement with approved guidelines.

Antibiotic administration. Mice were given one of three antibiotics, cefoperazone, clindamycin, or streptomycin. Cefoperazone (0.5, 0.3, or 0.1 mg/ml) and streptomycin (5, 0.5, or 0.1 mg/ml) were delivered via drinking water for 5 days. Clindamycin (10 mg/kg) was administered through intraperitoneal injection.

***C. difficile* challenge.** Mice were returned to untreated drinking water for 24 hours before challenging with *C. difficile* strain 630Δerm spores. *C. difficile* spores were aliquoted from a single spore stock stored at 4°C. Spore concentration was determined one week prior to the day of challenge (110). 10^3 *C. difficile* spores were orally gavaged into each mouse. Once the

gavages were completed, the remaining spore solution was serially diluted and plated to confirm the spore concentration that was delivered.

Sample collection. Fecal samples were collected on the day antibiotic treatment was started, on the day of *C. difficile* challenge and the following 10 days. For the day of challenge and beyond, a fecal sample was also collected and weighed. Under anaerobic conditions a fecal sample was serially diluted in anaerobic phosphate-buffered saline and plated on TCCFA plates. After 24 hours of anaerobic incubation at 37°C, the number of colony forming units (CFU) were determined (111).

DNA sequencing. Total bacterial DNA was extracted from each fecal sample using MOBIO PowerSoil-htp 96-well soil DNA isolation kit. We created amplicons of the 16S rRNA gene V4 region and sequenced them using an Illumina MiSeq as described previously (112).

Sequence curation. Sequences were processed using mothur(v.1.43.0) as previously described (112). Briefly, we used a 3% dissimilarity cutoff to group sequences into operational taxonomic units (OTUs). We used a naive Bayesian classifier with the Ribosomal Database Project training set (version 16) to assign taxonomic classifications to each OTU (114). With the fecal samples, we also sequenced a mock community with a known community composition and their true 16s rRNA gene sequences. We processed this mock community along with our samples to determine our sequence curation resulted in an error rate of 0.019%.

Statistical analysis and modeling. Diversity comparisons were calculated in mothur. To compare α-diversity metrics, we calculated the number of OTUs (S_{obs}) and the Inverse Simpson diversity index. To compare across communities, we calculated dissimilarity matrices based on metric of Yue and Clayton (115). All calculations were made by rarifying samples to 1,200

sequences per sample to limit biases due to uneven sampling. OTUs were subsampled to 1,200 counts per sample and remaining statistical analysis and data visualization was performed in R (v3.5.1) with the tidyverse package (v1.3.0). Significance of pairwise comparisons of α-diversity (S_{obs} and Inverse Simpson), β-diversity (θ_{YC}), OTU abundance, and network centrality (betweenness and degree) were calculated by pairwise Wilcoxon rank sum test and then *P* values were corrected for multiple comparisons with a Benjamini and Hochberg adjustment for a type I error rate of 0.05 (117). Logistic regression models were constructed with OTUs from all day 0 samples using half of the samples to train and the other half to test the model. The model was developed from the caret R package (v6.0-85) and previously developed machine learning pipeline (139). For each antibiotic treatment, conditional independence networks were calculated from the day 1 through 10 samples of all mice initially colonized using SPIEC-EASI (sparse inverse covariance estimation for ecological association inference) methods from the SpiecEasi R package after optimizing lambda to 0.001 with a network stability between 0.045 and 0.05 (v1.0.7) (129). Network centrality measures degree and betweenness were calculated on whole networks using functions from the igraph R package (v1.2.4.1).

Chapter IV

Simplified Fecal Community Transplant Restores *Clostridioides difficile* Colonization Resistance to Antibiotic-Specific Perturbed Murine Communities

4.1 Notes

This work was reprinted and modified with permission from Lesniak NA, Tomkovich S, Henry A, Taylor A, Colovas J, Bishop L, McBride K, Schloss PD. 2022. Simplified fecal community transplant restores *Clostridioides difficile* colonization resistance to antibiotic-specific perturbed murine communities. doi:10.1101/2022.03.XX.XXXXXX. This work was in preparation for submission in March 2022.

Conceptualization: N.A.L., S.T., P.D.S.; Data curation: N.A.L., L.B., K.M.; Formal analysis: N.A.L., ; Investigation: N.A.L., S.T., A.H., A.T., J.C., L.B., K.M., P.D.S.; Methodology: N.A.L., S.T., P.D.S.; Resources: N.A.L., S.T., L.B., K.M., P.D.S.; Software: N.A.L.; Visualization: N.A.L., P.D.S.; Writing - original draft: NAL; Writing - review & editing: N.A.L., ST AH AT JC LB KB P.D.S.; Funding acquisition: P.D.S.; Project administration: P.D.S.; Supervision: P.D.S.

4.2 Abstract

Fecal communities are transplanted into individuals to eliminate recurrent *Clostridioides difficile* infection (CDI) with high efficacy. However, this treatment is only used once CDI becomes recalcitrant to antibiotics or has recurred at least twice. We sought to investigate whether a fecal community transplant (FCT) pre-treatment could be used to prevent CDI altogether. We treated male C57BL/6 mice with either clindamycin, cefoperazone, or streptomycin, and then inoculated them with FCT from untreated mice before challenging them with *C. difficile*. We measured colonization throughout the experiment and sequenced the V4 region of the 16S rRNA gene to understand the dynamics of the murine fecal community in response to FCT and *C. difficile* challenge. Clindamycin-treated mice became colonized with *C. difficile* but cleared it naturally and did not benefit from FCT. Cefoperazone-treated mice became colonized by *C. difficile*, but FCT enabled clearance of *C. difficile*. In streptomycin-treated mice, FCT was able to prevent *C. difficile* from colonizing. Then we diluted FCT and repeated the experiments. Cefoperazone-treated mice no longer eliminated *C. difficile*. However, streptomycin-treated mice colonized with $1{:}10^2$ dilutions resisted *C. difficile* colonization. Streptomycin-treated mice that received a FCT diluted $1{:}10^3$, *C. difficile* colonized but later was eliminated. In streptomycin-treated mice, inhibition of *C. difficile* was associated with increased relative abundance of a select group of operational taxonomic units related to *Porphyromonadaceae* and *Lachnospiraceae*. These data have demonstrated *C. difficile* colonization resistance can be restored to a community susceptible via FCT but is dependent on gut community changes causing the susceptibility.

4.3 Importance

Antibiotic use, ubiquitous within the healthcare environment, is a major risk factor for *Clostridioides difficile* infection (CDI), the primary nosocomial infection. When CDI becomes recalcitrant to antibiotics, a fecal microbiota transplant from a healthy individual can effectively restore the gut bacterial community and eliminate the infection. While this relationship between the gut bacteria and CDI is well established, there are no therapies to treat a perturbed gut community to prevent CDI. This study explores the potential of restoring colonization resistance to antibiotic-induced susceptible gut communities. We describe the effect gut bacteria community variation has on the effectiveness of a fecal community transplant for inhibiting CDI. These data demonstrate that communities susceptible to CDI can be supplemented with fecal communities but the effectiveness is sensitive to the antibiotic perturbation. Thus, it may be possible to use a simplified bacterial community to recover colonization resistance to patients on antibiotics.

4.4 Introduction

The process by which gut bacteria prevent *Clostridioides difficile* and other pathogens from infecting the intestine is known as colonization resistance. Antibiotic-induced disruption of the gut bacterial community breaks down colonization resistance and is a major risk factor for *C. difficile* infection (CDI) (140). Gut bacteria inhibit *C. difficile* through the production of bacteriocins, modulation of available bile acids, competition for nutrients, production of short-chain fatty acids and altering the integrity of the mucus layer (14). After the initial CDI is cleared via antibiotics, the patient may become reinfected. When CDI recurs more than once, the gut bacterial community from a healthy person typically is used to restore the gut community in the

patient with recurrent CDI. Fecal microbiota transplant (FMT) has high cure rate, but 10 - 20% of people that receive a FMT will have another recurrence of CDI (141). Additionally, transfer of a whole fecal community can incidentally transfer pathogens and cause adverse outcomes (20). While the FMT is effective at curing recurrent CDI, it also has risks that must be considered.

The benefits and risks of the FMT have led to the development of simplified bacterial communities to treat CDI. Synthetic communities allow for more control and testing necessary for regulatory approval of this type of treatment. Tvede and Rask-Madsen were the first to successfully treat CDI with a community of isolates cultured from human feces (142). More recently, Lawley *et al.* analyzed murine experiments and the fecal communities from patients with CDI to develop a synthetic community of six isolates to inhibit *C. difficile* colonization (44). Recently developed simplified communities derived from human fecal communities, by methods such as selective isolation of spores or culturing bacteria, cured recurrent CDI in their initial application (143–145). Although, a recent phase 2 trial of SER-109, a spore-based treatment, failed its phase 2 clinical trial (146), these therapies have the potential to offer the benefits of the FMT without the associated risks but are only used once a patient has had multiple CDIs. For these to be successful, we need a better approach to identify candidate bacterial populations. Recently, an autologous FMT was shown to be effective at restoring the gut microbiota in allogeneic hematopoietic stem cell transplantation patients and prevented future complications, such as systemic infections (147). It is unclear whether a similar technique or simplified bacterial communities could be used to restore susceptible communities and prevent CDI (148). Since *C. difficile* colonizes different niches and the gut bacterial community responds differently to clear *C. difficile*, a treatment may be effective for one community and not another (25, 59).

FMT is sufficient to restore colonization resistance to people with a current infection. Thus, a fecal community should be sufficient to restore colonization to an uninfected community. We hypothesized that pre-treatment with a fecal community could restore colonization resistance to a subset of perturbed gut microbiota. Therefore, we tested whether a fecal community transplant (FCT) pre-treatment would prevent *C. difficile* colonization and how variation in susceptibility to *C. difficile* infection would alter the effectiveness of FCT pre-treatment. After testing the same FCT pre-treatment across different antibiotic-induced susceptibilities, we sought to determine whether diluted FCT pre-treatment could maintain the inhibition of *C. difficile* colonization.

