# Investigating host-microbiota interactions in IBD and CDI

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December 18th, 2020

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**INTRODUCTION:**

*Clostridioides difficile* (*C. diff*) is a spore-forming bacterium that causes the highest number of healthcare-associated infections in the developed world (1). The bacterium produces enterotoxins that can damage the lining of the intestines. *C. diff* infection (CDI) can be connected to a number of symptoms such as diarrhea, fever, nausea, and death. One of the reasons that *C. diff* poses such as a large danger is that colonization occurs mainly in patients who are receiving antibiotic treatments. People with intact intestinal microbiota populations have been shown to be resistant to CDI. However, since antibiotics disrupt the gut microbiota, CDI occurs most commonly in patients who are being treated with antibiotics.

However, there are factors other than antibiotic treatment that can cause increased susceptibility to *C. diff* such as inflammatory bowel disease or IBD. IBD is a disease that affects more than 5 million people around the world (2). The disease manifests itself in two forms: Crohn's disease and ulcerative colitis. As the name suggests, both diseases result in chronic intestinal inflammation with symptoms varying from abdominal pain, to bleeding and diarrhea. Crohn's disease can cause inflammation anywhere in the gastrointestinal (GI) tract, while ulcerative colitis mainly affects the colon and rectum. It should be noted that the symptoms for IBD can mirror those for CDI, which can make clinical diagnosis difficult without screening the gut microbiota.

Studies have shown that the abundance of CDI has been significantly increasing among hospitalized patients with IBD (3,4). This is an expected phenomenon since IBD has been associated with perturbations in the gut microbiota (5). The combined cases of IBD-CDI pose a particular clinical challenge because the treatments for CDI are limited. One of the common methodologies used to treat CDI is fecal microbiota transplantation or FMT (6). However, one

study found that the ability of FMT to restore the microbial ecology was inhibited in IBD patients. Given that IBD can cause increased susceptibility to CDI, even without antibiotic exposure, and that IBD can limit the treatment regimens used for CDI, the comorbid conditions of IBD-CDI present a unique and relevant topic of study.

When looking at susceptibility to *C. diff* there are three cases of interest. As stated earlier, (1) people with an intact intestinal microbiota remain resistant, while (2) antibiotic-treated and (3) IBD-affected patients are susceptible to CDI. The lab that I have been working in has developed a mouse model that can illustrate these three relationships. The model is described in full detail in the paper by Abernathy-Close et al. (7). In order to properly mimic the condition of IBD, the model required subjects who had a genetic susceptibility to having their microbial ecology disturbed under specific conditions. *Helicobacter hepaticus* (*H. hepaticus*) is a bacterium that has been used to trigger colitis in mice that are genetically deficient for the anti-inflammatory cytokine IL-10. When IL-10-deficient mice, otherwise referred to as IL-10 -/- mice, are colonized with *H. hepaticus* they develop colitis. This is similar to the expression of IBD in human patients. The model also takes advantage of earlier research to model the case of antibiotic-induced CDI. Previous mouse models have administered antibiotics such as cefoperazone to mice in order to mimic antibiotic-induced CDI in humans (8). With all three cases accounted for, the mouse model can be used to study the relationship between IBD and CDI.

The overall goal of the project was to use the mouse model created in previous experiments to investigate why IBD patients were susceptible to CDI in the absence of antibiotic exposure. In order to answer this question, two main aims were constructed. The first aim dealt with investigating the gut microbiota diversity in the two distinct models of CDI. The second

aim focused on determining the relationship between the degree of *C. difficile* activity, as defined by colonization burden and toxin levels, and host-microbiota responses in two distinct cases of CDI.

