# Predicting Adverse Outcomes in *Clostridioides difficile* Infections and Identifying Associated Host and Microbial Drivers of Disease Severity

by

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For Juliette

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# TABLE OF CONTENTS

# LIST OF FIGURES

**Figure**

# LIST OF TABLES

**Table**

# ABSTRACT

*Clostridioides difficile* is an anaerobic, toxigenic bacterium that causes nearly 500,000 gastrointestinal infections annually in the United States of America, with disease ranging from mild diarrhea to severe colitis and death. The goal of my thesis is to utilize a systems biology approach to examine *C. difficile* infection (CDI) pathogenesis in order to identify drivers of the variation seen in clinical outcome. The initial status of the system (host, microbiome, and pathogen) determines the trajectory of the infection, and fully exploring the variation seen in specific initial conditions allows for the discovery of emergent properties of the infection leading to adverse CDI outcomes.

CDI is a high concern for public health as it is rising in incidence, carries a high mortality and morbidity, and is difficult to treat, resulting in a multitude of emerging therapies currently being produced and tested in clinical trials for preventing and treating CDI. The wide variation of disease severity and high infection rate makes it imperative to identify patients that are at high risk of adverse outcomes early in the course of infection with *C. difficile*. We illustrate the ability of models utilizing serum inflammatory markers to predict which patients, at time of diagnosis, will develop adverse outcomes including ICU admission, colectomy and death. We also show that models produced on human data validate in experimentally infected mice with mild and severe CDI.

As there are high variations in patients in the clinic, we can use model systems to limit the variation we are exploring to identify what factors are associated with severe CDI outcomes. A majority of deaths from CDI occur in older individuals, indicating a relationship between age and the risk of developing severe CDI disease outcomes. We show that aging impacts the immune response to severe CDI in mice by altering serum inflammatory mediators and

the mobilization of neutrophils and eosinophils.

The microbiota is a key player in the development of CDI and the progression of the disease. We utilize a systems biology approach to examine *C. difficile* infection pathogenesis to identify the host and microbial drivers of variation in clinical outcome. We assembled a cohort of mice that includes a limited degree of variation in age and sex. Using this cohort, we examined the microbial community types ("enterotypes") that exist before and after antibiotics, during initial colonization with *C. difficile*, and at peak disease severity in mice. We describe the association of specific enterotypes with age, sex, colonization and subsequent disease severity.

The findings of this research highlight the complexity of *C. difficile* infections and the importance of a systems biology approach to its understanding. We find promising results for utilizing serum biomarker models for the determination of high-risk individuals at time of diagnosis, a potential method for allocating expensive and higher-risk emerging therapies to individuals who will develop adverse CDI outcomes. Our mouse cohort findings highlight the importance of age, sex, and microbial community structure in CDI pathogenesis and the prediction of adverse outcomes in CDI.

# CHAPTER I

# The Recent History of *C. difficile* Infections and their Impacts

There are many inherent difficulties in studying a complex system with multiple drivers and sources of variation. In *C. difficile* infections, the disease progression is affected by the host physiology, intestinal microbial community structure, and the pathogen virulence. Each core player of this complex relationship (host, intestinal community, and pathogen) has different initial drivers of variation (age, sex, immune state, diet, genetic composition, microbial exposure, etc), but these core players affect each other and lead to changes in one another throughout the course of infection. This wide range of initial conditions is carried throughout the infection leading to a wide range of disease outcomes.

The difficulty with managing *C. difficile* infections is that infection outcomes depend on four broad factors: infecting strain characteristics, intestinal microbial composition, host physiology and host immune response (Kyne et al., 2001; Sorg & Sonenshein, 2008, 2009; Islam et al., 2014; Ridlon et al., 2016; Theriot et al., 2016; Chilton et al., 2016; Thanissery et al., 2017). There are many different strains of *C. difficile*, each of which can carry a different set of enterotoxins. The intestinal microbiome differs significantly between individuals and antibiotics impact their microbial communities differently (Theriot et al., 2014). Lastly, host physiology and immune responses change as humans age, a process affected by immune deficiencies, immune reduction therapies, or other diseases that impact mucosal integrity

and the immune response such as inflammatory bowel disease (Dorshkind & Swain, 2009). These variations make it difficult to characterize individual components affecting pathogenesis and disease severity in humans (Rao et al., 2013, 2014). For this reason, modern research generally uses animal models to study CDI. The complete understanding of CDI requires the investigation into three areas of pathogenesis: prevention, severity of acute disease, and recurrence.

## 1.1 The Pathogenesis of *Clostridioides difficile* Infections and their Subsequent Healthcare Impacts

*Clostridioides difficile* is a gram-positive, spore-forming bacterium that produces a variety of enterotoxins, including TcdA, TcdB and Binary Toxin (CDT) (Chen et al., 2015; Chandrasekaran & Lacy, 2017; Di Bella et al., 2016). *Clostridioides difficile* infections (CDI) place a heavy burden on the health care system, with around 500,000 cases and a predicted cost of up to $1.5 billion in the US annually (Dubberke & Olsen, 2012; Zimlichman et al., 2013). Although *C. difficile* was first believed to be a nuisance disease that did not require new therapies (Bartlett, 1984), in the early 21st century, CDI incidence and morbidity increased drastically, galvanizing research into CDI pathogenesis and treatment (Loo et al., 2005; McDonald et al., 2005; Kyne et al., 2001).

### 1.1.1 Pathogenesis of *Clostridioides difficile*

*Clostridioides difficile* is a toxigenic, gram-positive, spore-forming bacterium. It can infect the gastrointestinal tract and cause mucosal damage. The pathogenesis of *Clostridioides difficile* infections is marked by interactions between the pathogen itself as well as the host and its native microbiota. Figure 1.1 illustrates the basic pathogenesis of the disease. Firstly, *Clostridioides difficile* infections (CDI) are spread by spores entering the gastrointestinal system. The spores can be transmitted by asymptomatic carriers or by active, symptomatic

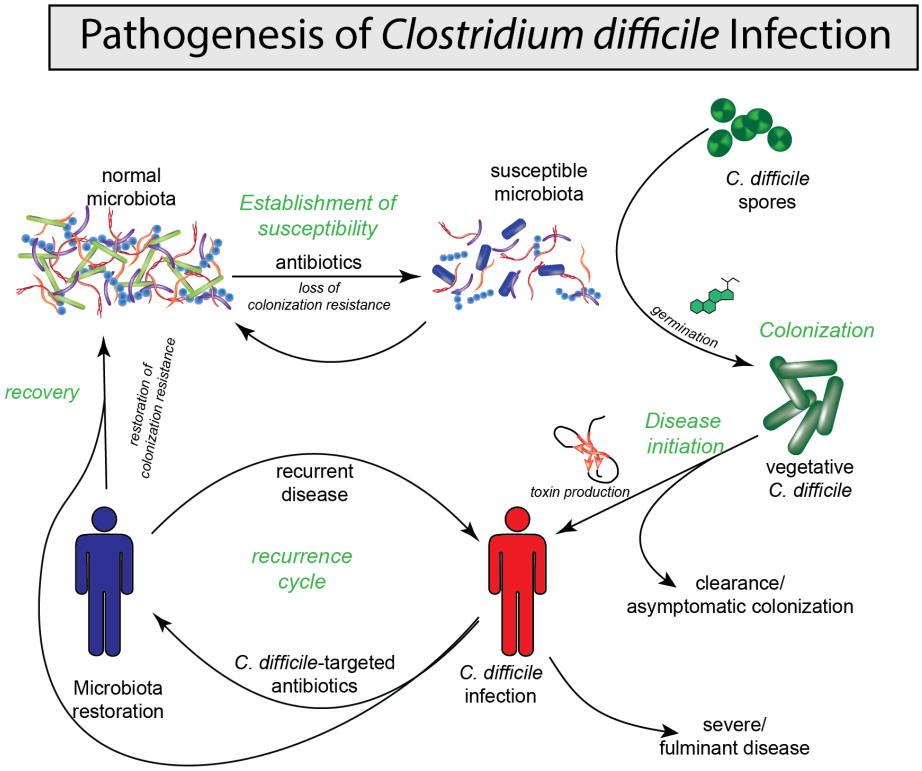



Figure 1.1 Pathogenesis of *Clostridioides difficile* Infection. Modified with permission from figure in (Britton & Young, 2014).

carriers. Common origins for the spores include soil, processed meats and food products, manure, as well as within healthcare settings such as hospitals and nursing homes.

Once *C. difficile* has entered the GI system, infection can occur if local factors cause germination and outgrowth of vegetative cells. Possible germinants include primary bile acids or glycine (Paredes-Sabja et al., 2014; Sorg & Sonenshein, 2008). In a healthy gut, the microbiota modifies host bile acids by deconjugation and dehydroxylation, generating secondary bile acids that inhibit growth of *C. difficile*. In the absence of native bacteria, however, C. difficle can cause opportunistic infections.

Once *Clostridioides difficile* has taken hold, it produces toxins: TcdA, TcdB, and/or binary toxin (Voth & Ballard, 2005; Chen et al., 2015; Di Bella et al., 2016; Chandrasekaran & Lacy, 2017). The abundance and ratios of these toxins depends on the strain of *C. difficile*. These toxins are released from the bacteria, bind to host cell receptors, get endocytosed,

and then translocate to the cytosol of host cells (Lyerly et al., 1985). By altering the cytoskeleton structure, the toxins increase epithelial permeability and disrupt cell to cell junctions. These factors lead to downstream damage, including diarrhea (Coffing et al., 2018), epithelial disruption (Riegler et al., 1995; Maciel et al., 2007), intestinal damage and colitis (Mitchell et al., 1986; Chumbler et al., 2016), or more severe systemic outcomes such as bowel perforation, peritonitis, septic shock, and/or organ failure (Carter et al., 2015). The eventual results of this damage can be death - CDI has a roughly 6.8% mortality rate (Luo & Barlam, 2018).

### 1.1.2 Occurrence of *C. difficile* Infections

People can become infected by *C. difficile* after intestinal microbiota disruption through mechanisms such as the usage of antibiotics. Once infected with *C. difficile*, clinical manifestations range from asymptomatic colonization to mild diarrhea and colitis to severe fulminant colitis and potentially fatal toxic megacolon.

One difficulty with studying CDI has been inconsistency of methods to identify the disease, as different hospitals and data sets have different testing methods and criteria used to identify CDI (Olsen et al., 2016). The first type of test commonly used to identify CDI were toxin enzyme immunoassays (EIA), which are relatively inexpensive, simple, and quick to run. However, EIA have low sensitivities (Tenover et al., 2011; Peterson et al., 2011), and often missed cases when they were used, presenting problems towards understanding the true incidence of CDI in patient populations (Polage et al., 2012). Over time, nucleic acid amplification tests were developed (Brecher et al., 2013; Cohen et al., 2014) which have improved sensitivities compared to the EIA, allowing a better characterization of the true incidence of CDI. However, some argue (Polage et al., 2015) that the nucleic acid amplification tests over-diagnosed cases not needing treatment, increasing the financial costs of CDI as patients received unneeded treatment. It remains unclear how to best determine which patients require which types of care from either a EIA or a nucleic acid amplifica-

tion test; instead, more analysis with additional data sources may be needed to securely predict patient outcomes (Leslie et al., 2012). Additionally, the method of diagnosis used (toxin enzyme immunoassays or nucleic acid amplification testing) must be considered when comparing data across sites (Gould et al., 2013).