4.5 Results

Effect of fecal transplant on *C. difficile* colonization was not consistent across antibiotic treatments. Our previous research had demonstrated that in mice perturbed with a different antibiotic, there was an antibiotic-specific change to the microbial community that also resulted in a difference in the ability to clear a *Clostridioides difficile* infection (59). We hypothesized that each of these treatments opened different niches that *C. difficile* exploited and that the resulting community varied in the types of bacteria required to recover colonization resistance. To test the ability of the murine communities to recover colonization resistance, we treated C57BL/6 mice with either clindamycin, cefoperazone, or streptomycin. After a short recovery period, the mice were given either phosphate-saline buffer (PBS) or a fecal community transplant via oral gavage (Figure 4.1). A day after receiving the oral gavage, the mice were challenged with 10^3 *C. difficile* 630 spores. Mice that were treated with either clindamycin or cefoperazone and received the fecal community transplant (FCT) pre-treatment had similar amounts of *C. difficile* colony forming units (CFU) as those which received PBS 1 day post

infection. In the clindamycin-treated mice, *C. difficile* colonization was cleared at similar rates between the FCT and PBS pre-treatments (Figure 4.2). For cefoperazone-treated mice, *C. difficile* colonized all mice, but mice that received the FCT pre-treatment cleared colonization (Figure 4.2). For the streptomycin-treated mice, the FCT pre-treatment resulted in either no detectable *C. difficile* colonization (8 of14) or an infection that the community cleared within 5 days (Figure 4.2). FCT treatment had different effects based on the antibiotic treatment and was only able to prevent colonization for some of the streptomycin-treated mice.

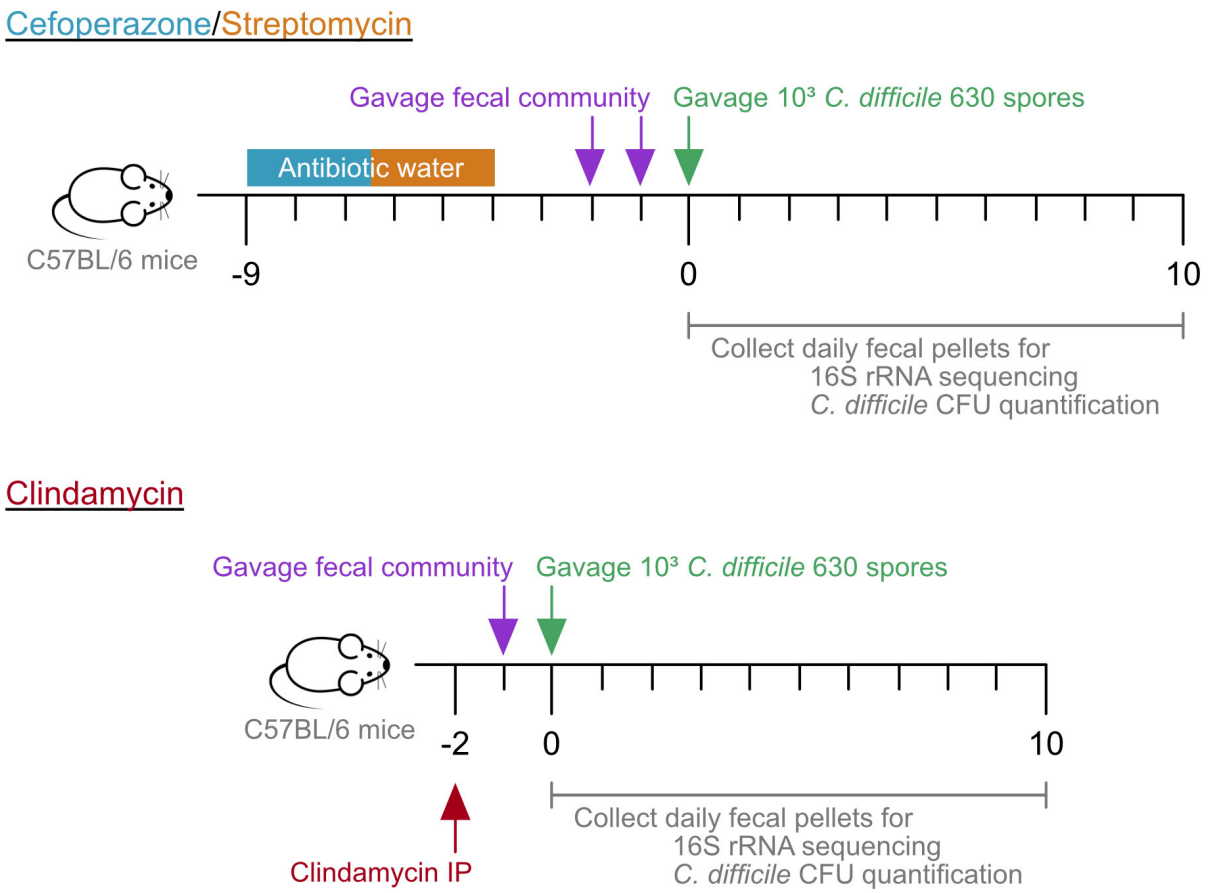


Figure 4.1. Mouse experiment timeline.

For cefoperazone and streptomycin, male C57BL/6 mice were given water with cefoperazone (0.5 mg/ml) or streptomycin (5 mg/ml) for 5 days. The mice were returned to untreated water for the remainder of the experiment. Two days after the antibiotic water was removed, mice were orally gavaged 100 μl of PBS or fecal community, once a day for two days. The following day, the mice were challenged with 10^3 *C. difficile* 630 spores. For clindamycin, male C57BL/6 mice were given an intraperitoneal injection of clindamycin (10 mg/kg) 2 days prior to *C. difficile* infection. 24 hours later, mice were orally gavaged with 100 μl of PBS or fecal community. The following day, the mice were challenged with 10^3 *C. difficile* 630 spores. Fecal pellets were collected prior to treatment (day -9 for cefoperazone/streptomycin, day -2 for clindamycin), cessation of antibiotics (day -2 for cefoperazone/streptomycin, day -1 for clindamycin), prior to *C. difficile* infection (day 0), and each of the following 10 days.

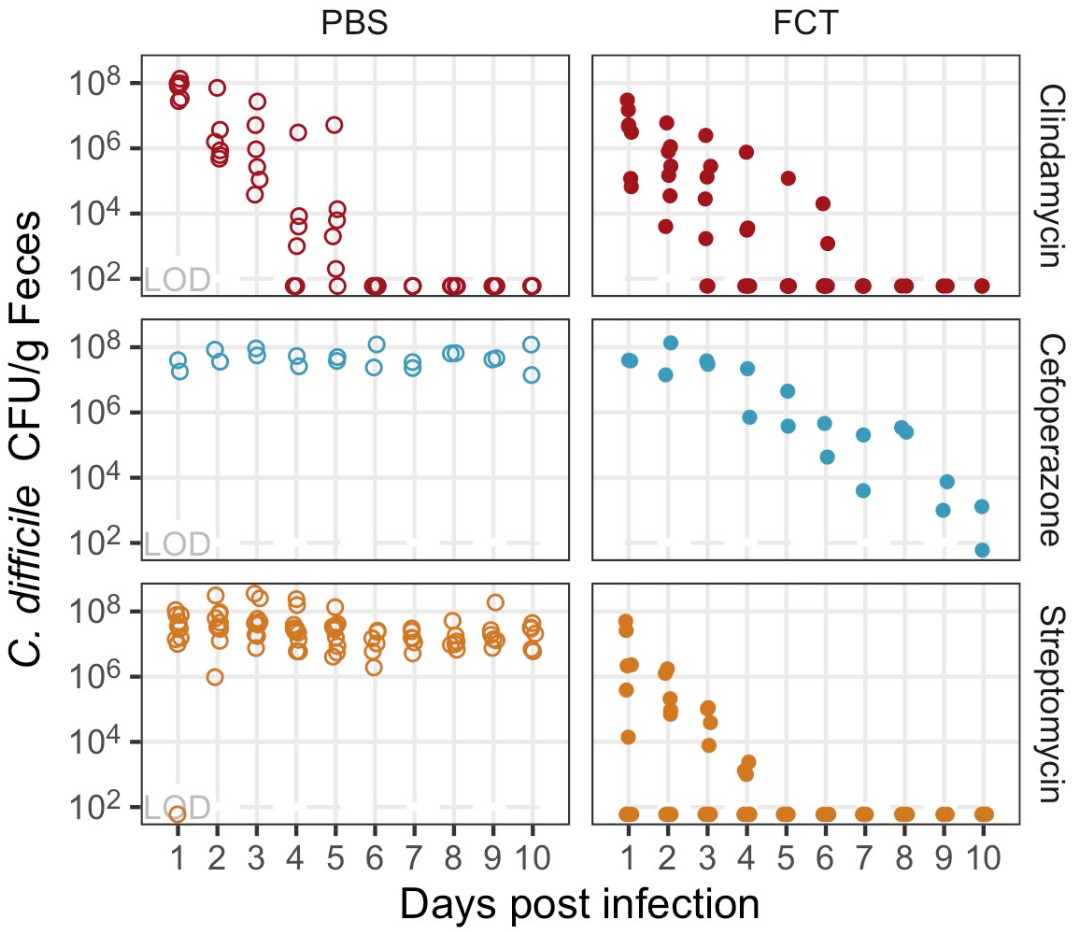


Figure 4.2. Fecal community transplant inhibited *C. difficile* colonization for mice treated with cefoperazone or streptomycin.

C. difficile CFU per gram of feces for mice treated with clindamycin (red points), cefoperazone (blue points), or streptomycin (orange points). Mice were orally gavaged either PBS (open circles) or FCT (fecal community transplant, filled circles) prior to the *C. difficile* infection. Each point represents an individual mouse. LOD = limit of detection.

Diluted fecal communities prevented colonization and promoted clearance for streptomycin-treated mice. Next, we sought to test whether diluted FCT pre-treatment could maintain its effect. To identify the minimally effective dilution, we repeated the same experimental design (Figure 4.1) with the FCT diluted serially down to $1:10^5$. Since FCT pre-treatment had no detected effect in clindamycin-treated mice, we did not study them further. Cefoperazone-treated mice pre-treated with diluted FCT, 1:10 and lower, were not affected and were colonized throughout the experiment (Figure 4.3). Streptomycin-treated mice pre-treated with diluted FCT either regained colonization resistance or were enabled to clear *C. difficile*. The streptomycin-treated mice pre-treated with FCT as dilute as $1:10^3$ cleared *C. difficile*. The streptomycin-treated mice pre-treated with FCT as dilute as $1:10^2$ had no *C. difficile* CFU detected throughout the length of the experiment. While more mice pre-treated with lower FCT dilutions were colonized (FCT 6 of 14 were colonized, 1:10 10 of 12 were colonized, $1:10^2$ 10 of 14 were colonized), the colonized mice that received the lower dilutions were still able to clear *C. difficile* (Figure 4.4). Thus, the simplified fecal communities of diluted FCT were able to restore colonization resistance and promote clearance of *C. difficile* in streptomycin-treated mice.