*Aim 1:* In order to study the gut microbiota diversity, a technique was needed to quantify the amount of diversity present within a particular ecosystem. For this project, the Shannon Diversity Index was used. The Shannon Diversity Index accounts for the abundance and evenness of the species present in an ecosystem (9). The higher the Shannon score, the higher the diversity of the ecosystem. The hypothesis for this aim was that the diversity would decrease in both the antibiotic and IBD groups. This was because lower gut microbiota diversity is associated with increased CDI susceptibility. Since both antibiotic treatments and IBD have been connected to increased CDI susceptibility, it was believed that both conditions would also result in lower gut microbiota diversity.

*Aim 2:* The second aim was created in order to compare the degree of *C. diff* colonization and the level of *C. diff* toxin produced in the two cases. It also sought to establish how the colonization burden and toxin levels would change the host-microbiota responses in both cases. From previous experiments with the mouse models, we know that IBD-induced CDI results in decreased disease severity when compared to antibiotic-induced CDI. Using this prior knowledge, I hypothesized that both the *C. diff* colonization burden and the toxin levels would be lower in the IBD group than in the antibiotic group. It was also hypothesized that the differing levels of *C. diff* activity in the two cases would lead to changes in the host-microbiota responses.

## METHODS

*Model Set-Up:*

The full model set-up is detailed in the paper by Abernathy-Close et al. which describes a few additional capabilities of the model (7). The mice for this experiment were obtained from a University of Michigan breeding colony originally derived from Jackson Laboratories. Both male and female wild-type or IL-10 -/- mice were obtained and kept in specific pathogen-free (SPF) environments. Each of the mice were at least 8 weeks old at the onset of the experiment. At the conclusion of the experiment, the mice were euthanized through $CO_2$ inhalation. The *H. hepaticus* strain was grown at 37°C for 3-4 days on tryptic soy agar (TSA) that was infused with 5% sheep's blood. The plates were stored in a microaerobic chamber that was at 1-2% oxygen in order to mimic the low oxygen concentrations of the gut. The *H. hepaticus* solutions for inoculation were prepared by harvesting the bacteria from the culture plates into trypticase soy broth (TSB). The solutions were diluted to $10^8$ colony-forming units (CFU) of *H. hepaticus* and then used to inoculate the mice via oral gavage. The colonization of the mice with *H. hepaticus* was confirmed using PCR of the *H. hepaticus cdtA* gene in DNA collected from fecal samples. The colonization status was confirmed before challenging the mice with *C. difficle* spores.

The *C. diff* spores were grown on pre-reduced taurocholate cycloserine cefoxitin fructose agar (TCCFA) plates. The plates were used to count the number of CFUs per mL for fecal and cecal samples as well as for the inoculation solutions. The plates were stored in an anaerobic chamber at 37°C for 18 hours before the number of colonies were counted.

The antibiotic-treated group was treated with 0.5 mg/mL of cefoperazone which was diluted in sterile drinking water. The water was provided to the mice for 10 days followed by 2 days of no

treatment. After the mice were pre-treated with *H. hepaticus,* a TSB vehicle, or antibiotics, they were orally gavaged with a solution of $10^3 – 10^4$ CFU *C. difficile* spores in 50 µL of water or a vehicle solution containing water alone. The full timeline of the experiment is provided in Figure 1 below.