In an effort to get better numbers on CDI infections, the CDC's Emerging Infections Program (EIP) began in 2009 monitoring seven initial locations. The monitoring program is intended to collect longitudinal, consistent data on the effectiveness of intervention strategies, characteristics of infecting strains, and changes in clinical outcomes (Cohen et al., 2014; Lessa et al., 2015). By monitoring a relatively large patient pool, the data set allows more resolved studies of patient sub-population outcomes (ex: Wendt et al., 2014). This effort has improved data on the incidence and causes of CDI (see Figure 1.2): in the 10 sites monitored by the CDC, community-acquired CDI is increasing as healthcare-acquired CDI decreases, although the total number of cases remain roughly constant.

Beyond the EIP, another way that researchers have tried to assess the impact of CDI is combining local disparate studies into single coherent analyses. Studies of CDI have also been frequently used in meta-analyses (ex: Marra et al., 2020) in an attempt to determine the true rate of CDI, accounting for differing diagnosing and accounting procedures between hospitals. The EIP and large number of smaller-scale studies illuminate CDI's effects on different sub-populations. Wendt et al. (2014) found that the incidence of CDI is roughly constant across all age groups for children, and generally (73% of the time) occurred with antibiotics use within 12 weeks previous to the identification of CDI in the child. Lessa et al. (2014) found that different geographical areas had different predictors of CDI incidence - most (but not all) or this effect can be explained by differences in populations in different geographical areas, with another potentially important factor being the types of antibiotics used in different areas. Olsen et al. (2016) found that the rates of CDI for patients 65 years or older are roughly ten times greater than those for patients younger than 65 years.

Together, all of these results paint a picture of CDI as a disease whose incidence depends

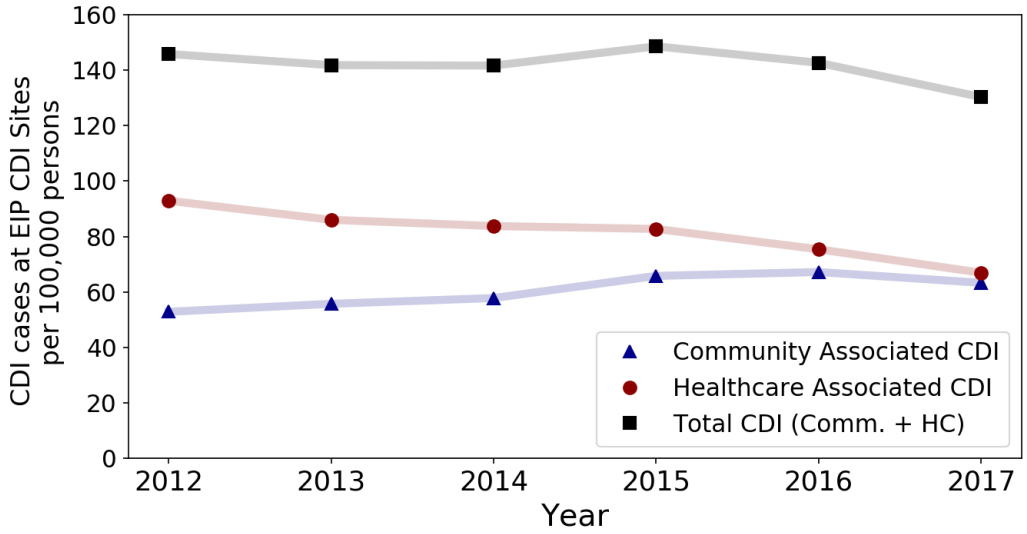



Figure 1.2 Community versus Healthcare Acquired CDI. The incidence of CDI at the 10 EIP sites monitored by the CDC for years in which data is available since the start of the program for the full 10 EIP sites. Data from `https://www.cdc.gov/hai/eip/cdiff-tracking.html`. The total number of CDI cases remains roughly constant over time, as those associated with transmission within the healthcare system are decreasing but community-associated cases are increasing. Note that the numbers from the EIP sites may not match those derived nationally due to differences in accounting.

strongly on host factors (Wendt et al., 2014; Olsen et al., 2016), the type of antibiotics used (Lessa et al., 2014), and the specific strain of the pathogen itself. All these factors must be considered in the effort to treat CDI.

### 1.1.3 Recurrence of *C. difficile* Infection

One characteristic of CDI is its tendency to recur. CDI recurrence is the reemergence of CDI symptoms two to eight weeks after the apparently successful treatment of the initial infection (McDonald et al., 2018) and occurs in up to 35% of adult patients with CDI (Marsh et al., 2012), with a median recurrence of 22% (Olsen et al., 2015; Zilberberg et al., 2014). Nicholson et al. (2015) found CDI recurrence for children to be 22%.

Patients can also experience multiple recurrences, and the infecting strain type correlates with the number of recurrences. Marsh et al. (2012) found that patients whose CDI recurred once were more likely to be reinfected with the same strain they were originally infected with, while patients with multiple recurrent episodes were equally likely to be afflicted by a new infection or the original infecting strain. Estimates for multiple (two or more) recurrences of CDI range from 8.2% to 9% (Abu-Sbeih et al., 2019; Sheitoyan-Pesant et al., 2016). Each subsequent recurrence increases the risk for additional recurrences (Olsen et al., 2015).

The CDC treatment guidelines suggest different treatments for the first recurrence of CDI and subsequent recurrences (see Figure 2.2). One newer and more risky therapy, fecal microbiota transplants (FMT), is suggested for only the second recurrence or beyond, but not for primary CDI or a first recurrence (McDonald et al., 2018), as it is difficult to standardize and the long-term effects are not known.

### 1.1.4 *C. difficile* Infection Mortality

In 1996, CDI incidence was roughly 31 cases per 100,000 patients (McDonald et al., 2005). By 2008, this number had increased to 61 per 100,000 (Hunt & Ballard, 2013). Of the most severe cases, the result is 15,000 attributable deaths annually. Along with the incidence of

the disease, the mortality of *C. difficile* has changed over the years as new strains emerged and new treatments were developed. An increase in the CDI mortality rate in the mid-2000s (a factor of four times greater mortality between 1999 and 2004; Redelings et al., 2007) was associated with the emergence of new hyper-virulent strains including the NAP1, BI, and 027 strains (Cookson, 2007; Hunt & Ballard, 2013). Subsequently, Luo & Barlam (2018) found in a study using 3,337,910 CDI cases from the Nationwide Inpatient Sample database that from 2004 to 2014, although CDI incidence continued to increase, the CDI mortality actually went down; in 2004, the 30-day mortality rate was 9.7%, and by 2014 it was only 6.8%. 80% or more of these deaths occur in patients 65 years or older.

### 1.1.5 Financial Costs of CDI

The burden of *C. difficile* infections is significant, as there are roughly 500,000 cases per year in the US (Lessa et al., 2015). The financial strain on the healthcare systems is also significant, as *C. difficile* infections cost up to $1.5 billion dollars annually in attributable health care expenses (Zimlichman et al., 2013). Disease outcomes vary greatly: colonization with *C. difficile* can result in outcomes ranging from asymptomatic carriage to diarrhea to severe colitis and death.

One reason that it is important to identify the cases of CDI requiring the highest degree of clinical intervention is that over-treatment leads to severe financial burdens on the healthcare system (Polage et al., 2015). Zhang et al. (2018) found that in the six months after CDI was identified in a patient, the typical cost of CDI treatment is $24,205 for primary CDI infection, with recurrent infections within the six month period costing an additional $10,580 to treat.

## 1.2 The Association of the Microbiome with Acute and Recurrent *C. difficile*

The native microbiome of the host plays an important role in preventing *C. difficile* from colonizing the host. Generally, the gut flora provides colonization resistance (Bohnhoff & Miller, 1962; Van der Waaij et al., 1971; Buffie & Pamer, 2013), and the native microbiota provides this resistance against *C. difficile* as well (Pérez-Cobas et al., 2015; Theriot & Young, 2015).

As discussed in Section 1.1.1, one of the ways that *C. difficile* colonization can be prevented is via the production of secondary bile acids, which hinder the growth of vegetative cells. For example, Buffie et al. (2015) found that resistance to CDI can be conferred by *Clostridium scindens*, which can synthesize secondary bile acids from primary ones due to its 7-alpha-dehydroxylase enzyme.

In more general ways, CDI can be prevented ecologically. The presence of a wide variety of other harmless of beneficial bacteria can starve *C. difficile* from the resources it would need to take hold. Studies of the gut diversity during infection seem to indicate that a healthy, diverse, robust microbiota could prevent the colonization of the gut by *C. difficile* (Wilson et al., 1986), particularly in cases of recurrent CDI (Chang et al., 2008). This is partially due to other bacterial species out-competing *C. difficile* for resources (Wilson & Perini, 1988). However, diversity alone is not enough to prevent *C. difficile* colonization (Ng et al., 2013); the specific species present in the gut are also important.

Resource competition also affects the ability of *C. difficile* to gain a foothold in the gut. Factors that increase the amount of resources available to *C. difficile* can include some types of antibiotics and some other species of gut bacteria. Immediately after use of antibiotics, increased levels of amino acids and sugar alcohols lead to increased susceptibility to CDI (Theriot et al., 2014). Some other gut bacteria may produce favorable conditions for *C. difficile* to take hold. For example, the presence of *Bacteroides thetaiotaomicron* in the gut

allows *C. difficile* to convert succinate to butyrate.

The complexity of the interplay between antibiotics, native bacteria, and *C. difficile* makes it difficult to build treatments using replacement bacteria. The underlying ecological connections are complex: the exact combinations of bacterial species that can prevent *C. difficile* colonization is not constant (Lawley et al., 2012).

## 1.3 Animal Models in *C. difficile* Research

The complete understanding of CDI requires the investigation into three areas of pathogenesis: prevention, severity of acute disease, and recurrence. These factors can be studied retrospectively using data compiled by health care providers, but there are limits to that data. Instead, animal models are used to study factors relating to disease.

Animal models have been used to determine the effect of *C. difficile* toxins and disease progression since the early study of *C. difficile* (Best et al., 2012). Animal models have allowed a deeper understanding of the interactions and functions of the three toxins, as well as a study of the disease response to particular community structures of microbiota and antibiotic types. The most common CDI models are hamsters (Chang et al., 1978; Rifkin et al., 1978; Larson & Welch, 1993), mice (Corthier et al., 1985; Wilson et al., 1986), and rabbits (Alcantara et al., 2001), with other animals such as prairie dogs (Muller et al., 1987) and zebra fish (Hamm et al., 2006) being less common. As more strains of *C. difficile* have been found, animal models also allow characterization of the differences between new and old strains. Lyerly et al. (1985) studied the effects of *C. difficile* toxins by delivering toxins to hamsters, mice, and rats intragastrically, and found toxins A and B interacted. Other groups, such as Triadafilopoulos et al. (1987) and Lima et al. (1988), used rabbit models to attempt to discern the difference in effect between toxin A and B.

A robust mouse model of CDI has been developed by the Young Lab (see for example Theriot et al., 2011) that characteristics well suited to tackle the CDI problem. This model has baseline colonization resistance against *Clostridioides difficile*, colonization resistance

modulated by antibiotic treatment, wide range in disease severity in mice infected with *C. difficile*, and colonization resistance that recovers after discontinuation of antibiotic therapy. Significant similarities exist between the mouse model of CDI and the human disease (Chen et al., 2008; Reeves et al., 2011).