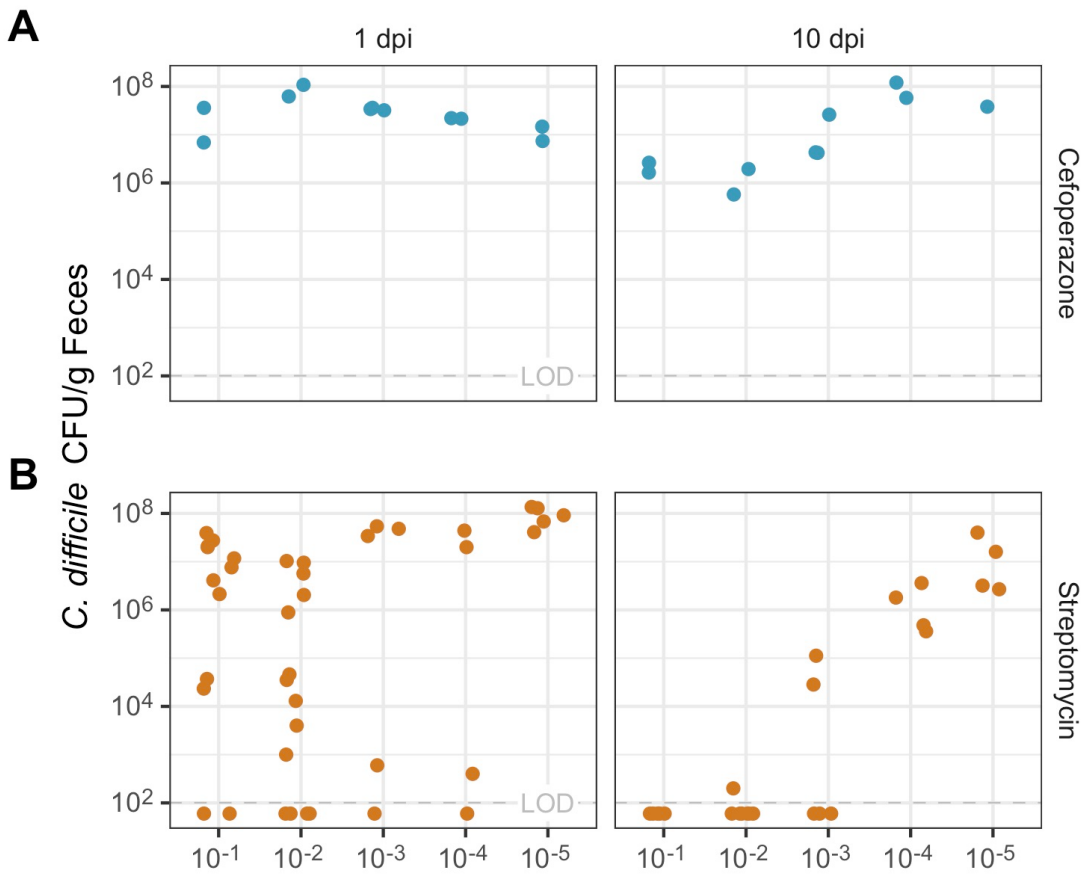


Figure 4.3. Diluted FCT inhibited *C. difficile* colonization for mice treated with streptomycin.

C. difficile CFU per gram of feces for mice treated with (A) cefoperazone (blue points) or (B) streptomycin (orange points). Mice were orally gavaged with a dilution of FCT (1:10 to $1:10^5$) prior to the *C. difficile* infection at one day post *C. difficile* infection (dpi) and 10 dpi. Each point represents an individual mouse. LOD = limit of detection.

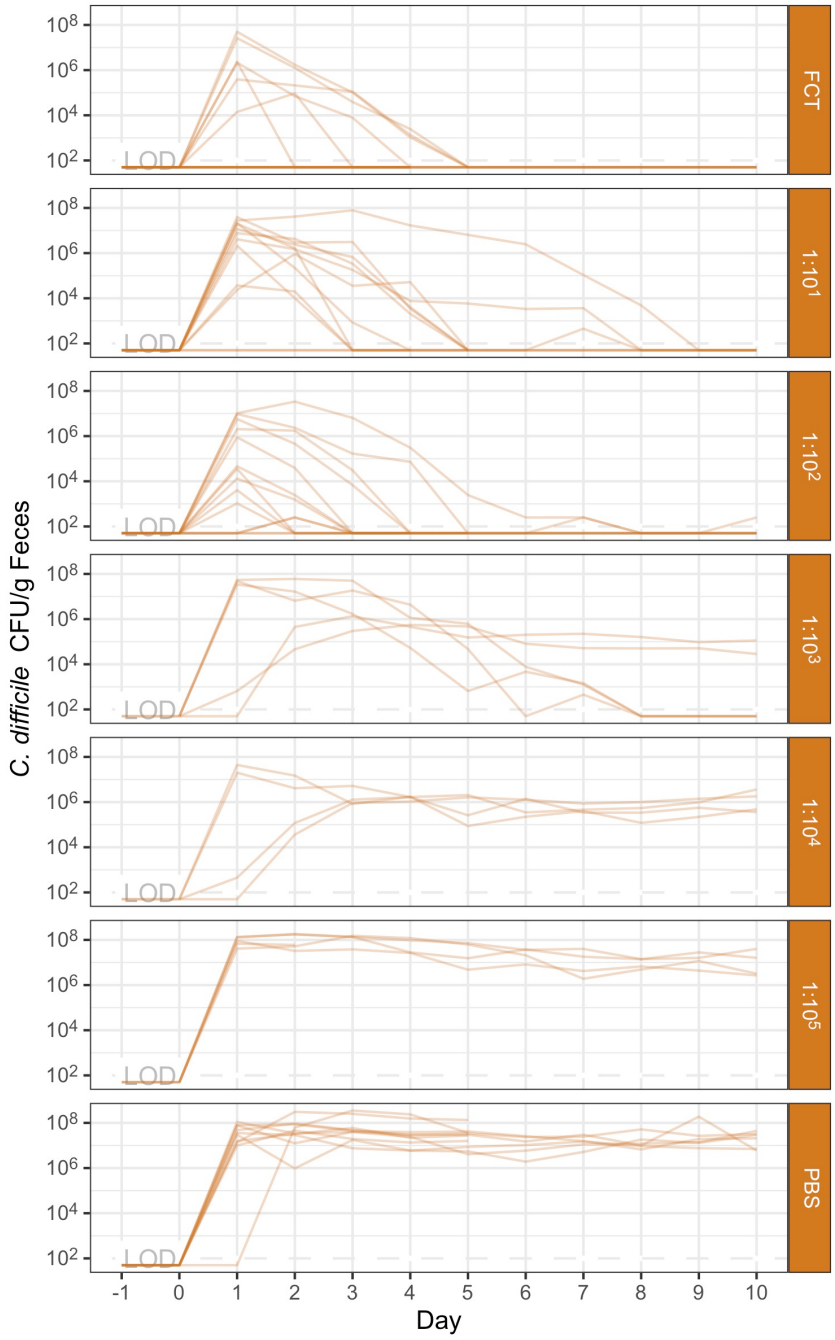


Figure 4.4. *C. difficile* colonization dynamics in streptomycin-treated mice across all prophylactic transplant treatments.

C. difficile CFU per gram of feces for streptomycin-treated mice orally gavaged PBS, fecal community transplant (FCT), or diluted FCT (1:10-1:10^5) prior to the *C. difficile* infection. Each semi-transparent line represents an individual mouse. Mice challenged with 10^3 *C. difficile* 630 spores on day 0. Lines grouped by the transplant treatment received. LOD = limit of detection.

Murine gut bacterial communities had not recovered their diversity by the time of *C. difficile* challenge. To elucidate the effects of the fecal community dilution on the murine gut bacterial community and *C. difficile* infection, we sequenced the V4 region of the 16S rRNA gene from the fecal community. The most significant difference between the communities of the FCT and its dilutions was the quantity of the 16S rRNA gene, which decreased monotonically (Figure 4.5D). The FCT dilutions of $1{:}10^3$ to $1{:}10^5$ had few samples with sufficient sequencing depth to provide bacterial community information. The FCT and its dilutions were not significantly different in either α-diversity (number of operational taxonomic units (OTUs) (S_{obs}) or Inverse Simpson) or β-diversity (θ_{YC}) (Figure 4.5A-C). Populations of *Acetatifactor*, *Enterobacteriaceae*, *Lactobacillus*, *Ruminococcaceae*, and *Turicibacter* correlated with the FCT dilution factor (Figure 4.5E). Overall, the abundance of the bacteria appeared to be the largest difference between FCT and its dilutions. For the gut communities, in comparison to the initial community (day -9), FCT pre-treatment did not result in significant diversity recovery at the time of *C difficile* challenge (day 0) for cefoperazone-treated mice (Figure 4.6) or streptomycin-treated mice (Figure 4.7). At the end of the experiment (day 10), the gut bacterial communities were more similar to their initial community in α-diversity (number of OTUs (S_{obs}) and Inverse Simpson) and β-diversity (θ_{YC}) diversity. The mice pre-treated with less dilute FCT were most similar to the initial community structure, whereas, the mice pre-treated with more dilute FCT resulted in little recovery of diversity, similar to the mice given PBS. Thus, the effect of FCT was not large enough at the time of *C. difficile* challenge to be detected in the community diversity but was sufficient to affect *C. difficile* colonization. The simplified FCT likely consist primarily the most abundant populations, since the less abundant populations are less likely to be

selected when subsampling the dilutions for gavaging the mice. This would suggest the effect was driven by the most abundant populations.