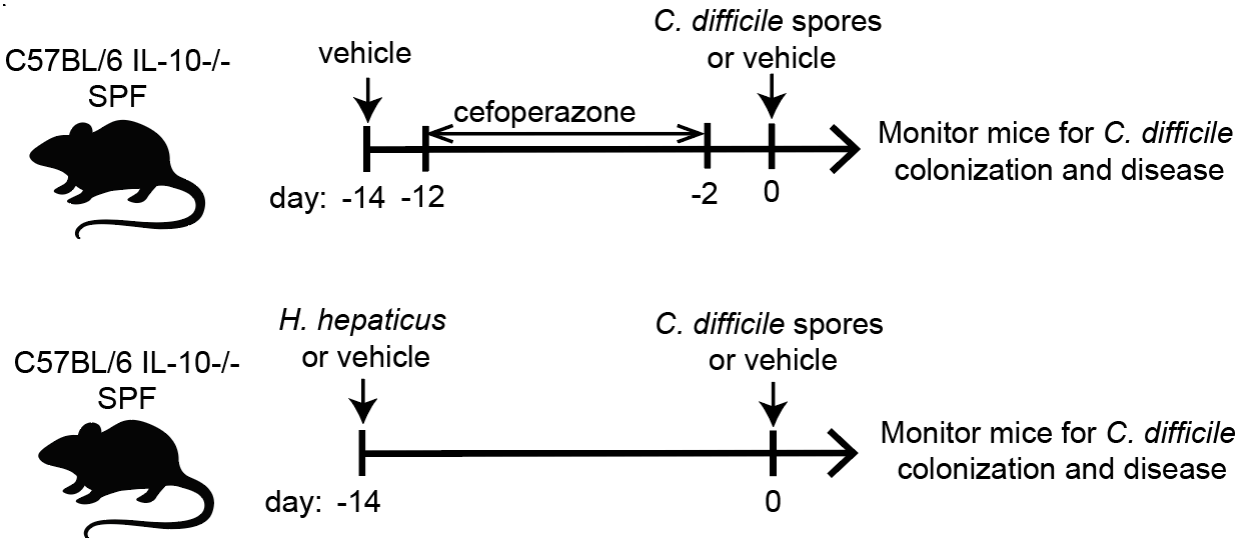


*Figure 1.* Experimental Timeline. The top drawing provides the timeline for the antibiotic treatment group, while the bottom drawing provides the timeline for the IBD treatment group.

*Data Collection*:

<u>*C. diff* colonization burden.</u> Fecal samples were collected from each mouse and weighed. The samples were passed into an anaerobic chamber where they were diluted 10% by volume with pre-reduced sterile phosphate buffered saline (PBS). The samples were then serially diluted onto pre-reduced TCCFA plates as stated earlier. After 18 hours, the number of colonies formed was counted by hand.

<u>*C. diff* toxin level quantification</u>. The amount of active *C. diff* toxin produced in each mouse was measured using a real-time cellular analysis (RTCA) assay (10). This methodology was

described in literature detailing a previous study. The RTCA assay measures the amount of active toxin present in the cecal contents of the mice. The cecal contents were collected from the mice when they were euthanized and then diluted by $10^{-3}$ with sterile 1X PBS. The toxin levels were found using the xCELLigence RTCA system and associated software.

16S rRNA gene sequencing. The cecal and colon contents were obtained from mice with and without IBD before they were challenged with *C. diff*. The contents were sent to the University of Michigan Microbiome Core which extracted total DNA from the samples (11). A region of the 16S rRNA gene was amplified in each sample. The amplicons were sequenced over 500 cycles with the Illumina MiSeq platform and MiSeq Reagent kit V2.

Statistical Analysis. All statistical analysis used in this project was done using RStudio. Here, p-values $\leq 0.05$ were considered to be significant.

## RESULTS

With regards to the first aim, the Shannon Diversity Scores were calculated using the 16S rRNA gene sequencing data. The 16S rRNA gene sequencing data provided information on the concentrations and types of species were present. Using the Shannon Diversity Index, the microbial diversity was plotted in a spaghetti plot. The spaghetti plots were produced for both the IBD treatment group (Figure 1) and the antibiotic treatment group (Figure 2). This type of plot allows us to track the gut microbial diversity in each mouse longitudinally for the entirety of the experiment. The Day 0 timepoint on both graphs is outlined since that is when the mice were challenged with *C. diff*, which is when the microbial diversity of the mice has the greatest impact on the outcomes.