Significant strides have been made utilizing this model of CDI, including characterization of the immune response during CDI, which results in increased cytokine expression in the tissue, histological damage, and recruitment of monocytes and neutrophils (Buonomo et al., 2013; Sadighi Akha et al., 2013; El-Zaatari et al., 2014; Buonomo et al., 2016; Tinoco-Veras et al., 2017). Many researchers have identified specific factors that are elevated during infection and studied the effect of knocking down these factors (Kim et al., 2014; McDermott et al., 2015, 2016). However, studies use different infecting strains, antibiotics, and mice populations (age, sex, etc). Even holding these factors constant, there are still substantial differences in the severity metrics used. Metrics used in past studies include clinical scores, weight loss, histopathology of the cecum, and of the colon. In histopathology, some groups study edema, epithelium damage, and infiltration, while others focus on only a single score or a subset of these. Additionally, certain studies will look at cecum alone while others will analyze colon score. Lastly, the length of infection studied will impact the resulting severity profile, as some CDI strains cause rapid disease (within 48 hours) while others cause an indolent course reaching peak severity at 96 hours (Pawlowski et al., 2010; Buonomo et al., 2013; Kim et al., 2014; McDermott et al., 2014, 2017; Shin et al., 2018).

## 1.4 Results of this Work

The goal of my thesis is to utilize a systems biology approach to examine *Clostridioides difficile* infection pathogenesis to identify the host and microbial drivers of variation in clinical outcome. The initial status of the system (host, microbiome, and pathogen) determines the trajectory of the infection. Fully exploring the variation seen in specific initial conditions will allow for the discovery of drives for disease outcomes.

CDI is a high concern for public health as it is rising in incidence, carries a high mortality and morbidity, and is difficult to treat. As such many new emerging therapies are being produced and research in clinical trials (Chapter II). Second, the wide variation of disease severity and high infection rate makes it imperative to identify patients that are at high risk of adverse outcomes early in the course of infection with *C. difficile*. Can we use serum-based biomarkers for predicting adverse outcomes in humans (Chapter III)? Third, as the there is a high variation in patients in the clinic, we can use model systems to limit the variation we are exploring to identify what factors are associated with severe CDI outcomes. Our mouse model of CDI exhibits similar immune response profiles as compared to humans affected by *C. difficile*. In order to perform the comparison between the immune responses of the mouse model and standard human immune responses, we need to model both mild and severe CDI. To do this in the mouse mode, we infect mice with either a high or low virulence strain. Fourth, age has been shown to be associated with the risk of developing severe CDI disease outcomes. We look at the impact of age on CDI in our mouse model and the associated immune response (Chapter IV). Fifth, the microbial community is a key player in the development of CDI and the progression of the disease. The end goal of my thesis is to utilize a systems biology approach to examine *C. difficile* infection pathogenesis to identify the host and microbial drivers of variation in clinical outcome. I have constructed a cohort of mice that includes a limited degree of variation in age and sex. I then use this cohort to examine the microbial community types that exist before and after antibiotics in mice. I then examine the associations of the resulting enterotypes with initial colonization with *C. difficile* and subsequent disease severity (Chapter V). I end with a summary of the results of this thesis, as well as a discussion of future directions, in Chapter VI.

# CHAPTER II

# Novel Therapies and Preventative Strategies for Primary and Recurrent *Clostridium difficile* Infections

Results in this chapter were published in: ***Dieterle, M. G.***, *Rao, K., Young, V.B. (2019). "Novel therapies and preventative strategies for primary and recurrent* Clostridium difficile *infections." Annals of the New York Academy of Sciences 1435 (1):110-138.* and are presented here with minor revisions.

## 2.1 Abstract

*Clostridium difficile* is the leading infectious cause of antibiotic-associated diarrhea and colitis. *Clostridium difficile* infection (CDI) places a heavy burden on the health care system, with nearly half a million infections yearly and an approximate 20% recurrence risk after successful initial therapy. The high incidence has driven new research on improved prevention such as the emerging use of probiotics, intestinal microbiome manipulation during antibiotic therapies, vaccinations, and newer antibiotics that reduce the disruption of the intestinal microbiome. While the treatment of acute *C. difficile* is effective in most patients, it can be further optimized by adjuvant therapies that improve the initial treatment success and decrease the risk of subsequent recurrence. Lastly, the high risk of recurrence has led to multiple emerging therapies that target toxin activity, recovery of the intestinal microbial community, and elimination of latent *C. difficile* in the intestine. In summary, CDIs illustrate the complex interaction among host physiology, microbial community, and pathogen that requires specific therapies to address each of the factors leading to primary infection and recurrence.

## 2.2 Introduction and Background

*Clostridium difficile* is a toxigenic, gram-positive, spore forming bacterium that can infect the gastrointestinal tract and cause mucosal damage. People can become infected by *C. difficile* after intestinal microbiota disruption through mechanisms such as the usage of antibiotics. Once infected with *C. difficile*, clinical manifestations range from asymptomatic colonization to mild diarrhea and colitis to severe fulminant colitis and potentially fatal toxic megacolon. *C. difficile* causes nearly half a million infections per year in the United States alone (Lessa et al., 2015), and costs up to $1.5 billion dollars annually in attributable health care expenses (Zimlichman et al., 2013).

While pseudomembranous colitis was first described in 1893, it was not known to be associated with antibiotic usage until 1974 (Bartlett, 2008; Tedesco et al., 1974). Even then, *C. difficile* was not known to be the causative agent. *C. difficile*, first isolated from newborns in 1935, was not identified as a leading cause of antibiotic-associated diarrhea and pseudomembranous colitis until 1978 (Bartlett, 2008; Hall & O'Toole, 1935). At this time, *C. difficile* infection (CDI) was seen as a treatable nuisance disease that did not necessitate specific therapy or the development of new treatments (Bartlett, 1984). However, the emergence of the CDI epidemics in the mid 1990s and early 2000s, caused by strains belonging to the type NAP1/BI/027, led to an increase in incidence and morbidity that galvanized the development for new therapeutics, monitoring, and testing (McDonald et al., 2005; Loo et al., 2005).

The first step of any treatment begins with the correct identification of the disease. While *C. difficile* is a leading cause of antibiotic associated diarrhea, it is not the only causative agent. As there are strains of nontoxigenic *C. difficile* that are incapable of causing disease and a high rate of asymptomatic carriage of toxigenic strains, accurate diagnosis cannot depend solely on identifying *C. difficile* in the stool. Instead, diagnosis of *C. difficile* is dependent on two factors: identification of toxigenic *C. difficile* or its toxins or histopathologic or colonoscopic evidence showing pseudomembranous colitis, and signs of clinical disease such as three or more unformed stools within 24 hours, radiographic evidence of ileus or toxic megacolon (McDonald et al., 2018). There are many diagnostic tests and algorithms for diagnosing *C. difficile*, each with strengths and weaknesses. Testing can include the following as either single tests or as part of a multi-step algorithm: EIA-

based toxin A/B tests, PCR based nucleic acid amplification tests (NAAT) for tcdB, or glutamine dehydrogenase tests (when paired with one of the prior toxin tests). The various testing algorithms have been summarized in the recent 2018 IDSA/SHEA Guidelines (McDonald et al., 2018). The main goal is to identify only the patients that require treatment and to classify them in terms of severity potential and post-therapy recurrence risk. A recurrent case of CDI is defined as symptom onset and stool specimen positive for *C. difficile* 2 to 8 weeks following the last positive specimen during previous treatment of primary CDI. Such classifications help guide therapy and determine which targeted therapeutics to use, since emerging therapies are being developed to specifically address prevention, CDI treatment and recurrence reduction.

Current therapies are mainly directed at addressing primary CDI with the use of antibiotics such as vancomycin, as well as treating recurrent disease with vancomycin or fidaxomicin. In repeatedly recurrent disease, additional current approaches use antibiotic tapers, antibiotic adjuvants, and fecal microbiota transplants (FMT) (McDonald et al., 2018). Newer therapies are being developed and put into practice to reduce initial infection; these include probiotics and vaccines. New treatments can also reduce the risk of recurrence and severe disease with narrow spectrum antibiotics, immunotherapies, and microbial replacement therapies. In this review, we will summarize the current therapy recommendations and indicate areas of improvement that new emerging drugs and treatments hope to address.

### 2.2.1 How the Pathogenesis of *C. difficile* Informs Treatment Approaches

The pathogenesis of *Clostridium difficile* infection represents the complex interaction between the pathogen, the host and the native microbiota (Figure 2.1). Spores can be spread by both asymptomatic carriers and symptomatic patients, necessitating the need to isolate infected individuals and appropriately clean the healthcare environment. The initial phase of *C. difficile* infection occurs when spores enter the gastrointestinal system and local environmental factors trigger germination and outgrowth of vegetative cells. Primary bile acids (e.g. taurocholic acid, cholate) act as germinants during in vitro experiments with glycine as co-germinant (Paredes-Sabja et al., 2014; Sorg & Sonenshein, 2008). Native members of the microbiota have the capacity to deconjugate and dehydroxylate primary bile acids into secondary bile acids, some of which have shown to be

inhibitory to vegetative *C. difficile* (Sorg & Sonenshein, 2009; Ridlon et al., 2016; Thanissery et al., 2017). Antibiotic-mediated alteration of the native bacteria can impair primary bile acid conversion to secondary bile acids, leading to an environment that promotes *C. difficile* sporulation and vegetative growth (Theriot et al., 2016).

Once vegetative cells have been produced from the spores in the colon, *C. difficile* cells produce the toxins TcdA, TcdB, and binary toxin. Specific strains of toxigenic *C. difficile* produce different levels and subsets of these toxins. For example, certain strains can produce TcdA and TcdB but not the binary toxin. TcdA and TcdB enter the cell by binding to specific cellular receptors found on intestinal epithelial cells (Chandrasekaran & Lacy, 2017). Once endocytosed, the acidification of the endosome leads to conformational changes of the toxin that releases the N-terminal glucosyltransferase domain into the cytoplasm. This domain acts to inactivate specific enzymes such as the Rho GTPases through glycosylation. This leads to both cytopathic and cytotoxic downstream effects by altering the cytoskeleton structure, epithelial permeability, and cell to cell junctions, as well as by activating the inflammasome and apopotosis (Di Bella et al., 2016; Chen et al., 2015). With the destruction of the epithelium, *C. difficile* infection can cause diarrhea and lead to complicated cases through systemic effects such as sepsis, shock, peritonitis and bowel perforation as the intestines becomes compromised.

Targeted antibacterial therapy can be used to reduce intestinal *C. difficile* levels. However, even with successful treatment and clearance, a median of 21.6% of patients will experience recurrent disease with an increased risk to reoccur following each recurrence (Deshpande et al., 2015). The high recurrence risk highlights the importance of restoring the colonization resistance against *C. difficile*.