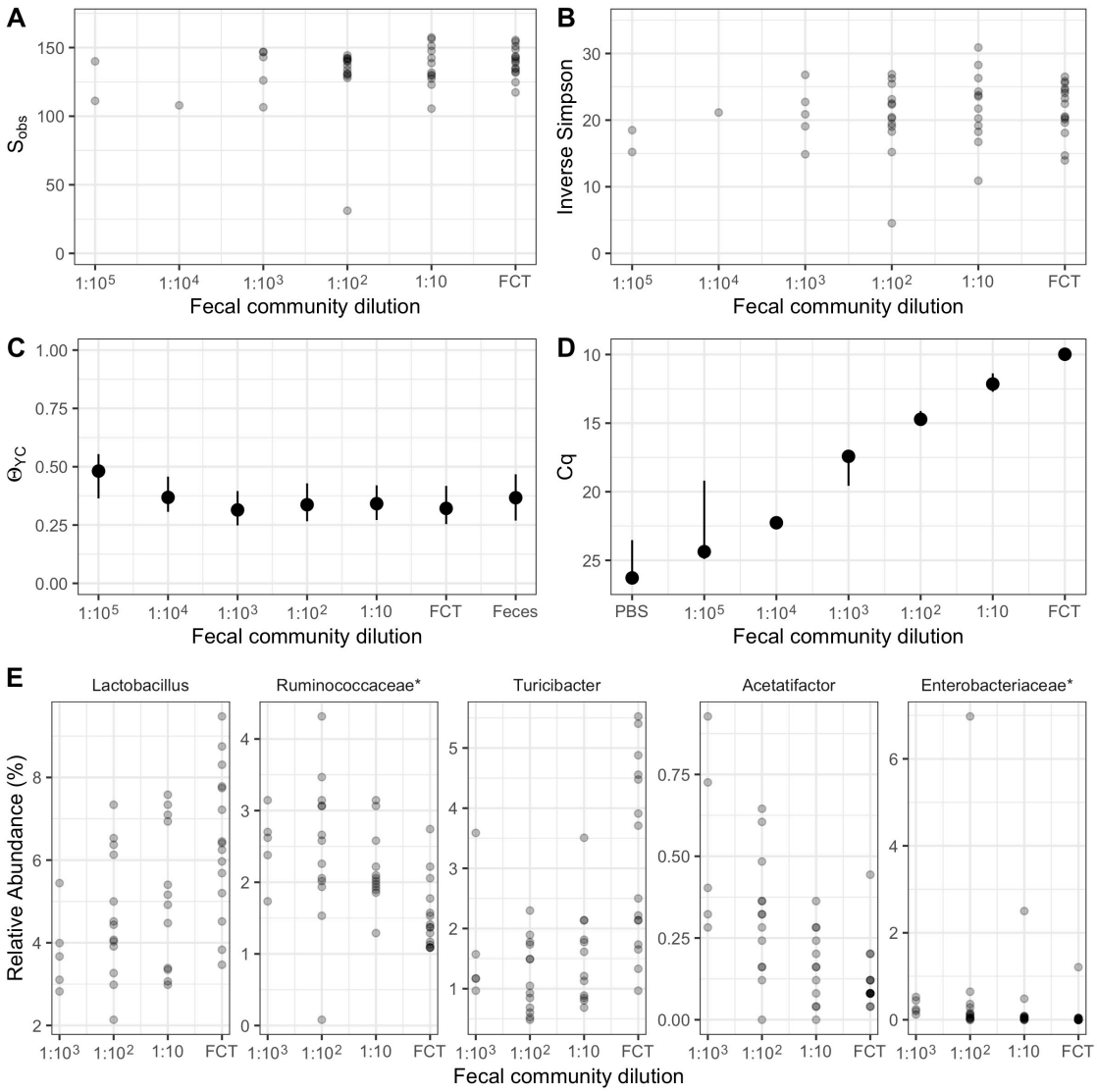


Figure 4.5. Diversity and quantification of fecal community dilutions used for prophylactic transplants in antibiotic-treated mice.

(A-C) Diversity of fecal community dilutions. α-diversity, measured by (A) S_{obs} and (B) Inverse Simpson for undiluted fecal community (FCT) and diluted fecal communities (1:10-1:10^5). Points are individual samples. (C) β-diversity, measured by θ_{YC}, community structure of feces collected from untreated mice, undiluted fecal community (FCT), and diluted fecal communities (1:10-1:10^5) compared to untreated feces. Points are median values and lines represent the interquartile range. (D) Cq values for qPCR of FCT and its dilutions for eubacterial 16S rRNA gene. Points are median values and lines represent the interquartile range. (E) Relative abundance of bacterial taxonomic groups that significantly correlate with fecal community dilutions (FCT-1:10^3) by Spearman correlation. Points are individual mice. * indicates that the bacterial taxonomic group was unclassified at lower classification rank.

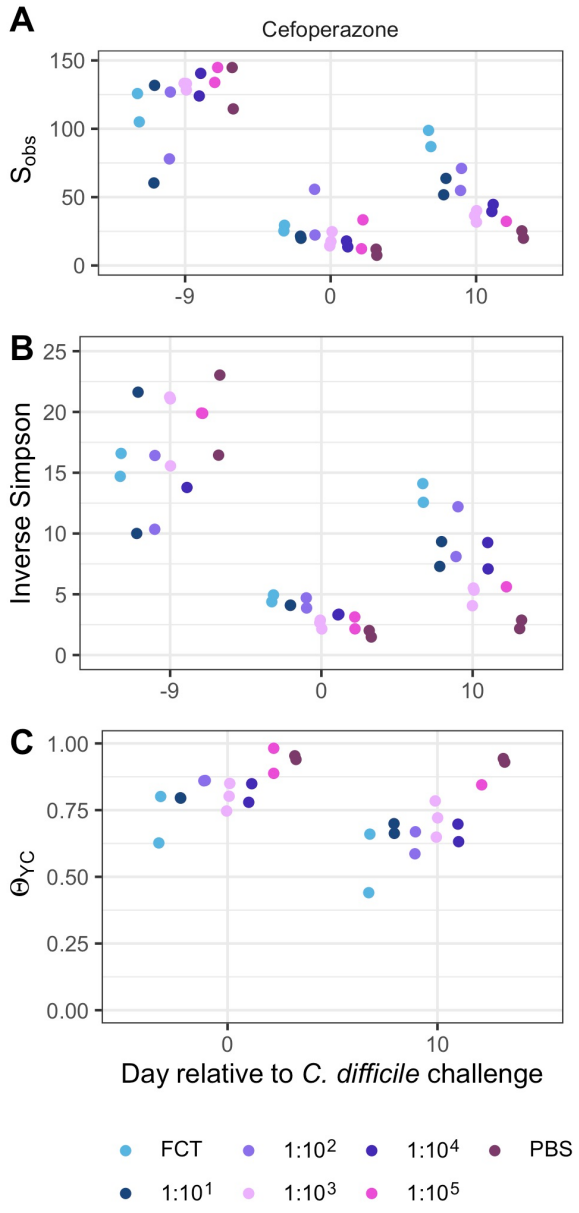


Figure 4.6. Diversity of murine gut bacterial community was not recovered at the time of *C. difficile* infection in cefoperazone-treated mice.

Diversity changes through experiments with cefoperazone-treated mice. α-diversity, measured by S_{obs} and Inverse Simpson, prior to beginning antibiotic treatment (day -9), after fecal community transplant on the day of *C. difficile* infection (day 0) and at the end of the experiment (day 10). β-diversity, measured by θ_{YC}, compare community structure on day 0 or 10 relative to the day -9 community of that individual. Data are grouped by the pre-treatment received, undiluted fecal community (FCT), diluted fecal community (1:10-1:10^5), or PBS. Points are individual mice.

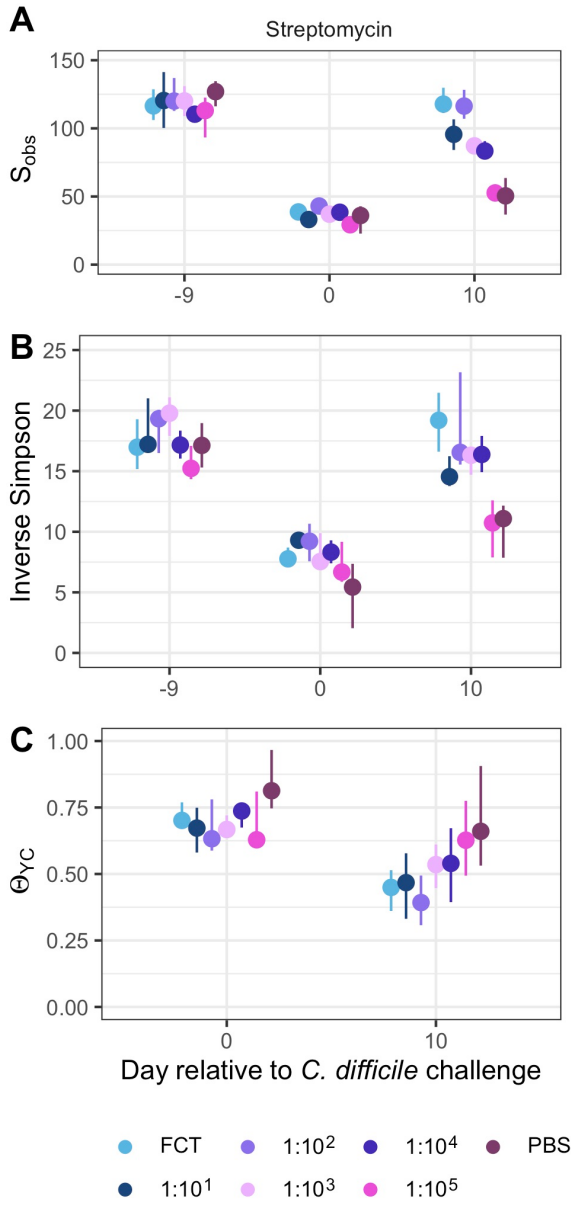


Figure 4.7. Diversity of murine gut bacterial community had not recovered at the time of *C. difficile* infection in streptomycin-treated mice.

Diversity changes through experiments with streptomycin-treated mice. α-diversity, measured by S_{obs} and Inverse Simpson, prior to beginning antibiotic treatment (day -9), after fecal community transplant on the day of *C. difficile* infection (day 0) and at the end of the experiment (day 10). β-diversity, measured by θ_{YC}, compare community structure on day 0 or 10 relative to the day -9 community of that individual. Data are grouped by the transplant received, undiluted fecal community (FCT), diluted fecal community (1:10-1:10^5), or PBS. Points are median values and lines represent the interquartile range.