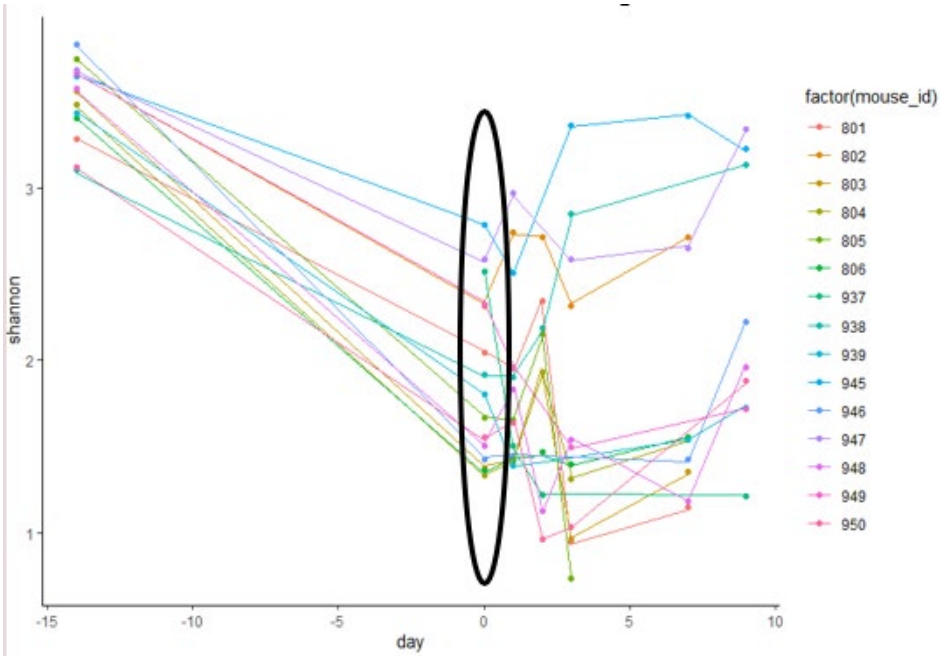


*Figure 1.* Shannon Diversity Scores Across Time for IBD Treatment Group.

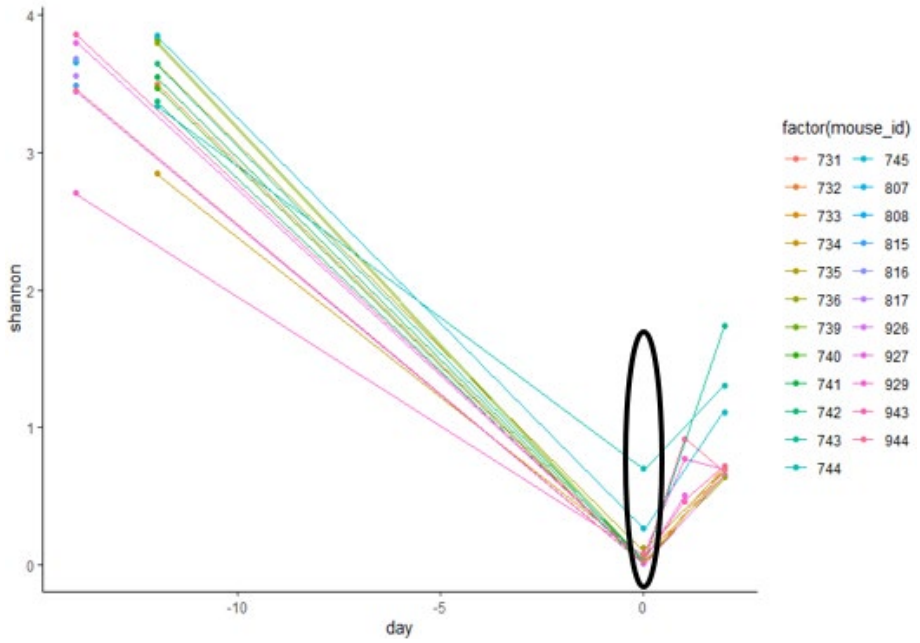


*Figure 2.* Shannon Diversity Scores Across Time for Antibiotic Treatment Group

As can be seen in Figures 1 and 2, the diversity of both groups decreases up to Day 0. It should be noted that the antibiotic treatment group decreases much more consistently and sharply than the IBD group. The average diversity at Day 0 for the antibiotic group is much lower than it is for the IBD group. It should also be noted that, at Day 0, the IBD group has a much greater deviation or variability in the diversity levels among mice. On a similar note, after the pre-treatment stops and the mice are challenged with *C. diff*, the antibiotic mice begin to recover some of their diversity, while the IBD mice have a more stochastic response, with some mice recovering their diversity and other mice losing even more diversity.