The interactions between the host, pathogen and microbiota in CDI presents multiple opportunities for the development of novel therapies that target specific steps in pathogenesis. For example, emerging therapies can reduce the risk of CDI by lowering the effect of systemic antibiotics, decreasing the levels of primary bile acids, increasing secondary bile acids, and restoring the native microbiota's ability to convert primary bile acids to secondary bile acids. While there is no universally accepted definition of narrow spectrum antibiotics, it is accepted in the field that this refers to antibiotics that affect a smaller range of bacterial groups, such as those that affect only gram

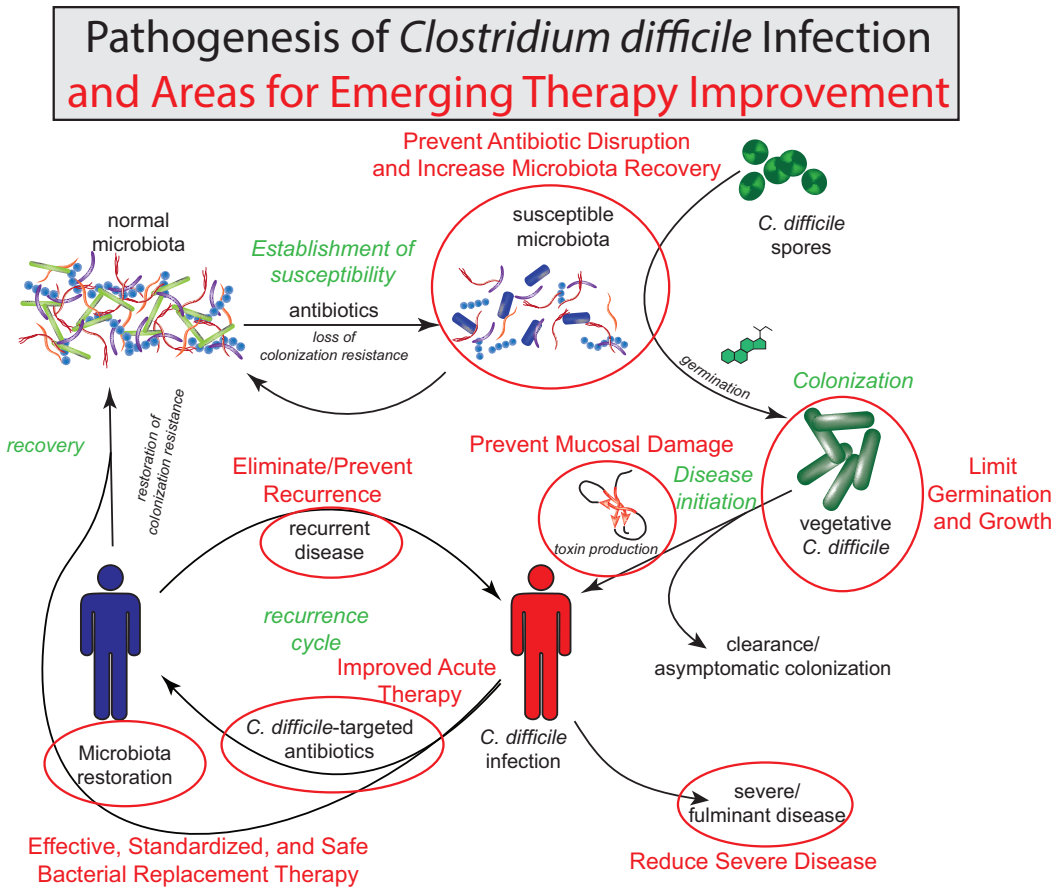



Figure 2.1 Starting with the normal microbiota, antibiotic disruption of the intestinal bacterial community results in a susceptible state, which can led to colonization with *C. difficile*. Once germinated, vegetative *C. difficile* produces a variety of toxins to cause mucosal damage. If the damage is severe, this may lead to severe disease. With effective antibiotic therapy *C. difficile* can be reduced and the natural colonization resistance can develop overtime as the natural microbial communities recover. Reinfection or recurrence may occur before this process is complete. Fecal microbiota transplant may expedite this recovery by directly replacing the missing microbial community members. Areas marked with a red circle are potential areas where new emerging therapies could improve clinical management.

positive bacteria. In contrast, broad spectrum antibiotics are those that affect multiple classes of bacteria, such as those that impact both gram-positive and gram-negative bacteria including anaerobic bacteria. It has been shown that specific broad spectrum antibiotics may cause further disruption of the native gut bacteria leading to increased risk of recurrence. As such, emerging therapies targeted to recovery of the native bacterial post CDI treatment include narrow spectrum antibiotics that allow for more rapid recovery of native bacteria and bacterial replacement therapies such as specific bacterial communities delivered by enema or oral ingestion. The *C. difficile* cells and toxins can also be targeted by preformed antitoxin and anti-*C. difficile* antibodies or by anti-*C. difficile* antibodies natively produced due to vaccination. These therapies have the potential to reduce risk of CDI, decrease disease severity, and prevent recurrence by targeting spores, vegetative cells, and toxins.

## 2.3 Conventional Management of CDI

### 2.3.1 Initial Management After Diagnosis

Once a patient is diagnosed with CDI, any inciting antibiotics should be discontinued if possible (Slimings & Riley, 2014; Bagdasarian et al., 2015). Initial management may also include decreasing proton pump inhibitors and anti-motility agents, which have been associated with the severity of CDI (Rao et al., 2014) and subsequently the risk for recurrence (Abou Chakra et al., 2014).

The next step is to determinate the severity of the illness as this will guide the therapeutic approach, with certain treatments recommended based on severity. In the 2018 IDSA/SHEA Guidelines, non-severe (mild to moderate) disease is defined as diarrhea occurring with white blood cell count <15,000 cells/mL and serum creatinine <1.5 mg/dL. Severe CDI is defined as CDI with white blood cell count $\geq$ 15,000 cells/mL or serum creatinine $\geq$ 1.5 mg/dL. Lastly, severe and complicated CDI is defined by systemic signs of infection and evidence of hypotension, ileus, or toxic megacolon (Cohen et al., 2010; McDonald et al., 2018).

### 2.3.2 Current Management Recommendations

*C. difficile* is resistant to many antibiotics, including fluoroquinolones and macrolides, with an increased resistance to rifampin as seen in 027 strains (Curry et al., 2009; von Muller et al., 2012). As indicated in the 2018 IDSA/SHEA guideline, the treatment of CDI is determined by severity and recurrence state. For primary CDI, non-severe disease is treated by vancomycin (125 mg orally 4 times daily for 10 days) or fidaxomcin (200 mg orally 2 times daily for 10 days). If neither is available or tolerated, metronidazole (500 mg orally 3 times daily for 10 days) can be used instead (McDonald et al., 2018). For severe CDI, the recommendation is vancomycin (125 mg orally 4 times daily for 10 days) or fidaxomcin (200 mg orally 2 times daily for 10 days). If CDI is severe and complicated, vancomycin (500 mg 4 times daily given by mouth or nasogastric tube) can be given with IV metronidazole (500 mg every 8 hours). If ileus is present, vancomycin can be given by rectal enema in addition to the oral vancomycin and IV metronidazole. If surgical intervention is necessary, rectal sparing subtotal colectomy or diverting loop ileostomy with colonic lavage followed by vancomycin flushes are recommended. For recurrent disease, first recurrence is treated with a standard course of vancomycin (125 mg orally 4 times daily for 10 days) if the previous CDI was treated with metronidazole. If the previous course was not metronidazole, the recurrent case is treated with vancomycin taper (e.g. 125 mg orally 4 times per day for 10-14 days, 2 times per day for a week, 1 per day for a week, and lastly every 2-3 days for 2-8 weeks) or fidaxomicin (200 mg orally 2 times daily for 10 days). For the second or subsequent recurrences, vancomycin taper, vancomycin (125 mg orally 4 times daily for 10 days) followed by rifaximin chaser (400 mg 3 times daily for 20 days), or fidaxomicin therapy (200 mg orally 2 times daily for 10 days) can be used. FMT can be considered for the second or subsequent recurrence, but is not recommended for primary CDI or a first recurrence (McDonald et al., 2018). Treatment management and dosing schemes are presented in Figure 2.2.

In the past, metronidazole was recommended for mild to moderate CDI for cost effective treatment, while vancomycin was used with metronidazole intolerance and/or if the patient has an increased risk of recurrence. However, recent clinical trials and a meta-analysis have shown that vancomycin is superior to metronidazole for non-severe CDI with a percentage resolution of 87% compared to 78% for metronidazole. The 2018 IDSA/SHEA guidelines indicate the use of van-

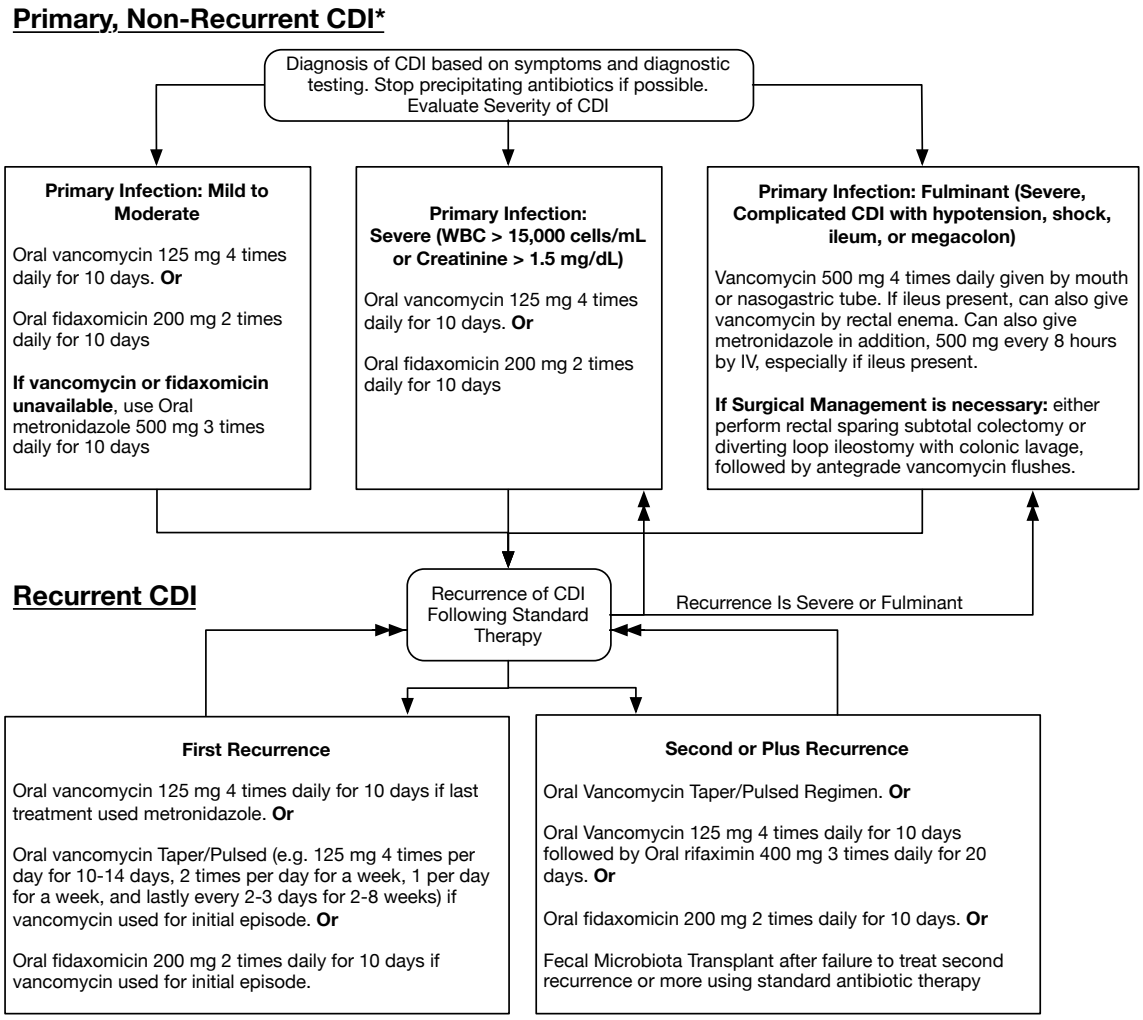



Figure 2.2 This diagram reviews the recommended treatment approaches for primary and recurrent CDI, depending on disease severity based on the 2018 IDSA/SHEA. Additionally, there is some evidence for fidaxomicin in place of rifaximin as a chaser for recurrent CDI and the EXTEND trial did show evidence for using extended-pulsed fidaxomicin for primary CDI however this is not currently reflected in guidelines (Johnson & Gerding, 2013; Soriano et al., 2014; Guery et al., 2018; McDonald et al., 2018). Primary *Clostridium difficile* infection is defined as a new episode of symptoms with no previous positive *C. difficile* test result within 8 weeks and conformation of CDI by diagnostic testing.

comycin or fidaxomicin for non-severe CDI, with metronidazole reserved for cases when the other two drugs are not available or tolerated (McDonald et al., 2018).