Gut bacterial community members differentially abundant in streptomycin-treated mice resistant to colonization. Although streptomycin-treated mice pre-treated with FCT varied in *C. difficile* colonization levels, at the time of challenge, FCT pre-treatment had little effect on the diversity of their gut bacterial communities regardless of the dilution. We used linear discriminant analysis (LDA) effect size (LEfSe) analysis to identify OTUs within the fecal bacterial communities from the streptomycin-treated mice that were differentially abundant between uncolonized and colonized mice and mice that remained colonized and those that cleared the colonization. The antibiotic treatment significantly altered 99 OTUs (Figure 4.6), but on the day of *C. difficile* challenge only 7 OTUs were differentially abundant between colonized and uncolonized communities (Figure 4.8A). Communities resistant to *C. difficile* colonization had more abundant populations of OTUs related to *Akkermansia*, *Clostridiales*, *Olsenella*, and *Porphyromonadaceae* and less abundant populations of an OTU related to *Enterobacteriaceae*. Thus, a small portion of OTUs, relative to the changes due to streptomycin treatment, were differentially abundant in mice that resisted *C. difficile* colonization compared to those that were colonized.

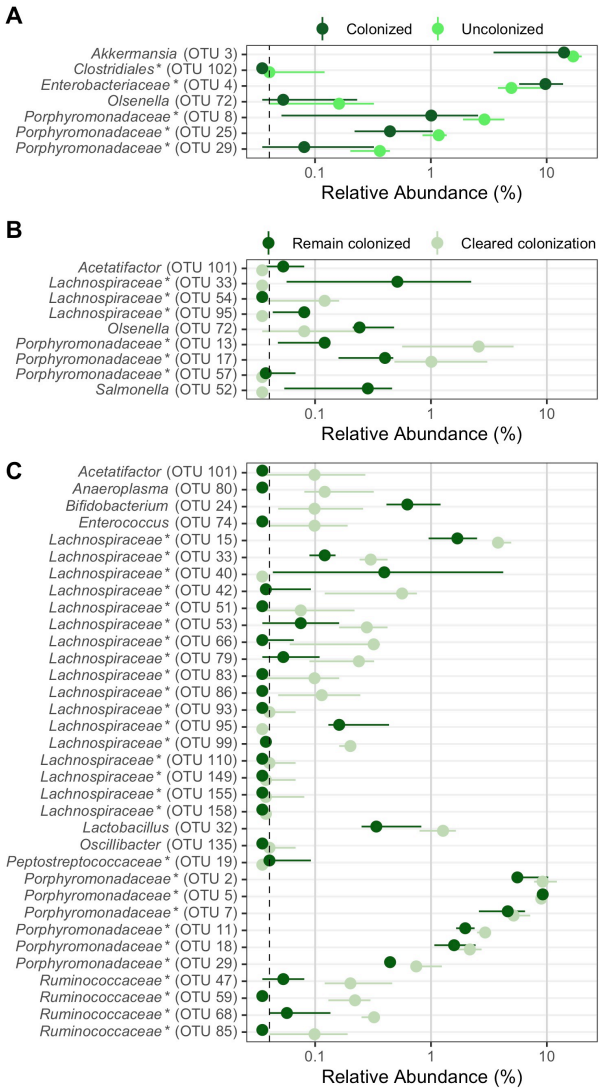


Figure 4.8. Bacterial community OTUs differentially abundant in streptomycin-treated mice which resisted or cleared colonization.

Murine gut bacterial community OTUs that were significantly different by LEfSe analysis. OTUs from streptomycin-treated mice at the time of *C. difficile* challenge (day 0) which were differentially abundant between (A) mice that were colonized (dark green) and those that were not (no detectable CFU throughout the experiment, bright green) or (B) mice that remained colonized (dark green) and those that cleared colonization (CFU reduced to below the limit of detection by the end of the experiment, faint green). (C) OTUs from streptomycin-treated mice at the end of the experiment (day 10) which were differentially abundant between mice that remained colonized (dark green) and those that cleared colonization (CFU reduced to below the limit of detection by the end of the experiment, faint green). Points are median values and lines represent the interquartile range. Dashed vertical line is the limit of detection. OTUs ordered alphabetically. * indicates that the OTU was unclassified at lower classification rank.

Murine gut bacterial communities that cleared *C. difficile* colonization were more similar to the initial community. To better understand the differences in streptomycin-treated murine fecal community that contributed to *C. difficile* clearance, we compared the communities that cleared *C. difficile* to those that did not at the time of challenge and 10 days post infection. At the time of *C. difficile* challenge, 9 OTUs were differentially abundant between communities that remained colonized and those that cleared colonization (Figure 4.8B). Communities that cleared *C. difficile* colonization had more abundant populations of OTUs related to *Porphyromonadaceae* and *Lachnospiraceae* and less abundant populations of OTUs related to *Acetatifactor*, *Lachnospiraceae*, *Olsenella*, *Porphyromonadaceae*, and *Salmonella*. At the end of the experiment, 29 of the 34 OTUs differentially abundant were more abundant in the mice that were able to clear the colonization (Figure 4.8C). Populations of OTUs related to *Acetatifactor*, *Anaeroplasma*, *Enterococcus*, *Lachnospiraceae*, *Lactobacillus*, *Porphyromonadaceae*, and *Ruminococcaceae* were increased in communities that cleared, recovering in abundance from the streptomycin treatment (Figure 4.9). Multiple OTUs related to *Lachnospiraceae* and *Porphyromonadaceae* (N = 14 and N = 5, respectively) were significant and accounted for greater portions of the community (more than 10%). However there were also one *Porphyromonadaceae* population (OTU 5) and two *Lachnospiraceae* related populations (OTU 40 and 95) more abundant in the mice that remain colonized. Thus, the more the gut bacterial members returned to their initial abundance resulted in a greater likelihood of clearing *C. difficile*.

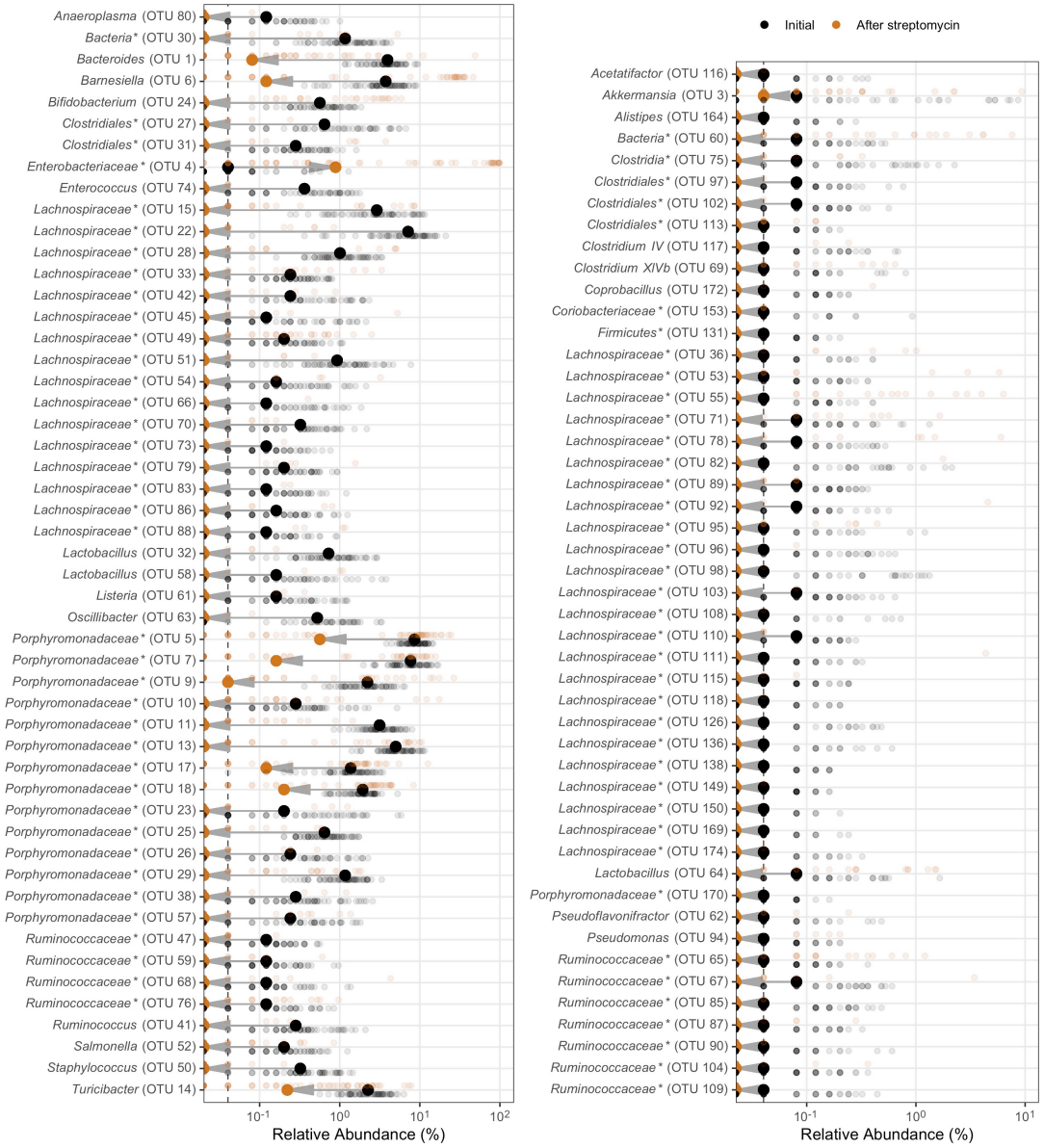


Figure 4.9. Murine gut bacterial community OTUs differentially abundant with streptomycin treatment.

Murine gut bacterial community OTUs that were significantly different by LEfSe analysis between untreated mice (Initial, black) and after 5 days of water with streptomycin (5 mg/ml) and 2 days of untreated water (After streptomycin, orange). Large bold points represent the group median. Small, semi-transparent points represent an individual mouse. Gray arrow indicates the direction the relative abundance shifted with the streptomycin treatment. Left plot displays OTUs with a median relative abundance greater than 0.1%, the OTUs lower are displayed in the right plot. Dashed vertical line is the limit of detection. OTUs ordered alphabetically. * indicates that the OTU was unclassified at lower classification rank.