For the second aim, the first goal was to quantify the differences in colonization burden and toxin levels between the two cases of CDI. The results for this aim were published in an earlier study in the lab (7). Both the colonization burden and toxin levels are displayed below in Figure 3. The colonization burden is expressed in the number of CFU's per gram of cecal

content while the toxin level is expressed in the relative amounts of active toxin per gram of cecal content.

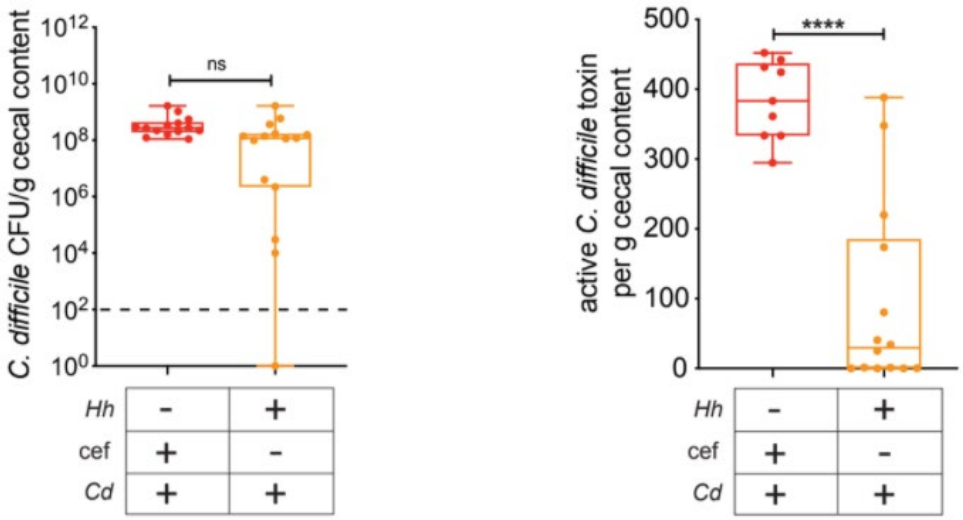


*Figure 3.* Differences in *C. diff* activity in the two cases of CDI. The red data points in both graphs represent the antibiotic treatment group, while the yellow data points represent the IBD treatment group.

From the left chart in Figure 3, it can be seen that the difference in the colonization burden between the 2 cases of CDI was not significant. However, as shown by the box-and-whiskers plot, the IBD group had more varied colonization burdens than the antibiotic group. From the right chart, it can be seen that the amount of active toxin is significantly higher in the antibiotic group than it is in the IBD group. Once more, we also see a greater amount of variability in the IBD group than in the antibiotic group.

The host-microbiota responses to the degree of *C. difficile* colonization in the antibiotic and IBD groups were also studied. The four categories used to look at the host-microbiota responses were disease severity, immune response, Shannon diversity, and toxin level. These categories were broken down into subcategories as shown in Table 1. In order to find

relationships between the level of colonization and the different host-microbiota responses a correlation study was used. The values for the different features were plotted against the colonization burdens of each mouse across all timepoints of the experiments. A statistical test was performed in RStudio to determine if there was a significant amount of correlation between the feature and the colonization burdens.