Fidaxomicin is a narrow spectrum, non-absorbable macrocyclic antibiotic which inhibits the bacterial RNA polymerase $\beta$ subunit (rpoB) that has been found to be noninferior to vancomycin in success rate in multiple phase 3 trials, but shows substantially reduced rate of recurrence (Kociolek & Gerding, 2016). Fidaxomicin has bactericidal activity against *C. difficile* by inhibiting RNA polymerase and disrupting RNA synthesis (Optimer, 2016). The narrow spectrum activity of fidaxomicin potentially maintains the stability of the intestinal microbiota at a higher level, leading to a more transient loss of colonization resistance and more robust recovery of the microbiota after treatment. Fidaxomicin shows similar clinical cure rates with reduced recurrence rates (Louie et al., 2011; Cornely et al., 2012; Fehér & Mensa, 2016; McDonald et al., 2018). While fidaxomicin comes at a higher cost than vancomycin, the treatment may lower the overall cost by reducing recurrence rates if used to treat patients with a high recurrence risk (Bartsch et al., 2013; Fehér & Mensa, 2016).

Additionally, studies have been done on using alternative fidaxomicin dosing regimens for treating primary and recurrent CDI looking at clinical cure rate and recurrence reduction. Soriano et al. (2014) showed in a case study of patients with multiple recurrent CDI that only 2 out of 11 (18%) patients had recurrence when given fidaxomicin in a tapering dose regimen as compared to 3 of 8 (38%) patients that were given only fidaxomicin as a chaser twice daily for 10 days. These patients received these chasing or tapering regimens after a standard CDI antibiotic therapy. This study indicated that fidaxomicin chasers and tapered regimens could help prevent recurrence in patients experiencing multiple recurrent CDI. However, the study was not randomized and had a low sample size, requiring future studies to further evaluate these findings (Soriano et al., 2014).

EXTEND, a recent randomized, controlled, phase 3b/4 trial, compared the effects of extended-pulsed fidaxomicin treatment (200 mg orally given twice daily for days 1–5, once daily every other day from days 7–25) to vancomycin treatment (125 mg orally four times a day for 10 days), for primary and recurrent CDI. 124 of 177 (70%) patients receiving the extended pulsed fidaxomicin treatment had sustained clinical cure at 30 days post treatment, while in comparison only 106 of 179 (59%) patients receiving the vancomycin treatment achieved sustained clinical cure at 30 days

post treatment (p = 0.030, odds ratio 1.62 [95% CI 1.04-2.54]). Additionally, the extended-pulsed fidaxomicin treatment resulted in lower recurrence at 90 days (6%) compared to the vancomycin treatment (19%) (p value = 0.00073, odds ratio 0.29 [95% CI 0.14-0.60]). This study showed that the extended-pulsed fidaxomicin treatment had better clinical cure for the patients as a whole with reduced recurrence (Guery et al., 2018). However, this study was not powered to look directly at this treatment for patients starting with recurrent CDI, and the study also showed that extended-pulsed fidaxomicin treatment may be inferior for severe CDI. Lastly, the study did not compare standard fidaxomicin therapy or extended-pulsed vancomycin therapy to the extended-pulsed fidaxomicin the study. Future study is needed to look at the effectiveness of this treatment for multiple recurrent CDI and to compare its effectiveness to standard fidaxomicin and extended-pulsed vancomycin therapy.

Lastly, rifaximin is a non-absorbable rifamycin formula that acts to inhibit bacterial RNA synthesis by binding to bacterial DNA-dependent RNA polymerase. Although rifaximin has a broad-spectrum of activity, it appears to only minimally disrupt the intestinal microbiota and has high activity against *C. difficile* (Hecht et al., 2007). While it has activity against *C. difficile*, resistance may develop rapidly. Rifaximin has shown potential as an adjuvant to conventional therapy for recurrent disease as a chaser with one study finding patients who received rifaximin after conventional therapy experienced recurrence at 15%, and those who received a placebo after conventional therapy experienced a 21% recurrence rate (Johnson et al., 2009; Garey et al., 2011). A small retrospective study of 32 patients treated with rifaximin for recurrent *C. difficile* infections found the treatment to be safe and with no recurrence of CDI after 12 weeks in 17 (53%) patients (Mattila et al., 2013). As such, rifaximin is not used for primary therapy, but can be used as a chaser to vancomycin to reduce recurrent diarrhea(McDonald et al., 2018; Garey et al., 2011; Salix Pharmaceuticals, 2018).

## 2.4 Areas for Improvement and Targets for Emerging Therapies

While current therapies lead to successful treatment of mild to moderate disease, there are needed improvements. CDI therapy success requires the resolution of diarrhea with absence of

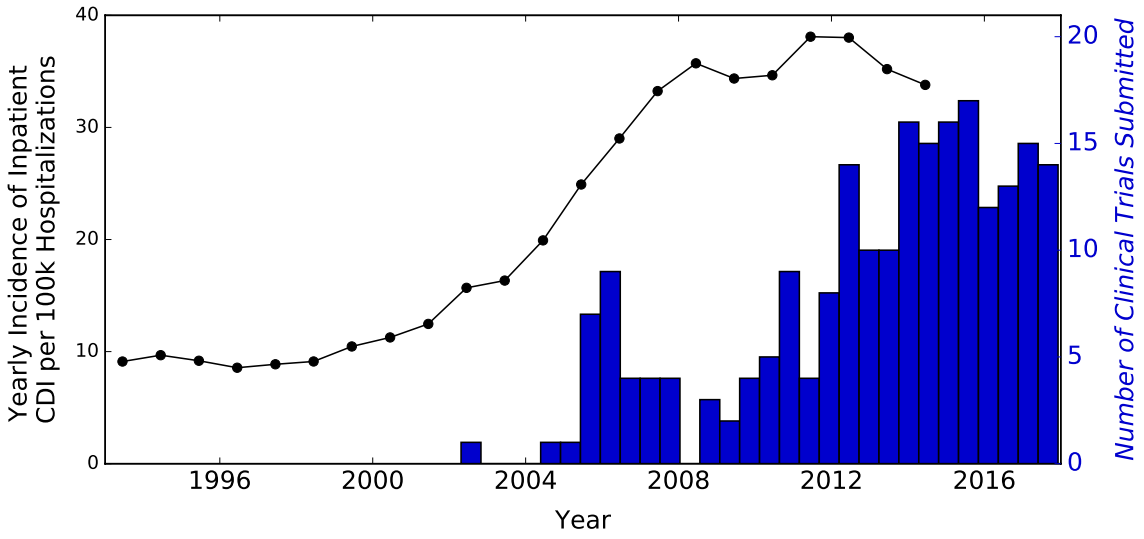



Figure 2.3 The submission of clinical trials increases following the increase in yearly incidence of CDI in the United States. Data on incidence was obtained from HCUPnet and clinical trial data was obtained from ClinicalTrials.gov. Only trials with *Clostridium difficile* in the condition category were included.

severe abdominal discomfort for more than 2 consecutive days. Although the successful treatment rates are high, as many as 18.9% to 27.3% of patients do not respond to treatment and experience treatment failure (Johnson et al., 2014). Additionally, the high severity disease and the significant rate of recurrence are promising targets for emerging therapies.

The US National Library of Medicine's records of clinical trials pertaining to *C. difficile* infections show a sharp increase in the number of clinical trials since the early 2000s which follows the increase in yearly incidence of inpatient CDI per 100,000 hospitalizations as observed from HCUPnet data (see Figure 2.3; data from HCUP, 2018; USGOV, 2018). This highlights the importance of developing new therapies to address the increase in incidence of CDI. While a large portion of the clinical trials are studying topics ranging from optimizing current antibiotic protocols and dosing and FMT, many trials are studying emerging therapies that target prevention, primary therapy, and/or reducing and treating recurrence (Figure 2.4). These emerging therapeutics are discussed below and are organized by which phase of pathogenesis they target: prevention, primary therapy, or recurrence (Figure 2.1).

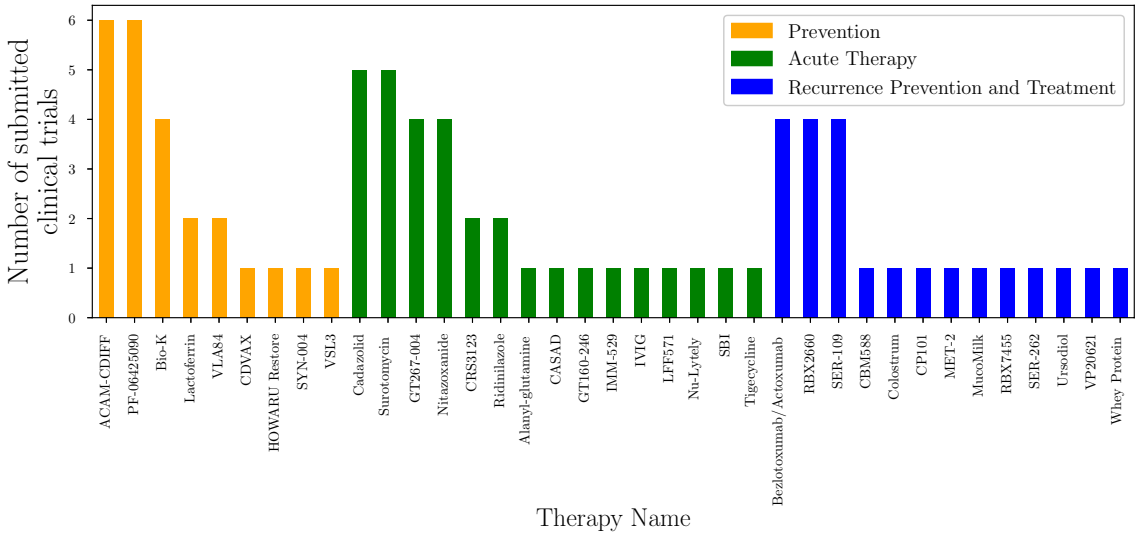



Figure 2.4 Clinical trial data was obtained from ClinicalTrails.gov. Only clinical trials listed on clinicaltrials.gov was included. If multiple therapeutic aims are being studied for a given drug, we sorted it by the categorization used in this article for the discussion section. Refer to drug Table 2.1 - Table 2.3 for specifics on each therapeutic (USGOV, 2018).

## 2.5 Prevention of CDI

The high incidence of CDI necessitates the development of strategies aimed at reducing intestinal microbial changes caused by systemic antibiotics, restoring colonization resistance and native bacterial communities, as well as reducing sporulation, colonization and toxin production by *C. difficile*. The current emerging therapies are: $\beta$-lactamases targeted at reducing systemic beta-lactam antibiotic disruption of intestinal bacteria; oral probiotics and bacterial replacement to restore bacteria associated with colonization resistance; vaccination to produce anti-*C. difficile* antibodies targeting spores, vegetative cells, and toxins; and therapies targeting toxin damage and CDI development once colonized (Table 2.1).