Negative associations dominated the interactions between the gut bacterial community and *C. difficile* in streptomycin-treated mice. In streptomycin-treated mice, pre-treatment with FCT and its dilutions had different effects on the bacterial community members which resulted in different community relative abundance and *C. difficile* colonization dynamics. We sought to capture the relationships occurring throughout this experiment using SPIEC-EASI (sparse inverse covariance estimation for ecological association inference) to construct a conditional independence network. Here, we focused on the associations of the *C. difficile* subnetwork (Figure 4.10). *C. difficile* CFU had positive associations with populations of OTUs related to *Enterobacteriaceae* (OTU 4) and *Peptostreptococcaceae* (OTU 19). OTUs related to *Clostridiales* (OTU 27), *Lachnospiraceae* (OTUs 15, 51, and 83), and *Porphyromonadaceae* (OTUs 23, 25, and 29) had negative associations with *C. difficile*, as well as the OTUs related to *Enterobacteriaceae*, and *Peptostreptococcaceae*. Overall, the majority of the associations between *C. difficile* and the gut bacterial community in streptomycin-treated mice were negative. This suggests this subset of the community may be driving the inhibition of *C. difficile* in streptomycin-treated communities.

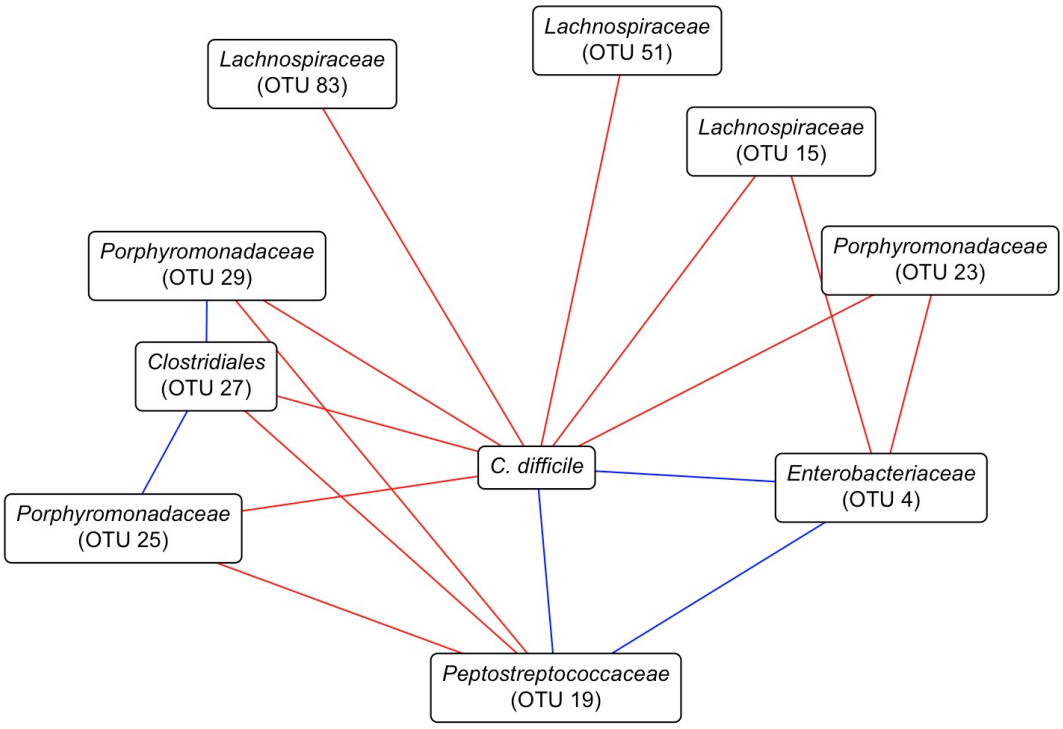


Figure 4.10. Streptomycin-treated murine fecal community associations with *C. difficile*.

Network constructed with SpiecEasi from the OTU relative abundances and *C. difficile* CFU data from 1 through 5 days post *C. difficile* infection. Red lines represent negative associations and blue lines indicate positive associations. *C. difficile* is based on CFU counts and *Peptostreptococcaceae* (OTU 19),the OTU most closely related to *C. difficile*, is based on sequence counts. Only *C. difficile* subnetwork shown.

4.6 Discussion

Transplanting the fecal community from untreated mice to antibiotic-treated mice prior to being challenged with *C. difficile* varied in the inhibition of *C. difficile* colonization based on the antibiotic treatment. This indicated that FCT pre-treatment can prevent *C. difficile* colonization in an antibiotic-specific manner. Additionally, by diluting the FCT we were able to narrow the community changes responsible for the effect to the most abundant OTUs. Overall, these results show that a simplified fecal community can assist a perturbed microbiota in preventing or resisting *C. difficile* colonization but is dependent on the perturbation.

Through diluting the FCT we were able to narrow the definition of the minimal community features that restored colonization resistance. Bacterial interactions with *C. difficile* were associated with the identity, abundance, and functions of adjacent bacteria. Ghimire *et al.* recently showed that individual species inhibited *C. difficile* in co-culture. However, when other inhibitory species were added, the overall effect on *C. difficile* was changed, in some cases increasing *C. difficile* growth (45). Based on this observation, we sought to find an inhibitory community using a top-down approach, beginning with an inhibitory community. Auchtung *et al.* recently developed a set of simplified communities from human fecal communities that were grown in minibioreactor arrays and tested for inhibition first *in vitro* then in a mouse model (43). They found four simplified communities that were able to reduce *C. difficile* colonization but with varied effect in a mouse model with the same gut microbiota. One way they simplified the community was through diluting the initial fecal sample. In our experiments, we began with a fresh whole fecal community to first determine if inhibition was possible. For cefoperazone and streptomycin treated mice, the treatments that inhibited *C. difficile*, we diluted the FCT to determine the minimal community which maintained inhibition. Cefoperazone-treated mice were

unable to maintain inhibition of *C. difficile* with diluted FCT pre-treatments and *C. difficile* maintained colonization of the intestine. Streptomycin-treated mice were able to maintain inhibition with diluted FCT pre-treatment. The diluted FCTs were only able to maintain the inhibition of the FCT on *C. difficile* and revealed the minimal changes associated with either colonization resistance or clearance for streptomycin-treated mice only.

We previously hypothesized that mice treated with either clindamycin, cefoperazone, or streptomycin would require unique bacterial communities to restore inhibition *C. difficile* (59). In that set of experiments, the dose of the antibiotic was varied to titrate changes to the community and determine what changes allow *C. difficile* to colonize and then be spontaneously cleared. We observed antibiotic-specific changes associated with *C. difficile* clearance. The data presented here complement those observations. For clindamycin-treated mice, there was no difference in colonization, clearance or relative abundance between PBS and FCT pre-treatment. *C. difficle* had similar colonization dynamics. It is possible that there was insufficient time for the FCT to engraft. However, when we added more time between clindamycin treatment and *C. difficile* challenge, *C. difficile* was unable to colonize (data not shown). Therefore, clindamycin-treated mice appear to naturally recover inhibition to *C. difficile*, which were unaffected by the FCT pre-treatment. For cefoperazone-treated mice, the FCT pre-treatment enabled the gut microbiota to eliminate the colonization but only in its most concentrated dose. This observation supports our previous report, indicating that the cefoperazone-treated community is more sensitivity to the amount of FCT it receives since cefoperazone reduced many bacterial groups and associations (Figure 4.11). As we previously hypothesized, streptomycin-treated mice were enabled to clear with a subset of the community, with the FCT pre-treatment diluted to $1{:}10^3$. Since we titrated the FCT dilutions, we could compare the bacterial communities of the mice which gained the

ability to clear *C. difficile* to the mice that received the next dilution which could not. This comparison revealed the minimal relative abundance differences associated with clearance. In agreement with previous studies, OTUs related to *Lachnospiraceae*, *Porphyromonadaceae*, and *Ruminococcaceae* increased with the clearance of *C. difficle* in the streptomycin-treated mice (35, 36, 46, 59, 149, 150). These data agree with our previous hypothesis that a simplified fecal community would only be able to promote clearance of *C. difficile* in streptomycin-treated mice.

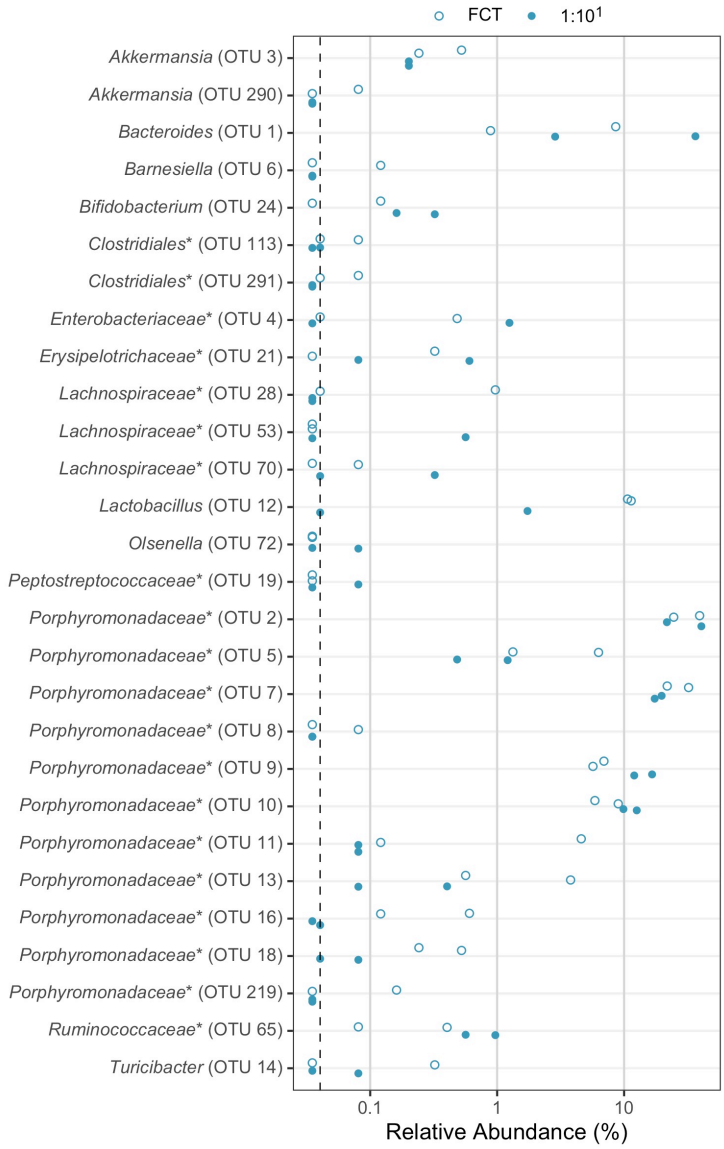


Figure 4.11. Murine gut bacterial community OTUs of cefoperazone-treated mice at the time of challenge.