**Table 1: Correlation of Host-Microbiota Responses with Colonization Burden**

| **Category** | **Feature** | **Significant Correlation with *C. difficile* burden** | | |
|---|---|---|---|---|
| | | Both Treatment Groups | IBD-CDI | Antibiotic-CDI |
| *Disease Severity* | Clinical Score | n.s. | n.s. | n.s. |
| | Cecum Histological Score | **Positive** | n.s. | n.s. |
| | Colon Histological Score | **Positive** | n.s. | n.s. |
| *Host/Immune Response* | RBC Count | n.s. | n.s. | **Positive** |
| | WBC Count | n.s. | n.s. | n.s. |
| | Neutrophil Count | n.s. | n.s. | n.s. |
| | Lymphocyte Count | n.s. | n.s. | n.s. |
| | Monocyte Count | n.s. | n.s. | n.s. |
| | Eosinophil Count | n.s. | n.s. | n.s. |
| | Basophil Count | n.s. | n.s. | n.s. |
| | Reticulocyte Count | n.s. | n.s. | n.s. |
| *Shannon Diversity* | Microbiota Alpha Diversity | **Positive** | **Positive** | n.s. |
| *Toxin Level* | *Cd* Pathogenesis | **Positive** | n.s. | n.s. |

*RBC: Red Blood Cell; WBC: White Blood Cell

Table 1 shows that the cecum and colon histological scores had a significant correlation with colonization burden when both treatment groups were combined. The same result was obtained for the Shannon diversity and toxin levels. The Shannon diversity was also significantly correlated in the IBD-CDI group, while the RBC count was significantly correlated in the antibiotic group. These features will be looked at in more detail in future experiments.

The host-microbiota responses were also compared to the toxin levels. Specifically, the Shannon diversity score was plotted against the active toxin levels of mice since that allows us to determine if the amount of microbial diversity affected the amount of active toxin produced. This relationship is plotted in Figure 4. The graphs were produced using k-means clustering which allowed us to partition our observations into "k" clusters that allow us to view the separation in the data more clearly. For this experiment a k-value of "3" was chosen to represent low, middle, and high toxin levels.