### 2.5.1 $\beta$-Lactamase

$\beta$-lactam antibiotics are associated with increased risk for subsequent CDI. By reducing the amount of active $\beta$-lactam antibiotic reaching the intestinal bacteria while preserving systemic drug activity, non-absorbable $\beta$-lactamases potentially prevent the loss of natural colonization resistance (Figure 2.5). SYN-004 (ribaxamase) is a recombinant $\beta$-lactamase manufactured by Synthetic

| Type | Treatment Name | Admin | Current or Last Phase Completed | Mechanism | Prevention | Acute Therapy | Recurrence Prevention/ Treatment |
|---|---|---|---|---|---|---|---|
| Beta Lactamase | SYN-004 | Oral | Phase 2 Ongoing | A recombinant $\beta$-lactamase from Bacillus licheniformi that acts to reduce the effect of systemic $\beta$-lactam antibiotics on the intestinal microbiota | X | | |
| Microbial | Bio-K | Oral | Phase 3 Ongoing | Probiotic containing three bacterial species: Lactobacillus acidophilus (CL1285), Lactobacillus casei (LBC80R), and Lactobacillus rhamnosus (CLR2). | X | | X |
| Microbial | VSL3 | Oral | Phase 2/3 Completed | A Refrigerated probiotic consisting of 8 different live strains of bacteria, including: Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus delbrueckii subsp. bulgaricus, Streptococcus thermophile | X | | |
| Microbial | HOWARU Restore | Oral | Phase 1 Complete | Probiotic containing four strains: Lactobacillus acidophilus, Bifidobacterium lactis Bl-04, Bifidobacterium lactis Bi-07, and Lactobacillus paracasei Lpc-37 | X | | |
| Toxoid Vaccine | VLA84 | IM | Phase 2 Completed | A recombinant vaccination composed of truncated portions of *C. difficile* toxins A and B. Given IM, this vaccine may induce anti-toxin antibody production | X | | |
| Toxoid Vaccine | ACAM-CDIFF | IM | Phase 3 Discontinued | Toxoid vaccine of Clostirdium difficile toxin A and B. Given IM, this vaccine may induce anti-toxin antibody production | X | | X |
| Toxoid Vaccine | PF-06425090 | IM | Phase 2 and 3 Ongoing | Genetically modified toxoid vaccine of *C. difficile* toxin A and B. Given IM, this vaccine may induce anti-toxin antibody production | X | | |
| Oral Vaccine | CDVAX | Oral | Phase 1 Terminated | An oral vaccine against that utilizes spores from a genetically modified bacterium to produce an oral vaccine may induce strong mucosal immunity. | X | | |
| Lactoferrin | Lactoferrin | Oral | Phase 2 Ongoing | May have the potential to reduce the cytotoxic damage of *C. difficile* toxin B and may delay Clostridium difficile growth and reduces toxin production | X | | |

Table 2.1 Emerging Therapies targeted at Prevention of CDI. For each emerging therapy, the administration route, current or last phase completed, and mechanism is listed. Additionally, each drug is marked for the applications it is being studied for in clinical trials, namely: Prevention, Primary Therapy, or Recurrence Prevention/Treatment. Clinical trial information was obtained from `ClinicalTrials.gov` (USGOV, 2018).

Biologics derived from P1A, a beta-lactamase isolated from Bacillus licheniformi, formulated for oral dosing that aims to reduce the effects on intestinal microbiota of $\beta$-lactam antibiotics that are given systemically. Preclinical trials in dogs showed that SYN-004 was well tolerated, minimally absorbed, and had no measurable effects on the systemic levels of co-administered intravenous ceftriaxone (Kokai-Kun et al., 2016). These results supported the progression of SYN-004 to clinical trials. Animal studies further showed that SYN-004 was capable of degrading ceftriaxone in GI system of dogs and reduced microbial changes in the gut of pigs treated with ceftriaxone (Kaleko et al., 2016). Two phase 1 clinical trials showed that SYN-004 was well tolerated and remained localized to the intestines (Roberts et al., 2016). Two phase 2 clinical trials confirmed that SYN-004

is capable of degrading the systemically given $\beta$-lactam antibiotics that enter the intestines (Kokai-Kun et al., 2017). A recently completed phase 2 clinical trial studied the ability of SYN-004 given orally in a 150 mg dose to prevent CDI in patients with a lower respiratory tract infection receiving IV ceftriaxone (NCT02563106). Results have not been posted. One limitation for $\beta$-lactamase treatment is that it will only be useful for patients receiving systemic $\beta$-lactam antibiotics. Non-$\beta$-lactam antibiotics that have a high risk for CDI, such as quinolones and clindamycin, would not be affected by a $\beta$-lactamase.

### 2.5.2 Probiotics

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). The aim of probiotics when used for CDI prevention is to restore bacteria associated with colonization resistance that may be disrupted by systemic antibiotic usage (Figure 2.5). While probiotics have the potential to prevent CDI, more research is needed to determine which patients would benefit from probiotic treatment and the long-term side effects of probiotic administration (Mills et al., 2018).

Bio-K is a probiotic manufactured by Bio-K Plus International containing three bacterial species: Lactobacillus acidophilus (CL1285), Lactobacillus casei (LBC80R), and Lactobacillus rhamnosus (CLR2). After an outbreak in the 284-bed community hospital Pierre-Le Gardeur (PLGH) in Quebec, every inpatient on antibiotics was prophylactically given Bio-K within 12 hours of the antibiotic prescription. For 10 years, 44,835 inpatients were observed and it was found that rates of CDI dropped from 18 cases per 10,000 patient-days to 2.3 cases per 10,000 patient-days. The rates for *C. difficile* infections at this hospital were found to be lower than comparable Canadian hospitals. No lactobacillus bacteremia was observed and it was concluded that Bio-K was safe and efficacious (Maziade et al., 2015). In a completed phase 3 trial, Bio-K prophylaxis after antibiotic usage reduced antibiotic-associated diarrhea and *C. difficile* associated diarrhea. Patients either received two capsules per day (Pro-2) or one capsule per day (Pro-1). Patients received the probiotics within 36 hours of antibiotic initiation and continued to receive the probiotics 5 days after antibiotics were concluded. Patients were followed up to 21 days after discontinuance of the probiotic. The incidence rates for antibiotic-associated diarrhea for Pro-2, Pro-1, and placebo were

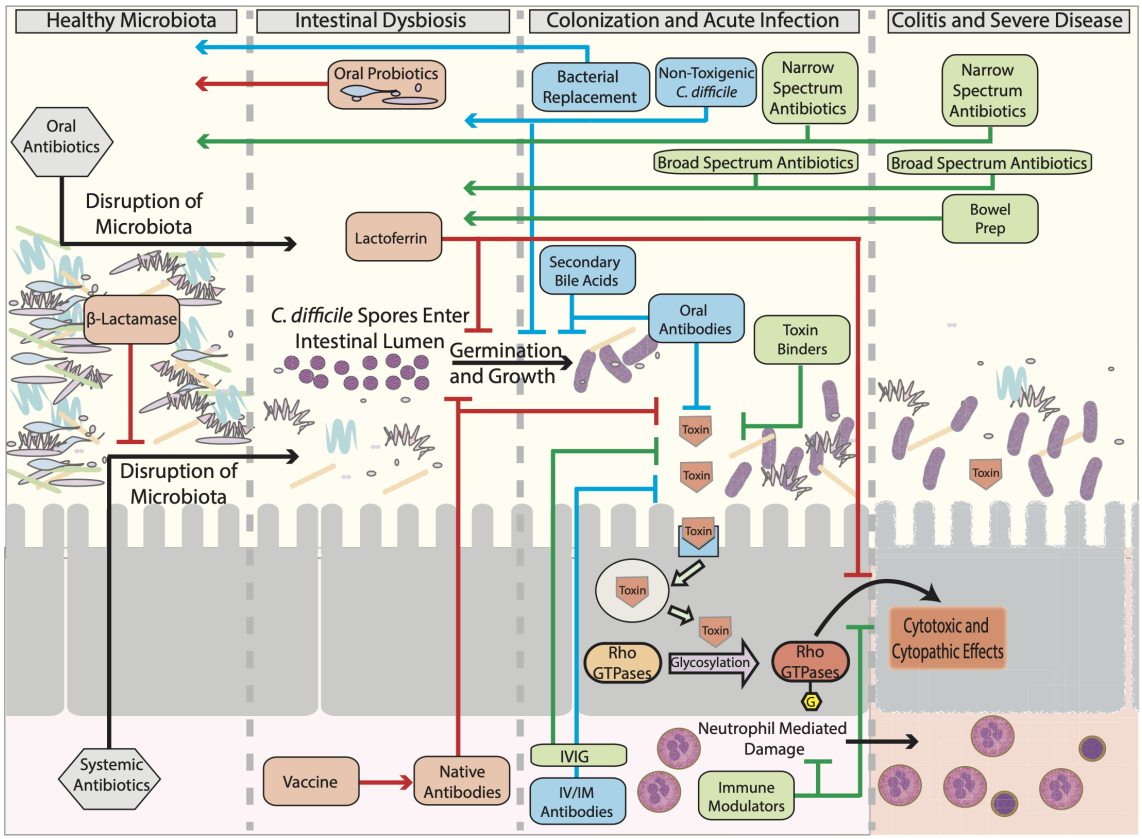



Figure 2.5 Boxes indicate therapies, while arrows indicate the effect of the therapies. Black arrows indicate events and steps in the development of CDI. Starting with the healthy microbiota, antibiotic alterations lead to a susceptible state where *C. difficile* spores can enter and germinate. This leads to colonization and infection. Toxin production can then trigger inflammation and cytotoxic/cytopathic effects on the mucosa, leading to colitis and severe disease. Red boxes and arrows indicate therapies aimed at preventing *C. difficile* infection. Green boxes and arrows indicate therapies aimed at treating primary CDI and reducing disease severity. Blue boxes and arrows indicate therapies aimed at reducing and treating recurrent CDI. IVIG = Intravenous Immunoglobulin

15.5%, 28.2% and 44.1% respectively. Rates of *Clostridium difficile* associated diarrhea for Pro-2, Pro-1 and placebo were 1.2%, 9.4% and 23.8% respectively. Additionally, the duration of antibiotic associated diarrhea was reduced (2.8 days for Pro-2, 4.1 days for Pro-1, and 6.4 days for Placebo Gao et al., 2010). These results indicate that Bio-K and similar probiotics could be efficacious for the prevention of CDI subsequent to antibiotic exposure.

VSL#3 (VSL3) is a refrigerated probiotic manufactured by Alfasigma that is consists of 8 different live strains of bacteria, including: Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus paracasei, Lactobacillus delbrueckii subsp. bulgaricus, and Streptococcus thermophiles (Selinger et al., 2013). VSL#3 was shown in a phase 2/3 trial (NCT00973908) to reduce the rate of antibiotic-associated diarrhea when given as a prophylaxis to average-risk hospitalized patients (0% VSL#3 vs 11.4% placebo). In this study, the VSL#3 patients received a VSL#3 sachet twice a day for the duration of their antibiotic course and one week after, while the placebo group received a placebo sachet twice daily instead. The study did not see any cases of *Clostridium difficile*-associated diarrhea in either the VSL#3 or placebo groups (Selinger et al., 2013). In this study, adverse event rates were found to be similar between the VSL#3 and placebo groups. Further studies are needed to determine which populations of patients would benefit from this prophylaxis therapy and to study the ability of VSL#3 to prevent CDIs in high risk patients. VSL#3 could also be tested for its ability to reduce recurrence.