Murine gut bacterial community OTUs that were present in at least one sample at the time of *C. difficile* challenge (day 0). Mice were pre-treated with either fecal community transplant (FCT, open circles) or FCT diluted 1:10 (filled circles). Points are individual samples. Dashed vertical line is the limit of detection. OTUs ordered alphabetically. * indicates that the OTU was unclassified at lower classification rank.

In addition to clearing *C. difficile*, a simplified fecal community restored colonization resistance to streptomycin-treated mice. Mice that received FCT pre-treatment as dilute as $1{:}10^2$ were not colonized to a detectable level. While restoring colonization resistance is not novel (53), here we have shown that the restoration of colonization resistance is dependent on the community perturbation and the fecal community being transplanted. As we identified community members associated with clearance, OTUs related to *Akkermansia*, *Olsenella*, and *Porphyromonadaceae* were more abundant and an OTU related to *Enterobacteriaceae* was less abundant at the time of *C. difficile* challenge. *Enterobacteriaceae* has been associated with *C. difficile* colonization and inflammation (59, 135, 137). Larger populations of *Akkermansia* were associated with preventing colonization, which we had previously observed, potentially indicating the maintenance of a protective mucus layer (59, 88, 89, 151). Increased populations of a select set of OTUs related to *Porphyromonadaceae* were also more abundant in mice that were resistant to colonization. *Porphyromonadaceae* has been previously associated with uncolonized gut communities and capable of buytrate and acetate production, which has been associated with successful FMT treatments (41, 59, 152–154). While *Porphyromonadaceae* was also increased in the mice that cleared colonization, colonization resistance may be due to OTU-specific functions or dependent on *Porphyromonadaceae* in addition to the differential abundances of *Akkermansia* and *Enterobacteriaceae*. With the simplified fecal community, we refined the gut bacterial community members that associated with preventing colonization in streptomycin-treated mice.

We have demonstrated that a simplified bacterial community can restore colonization resistance but the effect of the community and the bacteria that colonized was dependent on the specific antibiotic-induced susceptibility. When transplanting the fecal community into

antibiotic-induced susceptible mice, only mice treated with streptomycin were able to restore colonization resistance. Diluted FCT pre-treatment was similarly effective to undiluted FCT pre-treatment and revealed the minimal bacterial differences associated with restoration of colonization resistance. These results reveal that variation in the disruption of the gut microbiota alters the effectiveness of a bacterial community to restore colonization resistance. Previous studies tested simplified communities in a murine model that they were developed in without any variation to the gut microbiota (36, 44). Treatments supplementing the gut microbiota would benefit from being tested in different communities susceptible to CDI. Further research is necessary to characterize the specific niches of susceptible communities and the requirements to fill those spaces. Then it may be possible to identify people with gut microbiota that are susceptible to CDI and develop targeted simplified bacterial communities to recover colonization resistance and reduce the risk of CDI.

4.7 Methods

Animal care. Mice used in experiments were 6- to 13-week old male C57BL/6 mice obtained from a single breeding colony at the University of Michigan. During the experiment, mice we housed with two or three mice per cage. All murine experiments were approved by the University of Michigan Animal Care and Use Committee (IACUC) under protocol number PRO00006983.

Antibiotic administration. Mice were given either cefoperazone, clindamycin, or streptomycin. Cefoperazone (0.5 mg/ml) and streptomycin (5 mg/ml) were administered via drinking water for 5 days, beginning 9 days prior to *C. difficile* challenge. Antibiotic water was replaced every two days. Clindamycin (10 mg/kg) was injected into the intraperitoneal space, 2

days prior to challenge with *C. difficile*. All antibiotics were filter sterilized with a 0.22 μm syringe filter prior to use.

Fecal community transplants. Fecal pellets were collected from similar aged C57BL/6 mice not being used in an experiment the day of the fecal community transplants. 15-20 pellets were collected and weighed. Then in anaerobic conditions, the fecal pellets were homogenized weight per weight in phosphate-saline buffer (PBS) containing 15% glycerol (fecal community transplant, FCT). FCT was then serially diluted in PBS containing 15% glycerol down to $1:10^5$ fecal dilution and aliquoted into tubes for gavaging into mice. One set of aliquots were frozen at -80°C to be used for the following day for the cefoperazone and streptomycin experiments. Aliquots used on the second day were thawed at 37°C for 5 minutes prior to being used. All fecal community dilutions were centrifuged at 7500 RPM for 60 seconds. Mice were inoculated with 100 uL of the fecal dilution oral via a 21 gauge gavage needle. Fecal community transplants were administered from most dilute to least, which began with mice receiving PBS and finished with mice receiving FCT. Aliquots were frozen at -80°C after use for sequencing.

16S rRNA quantitative real-time PCR. Quantitative analysis of 16S rRNA in fecal community dilutions used for FCT was carried out using quantitative real-time PCR using primers and cycler conditions specified previously (155). Reaction volumes were prepared using 6 uL of SYBRTM Green PCR Master Mix (Applied Biosciences Ref 4344463), 1 uL each forward and reverse primer, and 2 uL sample DNA template. All qPCR reactions were run on a LightCycler 96 (Roche Ref 05815916001) using instrument-specific plates and sealers.

***C. difficile* challenge.** For streptomycin and cefoperazone, mice were given untreated drinking water for 96 hours before challenging with *C. difficile* strain 630Δerm spores. For

clindamycin, mice were given untreated drinking water at the time of the intraperitoneal injection, for 48 hours before challenging with *C. difficile* strain 630Δerm spores. *C. difficile* spores were aliquoted from a single spore stock stored at 4°C. Spore concentration was determined two days prior to the day of challenge (110). Mice were inoculated with 10^3 *C. difficile* spores via oral gavage. After inoculating the mice, remaining spore solution was serially diluted and plated to confirm the spore concentration.

Sample collection. Fecal samples were collected prior to administering antibiotics, after antibiotics were removed, prior to *C. difficile* challenge and on each of the 10 days post infection. Approximately 15 mg of each fecal sample was collected and weighed for plating *C. difficile* colony forming units (CFU) and the remaining sample was frozen at -80°C for later sequencing. The weighed fecal samples were anaerobically serially diluted in PBS, plated on TCCFA plates, and incubated at 37°C for 24 hours. The resultant colonies were enumerated to determine the *C. difficile* CFUs (111).

DNA sequencing. Total bacterial DNA was extracted from the frozen samples by the MOBIO PowerSoil-htp 96-well soil DNA isolation kit. We amplified the 16S rRNA gene V4 region and the amplicons were sequenced on an Illumina MiSeq as described previously (112).

Sequence curation. Sequences were processed using mothur(v.1.44.1) as previously described (112, 113). We used a 3% dissimilarity cutoff to group sequences into operational taxonomic units (OTUs). We used a naive Bayesian classifier with the Ribosomal Database Project training set (version 16) to assign taxonomic classifications to OTUs (114). We sequenced a mock community of a known 16S rRNA gene sequences and composition. We

processed this mock community in parallel with our samples to determine the error rate for our sequence curation, which resulted in an error rate of 0.029%.

Statistical analysis and modeling. We calculated diversity metrics in mothur. For α-diversity comparisons, we calculated the number of OTUs (S_{obs}) and the Inverse Simpson diversity index. For β-diversity comparisons, we calculated dissimilarity matrices based on metric of Yue and Clayton (θ_{YC}) (115). We rarefied samples to 2,480 sequences per sample to limit uneven sampling biases. OTUs were subsampled to 2,480 counts per sample. We tested for differences in relative abundance between outcomes with LEfSe in mothur. All other statistical analyses and data visualization was completed in R (v4.0.5) with the tidyverse package (v1.3.1). Pairwise comparisons of α-diversity (S_{obs} and Inverse Simpson), β-diversity (θ_{YC}), were calculated by pairwise Wilcoxon rank sum test. Correlations between bacterial genera and fecal community dilution were calculated using the Spearman correlation. *P* values were corrected for multiple comparisons with a Benjamini and Hochberg adjustment for a type I error rate of 0.05 (117). For streptomycin experiments, conditional independence networks were calculated from the day 1 through 5 samples of all mice using SPIEC-EASI (sparse inverse covariance estimation for ecological association inference) methods from the SpiecEasi R package after optimizing lambda to 0.001 with a network stability between 0.045 and 0.05 (v1.0.7) (129).

Chapter V

Discussion

C. difficile infection can occur once the indigenous gut microbiota has been disrupted. The bacteria in the gut provide the host with resistance to pathogen invasion through numerous mechanisms. Once this community is disturbed those mechanisms can be lost. Depending on the perturbation, different inhibitory mechanisms are affected, exposing perturbation-specific niches for *C. difficile* to colonize. We can treat CDI or the susceptible gut microbiota by supplementing the community with a consortium of bacteria. However, it is unclear whether *C. difficile* colonizing different niches affects disease outcome or the bacteria necessary to inhibit *C. difficile* colonization. The work presented in this dissertation investigated the effect of variation in the gut microbiota on *C. difficile* disease severity, clearance of *C. difficile* and restoration of colonization resistance.

5.1 Summary

Mouse models with diverse gut microbiota susceptible to CDI allowed us to uncover the role of bacteria in modulating CDI disease severity as well as understanding the relationship between bacterial groups and the clearance or prevention of *C. difficile* colonization. We showed that the perturbation affected the ability of simplified bacterial communities to restore clearance. These data demonstrate the importance of further elucidating the niches of susceptible communities and the specific bacteria capable eliminating them to prevent *C. difficile* colonization.