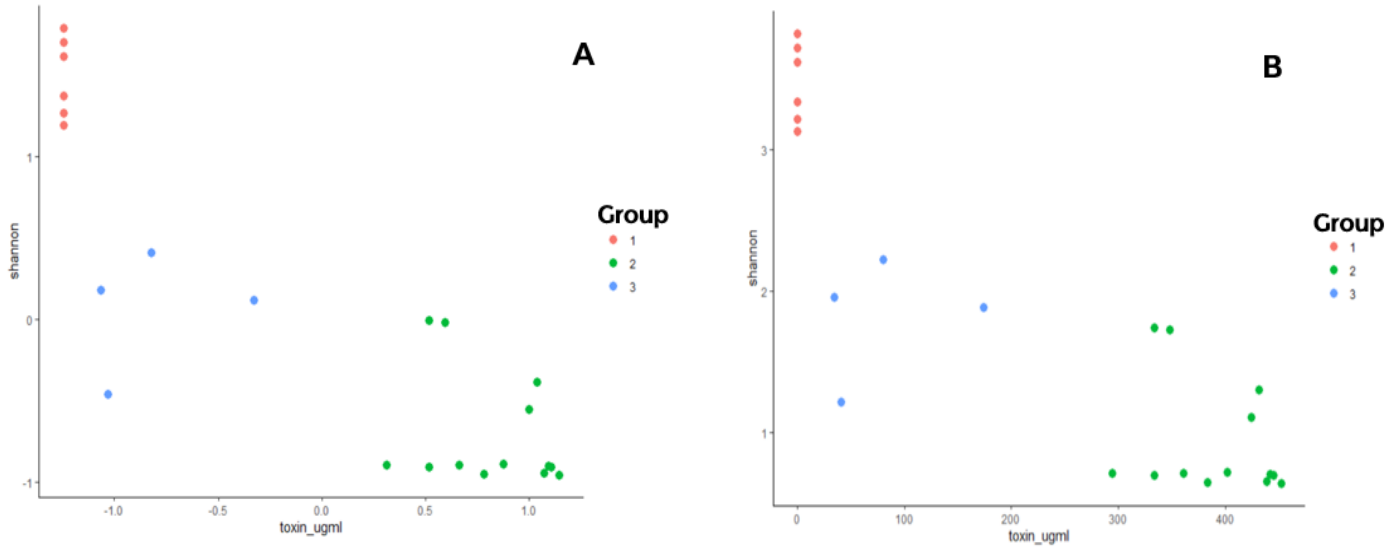


*Figure 4*. Plot of Shannon Diversity Score vs. Toxin Level. Figure 4A provides the standardized version of the data, while Figure 4B provides the raw data values.

As can be seen from Figure 4, a higher Shannon score was associated with a lower toxin level. It should be noted that differing k-values were used to generate graphs. However, k=3 provided the best clustering effect to view the data.

**DISCUSSION:**

For the first aim, it was found that both groups had decreased gut microbiota diversity, while the IBD group had a more variable response. Both models were susceptible to CDI, which was what was expected. For the second aim, the difference in colonization burden between the IBD and antibiotic group was not found to be significant. Instead, the key difference between the groups was that the colonization burden was more stochastic in the IBD group when compared to the antibiotic group. This could suggest that IBD shapes the gut microbiota in a more random way than the antibiotics do. This is supported by the fact that the gut microbiota diversity was more variable in the IBD group at Day 0. Interestingly, we also found that, although both groups were colonized to the same extent, the toxin level was significantly lower in the IBD group.

The correlation studies performed allowed us to identify which host-microbiota responses were correlated to the level of colonization of *C. diff.* However, based on feedback received during lab meetings, it became evident that the correlation study provided flawed results. Since the datapoints were provided from the same mice across multiple timepoints, each datapoint was not independent of the other datapoints. As such, outlier cases could have unduly influenced the level of correlation. However, this study still allowed me to identify important trends that should be studied further, such as the trend between colonization burden and toxin levels, cecum/colon histological scores, and red blood cell counts.

Finally, we found that higher Shannon scores were associated with lower toxin levels while lower scores were associated with higher toxin levels. This would seem to indicate that a higher gut microbiota diversity prevents *C. diff* from producing more active toxin. Overall, the data suggests that, similar to antibiotics, IBD causes increased CDI susceptibility by decreasing

the gut microbiota diversity. However, the response is more stochastic in the IBD than the antibiotic cases, which may suggest that a different mechanism is used to decrease microbial diversity in both situations. Given that IBD is associated with inflammation, we may need to study how exactly inflammation can shape microbial ecology and compare that effect to the effect of antibiotics.

The next steps for this project will involve looking more closely at the microbial diversity of both treatment groups. It was established that while the microbiota diversity of both the antibiotic and IBD groups decreased, the IBD group had a more stochastic response. However, the actual species present in the gut microbiota before and after the triggering of IBD and the treatment with antibiotics are still unknown. One potential reason for the increased susceptibility to CDI could be the disappearance of a key species that competitively occupied the niche that *C. diff* requires to survive within the gut. In order to establish what species had the biggest impact on the clinical differences of the treatment groups (colonization burden and toxin levels), a technique called LEfSe will need to be employed. This will allow us to characterize which species is most likely responsible for the differences in outcomes for the antibiotic and IBD groups. Overall, the mouse model can be used to further characterize the relationship between IBD and CDI, which will allow for the eventual development of new clinical treatments for CDI.

I would like to acknowledge Dr. Christine Bassis, Dr. Jonathan Golob, Dr. Ingrid Bergin, and Maddie Barron for their expertise and advice on the project. Additionally, I would like to thank Dr. Young for allowing me to work as a research assistant in his lab for the last few years. Finally, I would like to offer special thanks to Dr. Close who has been my personal and direct research mentor for the last 3 years.

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