HOWARU Restore is a four-strain probiotic manufactured by DuPont consisting of Lactobacillus acidophilus, Bifidobacterium lactis Bl-04, Bifidobacterium lactis Bi-07, and Lactobacillus paracasei Lpc-37. In a randomized dose response study, patients treated with antibiotics were given either high dose HOWARU restore, low dose HOWARU restore, or placebo as a preventative measure. Antibiotic associated diarrhea was lower in the probiotic groups, with rates of 12.5, 19.6, and 24.6% for high dose consisting of $1.70 \times 10^{10}$ Colony Forming Units (CFU), low dose consisting of $4.17 \times 10^{9}$ CFU, and placebo respectively. Both probiotic groups had reduced levels of *Clostridium difficile* associated diarrhea with rates of 1.8% in the probiotic groups and 4.8% in the placebo group (Ouwehand et al., 2014). No adverse events were found to be related to HOWARU restore, with the adverse event rate being 4.2%, 4.2% and 7.2% in the high dose, low dose, and placebo

groups respectively. An early phase 1 trial was recently completed with results pending studying the effects of HOWARU Restore probiotics on healthy elderly patients and the resulting levels of *Clostridium difficile* and changes in microbial diversity in fecal samples (NCT02207140).

Additional clinical trials have been or are being performed looking specifically at the ability of Lactobacillus reuteri probiotics in the prevention of antibiotic associated diarrhea or *Clostridium difficile* infection. A phase 3 trial was recently completed and another clinical trial is currently recruiting (NCT01295918, NCT02127814). In the phase completed phase 3 trial, patients in the treatment arm were given a chewable tablet containing 1 x $10^8$ CFU of L. reuteri, produced by BioGaia AB once per day during their antibiotic treatment and then continued for seven days after their antibiotic treatment was concluded. Results have not been posted.

### 2.5.3 Vaccines

Asymptomatic carriage of *C. difficile* is associated with a decreased risk of developing CDI. Additionally, a patient's levels of antibodies against *C. difficile* toxins correlates inversely with the development of recurrent disease (Kyne et al., 2001; Sanchez-Hurtado et al., 2008). As such, the development of vaccinations against *C. difficile* and its toxins has the potential to prevent not only initial disease but recurrence as well (Figure 2.5). Vaccines have similar advantages and limitations to probiotics. As they are preventative in nature, it is important to determine which patients would benefit from vaccination.

VLA84 (IC84) is a recombinant vaccine developed by Valneva that is composed of truncated portions of *C. difficile* toxins A and B. In a phase 1 trial of 51 adult and 50 elderly patients, VLA84 was well tolerated and induced high levels of antibodies against *C. difficile* toxins A and B (Bezay et al., 2016). A phase 2 trial was completed in 2015, in which patients were divided into four groups receiving either VLA84 75 $\mu$g without Alum (150 patients), VLA84 $\mu$g without Alum (150 patients), VLA84 200 $\mu$g with Alum (150 patients), or placebo (50 patients). The vaccination schedule consisted of intramuscular injections into alternating deltoid regions at day 0, 7 and 28. The results again showed that it induced seroconversion in up to 83% of participants for antibodies against both toxins A and B, and up to 97% against toxin A alone (NCT02316470; Dubischar, 2016). If successful, VLA84 and similar vaccinations would be potential preventative treatments

that could be given to high risk populations to protect against future CDIs.

ACAM-CDIFF is a toxoid A and B vaccine developed by Sanofi that is given parenterally. It was found to be safe in adults and elderly patients in two phase one trials (NCT00127803, NCT00214461 Greenberg et al., 2012). The optimal dosing was tested in phase two trials, with one finding that giving a high dose with adjuvant (100 ug antigen+AIOH) at 0-7-30 days resulted in the greatest immune response (de Bruyn et al., 2016). A phase 3 trial was discontinued and the vaccine research as a whole was discontinued by Sanofi following discouraging interim analysis (NCT01887912).

PF-06425090 is a vaccine developed by Pfizer composed of genetically modified toxoids A and B from *C. difficile* (Vidunas et al., 2016; Donald et al., 2013). The drug has completed multiple phase 1 and a single phase 2 trial so far, with another phase 2 trial active (NCT02561195) and a phase 3 (NCT03090191) trial currently recruiting. The current phase 2 trial has three groups: a high dose 200 $\mu$g vaccine, low dose 100 $\mu$g vaccine, and placebo. In addition, these three groups are split into two vaccination schedules. The vaccine is given in a 3-dose vaccination schedule by 0.5 mL intramuscular injection. The first vaccination schedule is day 1, 8 and 30 and the second schedule is month 0, 1, and 6. Results from the phase 2 trial are currently being reported with indication that the vaccination is leading to increased anti-toxin A antibody titers although no statistical analysis has been performed yet. Further study is required to test its efficacy in preventing CDI.

CDVAX is a novel oral vaccine against *C. difficile* that utilizes spores from a genetically modified bacterium to produce an oral vaccine that may induce strong mucosal immunity. This differs from injectable vaccines as the antigens will be presented from the mucosal side of the GI system. Recently, a phase 1 trial (NCT02991417) for CDVAX vaccination against *C. difficile* was terminated. More clinical research is needed to test CDVAX efficacy and safety.

### 2.5.4 Lactoferrin Therapy

For CDI to develop, *C. difficile* has to colonize the intestines and produce mucosal damage leading to symptoms such as diarrhea. If therapies are developed to target *C. difficile* growth, toxin production and mucosal damage, CDI can be prevented even if the intestinal microbial community is disrupted (Figure 2.5). Elevated levels of fecal lactoferrin and increased WBC count has been shown

to be associated with increased CDI severity (Boone et al., 2013). Utilizing rat intestinal epithelia cells (IEC-6), Otake et al. showed that lactoferrin has the potential to reduce the cytotoxic damage of *C. difficile* toxin B (Otake et al., 2017). In an in vitro chemostat gut model, Chilton et al. show that lactoferrin delays *C. difficile* growth and reduces toxin production (Chilton et al., 2016). There are two currently recruiting clinical trials testing lactoferrin. The first is looking at using lactoferrin prophylactically during initial antibiotic usage for pediatric patients to reduce antibiotic associated diarrhea (NCT02626104). In this study, children will receive either 100 mg of oral lactoferrin twice daily for the duration of antibiotic treatment or they will receive a placebo of 100 mg of oral maltodextrin twice daily. The second study is looking at the use of lactoferrin to prevent CDIs in long-term care patients with feeding tubes who require broad spectrum antibiotics (NCT00377078). In this study, patients in the treatment arm will be given lactoferrin at a concentration of 5 mg/mL given during the enteral feeding system flush cycle, consisting of 600 mL per day. This will be started at the beginning of the first day of antibiotic treatment and continued 8 weeks following the last antibiotic dose. The results from this study will hopefully determine if lactoferrin will prove to be an effective treatment worthy of additional study.

## 2.6 Treatment of Primary CDI: Reducing Severity and Increase Clinical Cure

If the onset of CDI cannot be prevented, additional emerging therapies are needed to reduce mucosal damage, disease severity, and the risk of recurrence. As the highest mortality is associated with severe and complicated CDI, therapies given shortly after the diagnosis of CDI, which aim to reduce the burden of *C. difficile* cells and toxins, reduce toxin damage at the mucosa barrier, and help restore the microbiota, can potentially reduce the progression to severe and complicated CDI (Table 2).

### 2.6.1 Antibiotics

One area of interest is the development of new narrow spectrum antibiotics that have less of an effect on native bacteria. This would allow for the native bacteria to recolonize and reestablish dur-

| Type | Treatment Name | Admin | Current or Last Phase Completed | Mechanism | Prevention | Acute Therapy | Recurrence Prevention/ Treatment |
|---|---|---|---|---|---|---|---|
| Antibiotic | Cadazolid | Oral | Phase 3 Completed | Inhibits protein synthesis and to some extent inhibiting DNA synthesis. Inhibits sporulation, toxin production, and is additionally bactericidal | | X | |
| Antibiotic | CRS3123 | Oral | Phase 1 Completed | Narrow spectrum antibiotic that acts by inhibiting bacterial methionyl-tRNA synthetase, with high activity against gram positive bacterial and *C. difficile* but low activity against gram negative bacteria | | X | |
| Antibiotic | LFF571 | Oral | Phase 2 Completed | Semisynthetic thiopeptide that acts on gram positive bacteria by blocking protein synthesis | | X | |
| Antibiotic | MCB3837/ MCB3681 | IV | Phase 1 Completed | Hybrid fluoroquinolone-oxazolidinone that has a water-soluble prodrug formulation termed MCB3837 which can be given by IV | | X | |
| Antibiotic | Nitazoxanide | Oral | Phase 3 Completed | Noncompetitive inhibitor of the pyruvate-ferredoxin / flavodoxin oxidoreductases with anti-*Clostridium difficile* activity. | | X | |
| Antibiotic | Ramoplanin | Oral | Phase 3, Phase 2b Ongoing | Ramoplanin is a glycolipodepsipeptide which disrupts cell wall biosynthesis by binding to peptidoglycan. It is non-absorbable, binds to spores and kill vegetative Clostirdium difficile cells in vitro. | | X | |
| Antibiotic | Ridinilazole | Oral | Phase 2 Completed | Narrow spectrum, nonabsorbable novel antibiotic that potentially impacts cell division, but the mechanism is not fully understood | | X | |
| Antibiotic | Surotomycin | Oral | Phase 3 Completed | Bactericidal cyclic lipopeptide that acts to dissipate the membrane potential of *Clostridium difficile* | | X | |
| Antibiotic | Tigecycline | IV | Interventional Completed | A glycylcyline which acts by binding to the 30S ribosomal subunit and inhibiting protein translation by blocking tRNA molecules from entering the A site of the ribosome. | | X | |
| Binder | CASAD | Oral | Phase 2 Trial Terminated | Sequesters clostridium toxin A and B with limited off target protein binding. | | X | |
| Binder | GT267-004 | Oral | Phase 3 Completed | A polystyrene binder that may sequester *Clostridium difficile* toxins A and B. | | X | |
| Binder | GT160-246 | Oral | Phase 2 Completed | A high-molecular-weight soluble anionic polymer that has been shown in hamsters to reduce mortality from *Clostridium difficile* infections and in vitro data suggests it can neutralize the activity of toxin A and B. | | X | |
| Alanyl-glutamine | Alanyl-glutamine | Oral | Phase 2 Terminated | A dipeptide that has been shown to have potential therapeutic effects in reducing *C. difficile* toxin damage in intestinal epithelial cells. | | X | |
| Antibody | IVIG | IV | Phase 4 Terminated | Intravenous Immunoglobulins derived from pooled human serum. | | X | |
| Antibody | SBI | Oral | Active | Serum-derived Bovine Immunoglobulins | | X | |
| Polyclonal Antibody | IMM-529 | Oral | Phase 1/2 Trial Ongoing | Polyclonal antibodies against TcdB, vegatative cells and spores | | X | |
| Bowel Prep | Nu-Lytely | Oral | Phase 4 Recruiting | A formulation of the osmotic laxative PEG 3350 and electrolytes that can be used to reduce luminal load of *C. difficile* spores and toxins | | X | |

Table 2.2 Emerging Therapies Targeted at Primary Management of CDI. For each emerging therapy, the administration route, current or last phase completed, and mechanism is listed. Additionally, each drug is marked for the applications it is being studied for in clinical trials, namely: Prevention, Primary Therapy, or Recurrence Prevention/Treatment. Clinical trial information was obtained from `ClinicalTrials.gov` (USGOV, 2018).

ing CDI therapy, potentially reducing disease severity and reducing recurrence in the future (Figure 2.5). Additionally, some CDI cases are resistant to standard antimicrobial therapy, necessitating

the development of novel antibiotic therapies for treating these resistant cases.