The gut bacterial community potentiates *Clostridioides difficile* infection severity. In Chapter II, we challenged germ-free mice colonized with one of fifteen human fecal communities with *C. difficile*. The resultant CDI had varied disease manifestation but was consistent within each donor group. The murine gut communities provided diverse compositions of bacterial members and relative abundances which revealed that a single isolate of *C. difficile* can have a range of disease severities. Typically, mouse models of CDI use a homogeneous gut microbiota and vary the strain of *C. difficile* to observe variation in the disease. However, recent studies have revealed different gut microbiota affected *C. difficile* clearance and severity (46, 53). Therefore we sought to explore the relationship between the gut microbiota and CDI disease severity. We associated gut bacterial relative abundances with the detection of toxin, cecal tissue histopathologic scores, and mortality. Less severe disease, non-moribund mice with no toxin detected and low histopathologic scores, was associated with increased populations of *Akkermansia*, *Anaerostipes*, *Coprobacillus*, *Enterocloster*, *Lactonifactor*, and *Monoglobus*. The relationship of *Anaerostipes*, *Coprobacillus*, *Lactonifactor*, and *Monoglobus* to reduced susceptibility to CDI, supported by previous studies, may be attributed to their dietary fiber fermentation, short-chain fatty acid production, or potential lantibiotic expression (79–88). *Akkermansia* has been associated with CDI but in our data was most abundant with in mice with less severe disease, although some moribund mice did have increased populations of *Akkermansia*. In those moribund mice, *Akkermansia* may have been crossfeeding and exposing a niche for *C. difficile* (90–92). *Enterocloster*, also more abundant in mice with less severe disease, can reduce *C. difficile* colonization (35, 93) but also has been associated with infections (94, 95). More severe disease, as judged by either a high histopathologic score or moribundity, was associated with increased populations of *Enterococcus*, *Klebsiella*, *Shigella/Escherichia*,

Staphylococcus, and *Streptococcus,* which have been previously associated with antibiotic use and CDI (41, 96–101), and increased populations of *Eggerthella*, *Prevotellaceae* and *Helicobacter*, which have been associated with intestinal inflammation (102–104). *Helicobacter* was detected only in mice with severe disease, which has recently been shown to be sufficient to induce CDI susceptibility in IL-10 deficient C57BL/6 mice (105). This relationship between *Helicobacter* and CDI should be investigated further to determine if *Helicobacter* is causing the susceptibility we observed here, as it had in the IL-10 deficient C57BL/6 mice. These data revealed that different community compositions can potentiate the disease caused by *C. difficile* infection. This finding highlights the contribution of both the gut microbiota and *C. difficile* to the infection and dynamics of CDI.

Clearance of *Clostridioides difficile* colonization is associated with antibiotic-specific bacterial changes. In Chapter III, we perturbed the gut microbiota of mice with either clindamycin or a series of dosages of either cefoperazone or streptomycin. These perturbations produced a set of mice which, when challenged with *C. difficile,* would become colonized, but then naturally eliminate *C. difficile* within 10 days. The community dynamics concurrent with *C. difficile* clearance showed that each antibiotic treatment group had unique community changes associated with eliminating CDI. In the gut communities of clindamycin-treated mice, the community at the time of infection was dominated by *Enterobacteriaceae*. Over the course of clearing *C. difficile*, *Enterobacteriaceae* returned to lower relative abundance and many of the genera returned to pre-clindamycin treatment relative abundances. Clindamycin treatment seems to transiently suppress the majority of the community, allowing *Enterobacteriaceae* to bloom and providing a niche for *C. difficile*. However, the community is still sufficiently established to recover their populations and outcompete *C. difficile*. Therefore, allowing the community to

recover naturally may be more effective than a treatment that may perturb the recovering community (34, 44, 130–132). In cefoperazone-treated mice, the community is characterized by prolonged suppression of a majority of the community and increased populations of a few groups of bacteria, namely *Enterococcus*, *Pseudomonas*, *Staphylococcus*, and *Enterobacteriaceae*. This community appears to be lacking many community members and interactions which could indicate many niches are available. Since the community is lacking so many members, it may need a more significant community recovery to ensure diversity to block all available niches and outcompete *C. difficile* via multiple FMT or an enriched community (97, 133, 134). In streptomycin-treated mice, there were few differences between the communities that cleared *C. difficile* and those that remained colonized. There was overlap in the differential abundances in each genera. Clearance may occur through the recovery of a few select OTUs of those genera, such as *Bifidobacterium* OTU 24 and *Turicibacter* OTU 18, or a specific *Porphyromonadaceae* OTU. Since there are few changes and many of the community members and interactions persist, a FMT or simplified bacterial community may be sufficient to recover colonization resistance. These data showed that each antibiotic resulted in specific changes associated with *C. difficile* clearance, which could help inform the necessary bacteria to colonize available niches to restore colonization resistance.

Simplified fecal community transplant restores *Clostridioides difficile* colonization resistance to antibiotic-specific perturbed murine communities. In Chapter IV, we treated mice with either clindamycin, cefoperazone, or streptomycin and then inoculated the mice with a fecal community transplant (FCT). When challenged with *C. difficile*, only the streptomycin-treated mice pre-treated with FCT were able to prevent colonization. Cefoperazone-treated mice pre-treated with FCT were able to clear the colonization. *C. difficile* colonization in clindamycin-

treated mice was not significantly different between PBS and FCT pre-treatment. We then performed similar murine experiments and pre-treated mice with diluted FCT to test whether simplified communities could maintain the inhibition of *C. difficile*. Diluted FCT pre-treatment did not have an effect on cefoperazone-treated mice. Streptomycin-treated mice were able to resist colonization when pre-treated with FCT diluted $1{:}10^2$ or clear *C. difficile* when pre-treated with FCT diluted $1{:}10^3$. Clindamycin-treated mice were unaffected by the FCT pre-treatment. We could not add more time for the FCT to colonize since mice not given a pre-treatment did not become colonized when more time was given. Thus, clindamycin treatment caused a transient susceptibility which cleared naturally. Cefoperazone-treated mice were only able to clear *C. difficile* colonization when pre-treated with undiluted FCT. FCT pre-treatment was insufficient to restore colonization resistance. Cefoperazone-treated mice likely required a more significant FCT pre-treatment, a more diverse FCT pre-treatment, or community enriched for specific niches. Streptomycin-treated mice were able to restore colonization resistance when pre-treated with a simplified fecal community, FCT diluted $1{:}10^2$. Colonization resistance is likely restored to streptomycin-treated mice through the supplementation of the more abundant community members. Populations of OTUs related to *Akkermansia*, *Olsenella*, and *Porphyromonadaceae* were more abundant and *Enterobacteriaceae* was less abundant at the time of *C. difficile* challenge. While *Akkermansia* has been associated with CDI, in our data as well as other studies it has been associated with preventing colonization (59, 88, 89, 151). There were many OTUs related to *Porphyromonadaceae* that were more abundant with the clearance of *C. difficile* but only three (OTUs 8, 25, and 29) which were associated with restored colonization resistance. Thus, there may be specificity to the function of those OTUs. *Enterobacteriaceae* has been associated with *C. difficile* colonization. FCT pre-treatment may either directly inhibit

Enterobacteriaceae or eliminate the niche promoting its expansion which may also be the same as *C. difficile* (135, 137). These data have shown that the minimal community to restore colonization resistance is antibiotic-perturbation specific. Each antibiotic perturbation had different requirements to inhibit *C. difficile*. Therefore, in order to more generally restore colonization resistance we test simplified bactierial communities in a set of diverse susceptible communities.

5.2 Future Directions

Overall, the results from these Chapters, II, III, and IV have shown that the variation of the gut microbiota affects CDI disease severity, community changes associated with *C. difficile* colonization clearance, and the community necessary to restore colonization resistance. Next, we can further elucidate the niches available to *C. difficile* in susceptible communities as well as use more diverse communities to test bacterial community-based therapies. Future studies could explore defined simplified communities to restore colonization resistance. There are a few studies that have identified simplified bacterial communities to treat recurrent CDI (43, 44). To ensure these communities are more broadly applicable, they could be tested in experimental models with heterogeneous gut microbiota and susceptibilities. Additionally, future studies could further elucidate susceptibility to CDI. In these studies, we primarily investigated the community membership, diversity, and relative abundance using 16S rRNA gene sequencing. While we observed different patterns in clearance and colonization resistance, different community members can perform similar functions or produce similar metabolites that may contribute to the these outcomes. Future studies could investigate metatranscript and metabolite differences associated with disease severity, during clearance of *C. difficile*, and restoration of colonization resistance. Metabolite information could offer insights into the molecules and nutrients present

mediating each of those actions. This information could also offer support in developing synthetic bacterial communities to inhibit *C. difficile*. These experiments may help us understand the importance of sialic acid, amino acids, glycine, bile salts, SCFAs, and indole in clearance and colonization resistance (22, 24–27). With defined mechanisms of gut community inhibition of *C. difficile*, we could work to develop synthetic communities to consume excess metabolites or resistant to indole to fill specific niches that *C. difficile* could occupy. As this map of niches available for *C. difficile* to colonize resolves, we could develop tests to detect available niches, such as excess metabolites in the feces of high-risk patients, and supplement them with a synthetic community to fill the available niches, reducing their risk of CDI during hospital stays. These studies would continue developing our understanding of CDI while bolstering the robustness of therapuetic communities by ensuring the findings are applicable to broader communities with variation in the gut microbiota closer to the variation among people and the multitude of potential perturbations we are exposed to (50, 156).

5.3 Conclusion

The composition and state of the gut microbiota holds great control over *C. difficle* infection. With an improved understanding of the relationship between *C. difficile* and the gut microbiota, we can better manage this healthcare threat and reduce the infections and deaths due to CDI. The findings of this work have helped to elucidate the relationship between the gut microbiota and *C. difficile*. While *C. difficile* is necessary for CDI, the composition and function of the gut microbiota can determine whether that infection progresses to severe disease. Furthermore, the gut microbiota at the time of infection affects whether the infection can naturally resolve or the extent of treatment needed to eliminate CDI. Finally, the niches available in the gut microbiota can allow *C. difficile* colonization unless filled with the appropriate

bacterial groups. Each gut microbiota has needs specific to their perturbation and disrupted mechanisms of colonization resistance. This work highlights the importance of understanding the niches of the specific susceptible communities in order to improve our ability to detect susceptible gut microbiota and treat them.

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