Cadazolid is a hybrid fluoroquinolone-oxazolidinone antibiotic produced by Actelion Pharmaceuticals Ltd which acts by inhibiting protein synthesis and to some extent inhibiting DNA synthesis (Locher et al., 2014). In phase 1 trials, cadazolid was well tolerated and minimally absorbed, with high concentrations found in the stool (Baldoni et al., 2014; Gehin et al., 2015). Cadazolid inhibits sporulation, toxin production, and is bactericidal. A phase 2 trial studied the effectiveness of different doses of cadazolid compared to vancomycin in patients with CDI. The four treatment groups were: 250 mg cadazolid twice daily (20 patients), 500 mg cadazolid twice daily (22 patients), 1000 mg cadazolid twice daily (20 patients), or 125 mg vancomycin four times daily (22 patients). All treatments were given for 10 days. In this study, cadazolid treatment showed lower recurrence rates compared to vancomycin treatment (18-25% compared to 50% recurrence) and cadazolid showed higher sustained clinical cure rates (46.7%-60% compared to 33.3%). There was no evidence for an effect of dosage of cadazolid (NCT01222702). In addition, cadazolid treatment was well tolerated (Louie et al., 2015). Two phase 3 studies have been completed, with a third currently suspended. Recently completed Phase 3 trials are being analyzed for efficacy with one appearing to achieve the primary endpoint and the other second failing to meet the primary endpoint of clinical cure rate (Maxwell-Scott & Goldenberg, 2017). The formal analysis of these two studies will be needed to determine the efficacy of cadazolid for the treatment of CDI.

CRS3123 (REP3123) is a novel narrow spectrum antibiotic produced by Crestone Inc that acts by inhibiting bacterial methionyl-tRNA synthetase, with high activity against gram-positive bacteria and *C. difficile* but low activity against gram-negative bacteria (Nayak et al., 2017; Citron et al., 2009). CRS3123 was reported to reduce spore formation and toxin formation in a hamster model (Ochsner et al., 2009). One phase 1 trial found CRS3123 to be well tolerated for multiple doses (100 mg, 200 mg, 400 mg, 800 mg, and 1200 mg) and the indicated safety of the drug supported further research into its efficacy (Nayak et al., 2017). Further research is necessary to test the efficacy of CRS3123 for the treatment of CDI.

LFF571 is a semisynthetic thiopeptide produced by Novartis Pharmaceuticals that acts on gram-positive bacteria by blocking protein synthesis (Leeds et al., 2012). In a phase 2 study (NCT01232595) completed in 2013, LFF571 was found to be minimally absorbed and with high

retention in the intestines (Bhansali et al., 2015). In this study, adults experiencing primary or first recurrent CDI were randomized to receive either LFF571 (200 mg) or vancomycin (125 mg) four times daily for 10 days. LFF571 had noninferior clinic cure rates (90.6%) compared to vancomycin (78.3%), with a potential to have a lower recurrence rate (19% vs 25%; Mullane et al., 2015). LFF571 treatment had more adverse events than vancomycin (76.1% vs. 69.2%) but had less adverse events suspected to be related to the treatment (32.6% vs 38.5%).

MCB3681 is a hybrid fluoroquinolone-oxazolidinone produced by Morphochem that has a water-soluble prodrug formulation termed MCB3837 which can be given by IV. As it can be delivered through IV, this therapy could be an option for severe and complicated *C. difficile* infections when oral therapy fails. It has been shown to have activity against gram-positive bacteria including *C. difficile*, but limited activity against gram-negative bacteria such as those native to the human gut (Rashid et al., 2014a,b; Freeman et al., 2017). A phase 1 study, where 12 healthy volunteers were given daily intravenous infusions of 6 mg/kg MCB3837 over 12 hours for five days, showed little impact on microbiota and suggested the drug was well tolerated (Dalhoff et al., 2015). Phase 2/3 trials are being planned currently and the FDA designated MCB3837 as a "Qualified Infectious Disease Product (QIDP) for the treatment of *Clostridium difficile* infection (CDI)".

Nitazoxanide is believed to be a noncompetitive inhibitor of the pyruvate-ferredoxin/flavodoxin oxidoreductases produced by Romark Pharmaceuticals (Hoffman et al., 2007). Currently, nitazoxanide is FDA approved for treatment of cryptosporidiosis and giardiasis, given by tablet orally (500 mg) every 12 hours for 3 days, with possible adverse side-effects of abdominal pain, nausea, headache or discolored urine. Nitazoxanide (500 mg twice per day for 7 or 10 days) was shown to have similar clinical cure and recurrence rates versus vancomycin (125 mg four times per day for 10 days) and metronidazole (250 mg four times per day for 10 days Musher et al., 2006, 2009). However, nitazoxanide is more expensive than metronidazole, limiting its use for primary CDI (Drekonja, 2014). A phase 3 trial with results currently being reported is looking at the use of nitrazoxanide (500 mg twice daily) as a treatment for CDI that has failed metronidazole or vancomycin (NCT00304356).

Ramoplanin is a glycolipodepsipeptide which disrupts cell wall biosynthesis by binding to peptidoglycan (Farver et al., 2005). It has been shown to be non-absorbable, and to bind to spores

and kill vegetative *C. difficile* cells in vitro. This may indicate a potential use for ramoplanin as a preventative measure to reduce risk of initial disease or reduce recurrence by binding to spores and then killing the ones that germinate (Kraus et al., 2015). A phase 2 trial comparing treatment with ramoplanin vs. vancomycin for CDI showed similar clinical response rates with 400 mg ramoplanin (71% vs 78%) and similar sustained clinical response rates (83% 200 mg ramoplanin, 85.2% 400 mg ramoplanin, 85.7% vancomycin). A phase 3 clinical trial has been approved by the FDA.

Ridinilazole (SMT19969) is a narrow spectrum, non-absorbable novel antibiotic produced by Summit Therapeutics that potentially impacts cell division, but the mechanism is still not fully understood (Basseres et al., 2016). A phase 1 trial studied the safety of oral doses of up to 2,000 mg of ridinilazole. Ridinilazole was shown to have minimal changes on the microbiota and was not measured at high serum levels in this phase 1 trial (Vickers et al., 2015). A phase 2 trial with 100 patients with CDI compared 10-day oral ridinilazole treatment (200 mg every 12 hours) to 10-day oral vancomycin treatment (125 mg every 6 hours). This trial showed that ridinilazole caused a higher sustained response rates compared to vancomycin (67% vs 42%) as well as a reduction in recurrence (14.3% vs. 34.8%) (Vickers et al., 2017). There were no adverse events that necessitated discontinuation of ridinilazole, and the adverse events were similar between ridinilazole and vancomycin treatment. An additional phase 2 trial was completed in 2016 comparing ridinilazole to fidaxomicin; study results are being analyzed (NCT02784002).

Surotomycin (CB-183,315) is a bactericidal cyclic lipopeptide, originated by Cubist Pharmaceuticals and currently developed by Merck & Co, that acts to dissipate the membrane potential of *C. difficile* (Alam et al., 2015). Surotomycin, which was given to two treatment groups either at 125 mg twice daily or 250 mg twice daily for 10 days, was shown in a phase 2 trial to reduce recurrence of *C. difficile* infection compared to vancomycin, given at 125 mg four times daily for 10 days (Chesnel et al., 2012; Lee et al., 2016). In this phase 2 trial, adverse events were similar between both surotomycin arms and the vancomycin arm. However, surotomycin (250 mg given orally twice daily for 10 days) failed to achieve noninferiority versus vancomycin (125 mg given orally four times daily for 10 days) in a recent phase 3 trial with initial cure rates of 79% vs 83.6% and sustained clinical response rate of 60.6% vs 61.4% (NCT01597505 Boix et al., 2017). An additional phase 3 was recently completed studying the effectiveness of surotomycin treatment (285 patients; 250 mg

twice daily for 10 days) compared to vancomycin treatment (292 patients; 125 mg vancomycin four times daily for 10 days) for patients with confirmed CDI (NCT01598311). The study showed that surotomycin was non-inferior to vancomycin for clinical response at the end of the trial (83.4% vs 82.1%), but surotomycin failed to demonstrate superiority to vancomycin in clinical response over time and sustained clinical response (Daley et al., 2017).

Tigecycline is a glycylcyline produced by Pfizer which acts by binding to the 30S ribosomal subunit and inhibiting protein translation by blocking tRNA molecules from entering the A site of the ribosome (Inc, 2013). It is FDA approved for treatment of community acquired pneumonia (100 mg by IV once, with 50 mg IV given every 12 hours thereafter for 7 to 14 days), complicated skin/subcutaneous infection (same dosing but for 5 to 14 days), and complicated abdominal infections (same dosing but for 5 to 14 days Wyeth, 2016). Tigecycline has cured CDI in case reports; however, pooled data may indicate an association with higher mortality (Tasina et al., 2011; Herpers et al., 2009). An interventional clinical trial where patients received standard *Clostridium difficile* associated diarrhea treatment of vancomycin or metronidazole with the addition of IV tigecycline (100 mg once, followed by 50 mg IV every 12 hours after) for the duration of hospitalization (approximately 7-14 days) was completed in 2013 (NCT01401023) with findings on clinical cure not yet reported. A recent retrospective observational study found tigecycline combination therapy with vancomycin  metronidazole to be safe and effective for the treatment of severe-complicated CDI (Bishop et al., 2018).

### 2.6.2 Toxin Binders

Toxin binders are molecules that have been shown to sequester toxins and some have the additional ability to bind to pro-inflammatory factors. While current studies into toxin binders have not proven successful to a large extent, it is important to consider them as they could potentially be effective if the right formulation is discovered. Given orally, these binders enter the lumen and can be used during CDI to reduce the mucosal damage done both by the toxins and by the host immune response. In this way, binders have the potential to reduce disease severity, hopefully lowering the number of cases of severe and complicated CDI cases (Figure 2.5). However, these binders could bind pharmaceuticals, so they should not be co-administered with standard therapy if found to

bind to standard drugs (Bagdasarian et al., 2015). As this is an important consideration, newer binders aim to reduce pharmaceutical cross binding.

Calcium Aluminosilicate Anti-Diarrheal (CASAD), developed by Salient Pharmaceuticals, has been shown to sequester Clostridium toxin A and B with limited off target protein binding (Sturino et al., 2015). Similar to other binders, if CASAD can preferentially bind to Clostridial toxins without binding to antibiotics, then this could reduce the severity of disease and potentially reduce recurrence risk. A phase 2 trial attempting to study the effectiveness of adding CASAD given in 500 mg capsules orally three times daily for 14 days to standard CDI treatment was terminated for low enrollment (NCT01570634), in which the authors describe the potential of Calcium Aluminosilicate to bind TNF$\alpha$, IL-1, IL-6, and IL-10 which could theoretically help reduce the immune response to the infection and decrease fever and leukocytosis (NCT01570634). Further research is needed to study the effectiveness and safety of CASAD for CDI treatment.

GT267-004 (tolevamer) is a polystyrene binder developed by Sanofi that is proposed to sequester *C. difficile* toxins A and B. It was shown in a phase 2 trial to be noninferior to vancomycin treatment for mild to moderate CDIs with potential to reduce recurrence (Louie et al., 2006). However, the analysis of two phase 3 trials showed that tolevamer (563 patients received 9 g loading dose followed by 3 g every 8 hours for 14 days) was inferior to both metronidazole (289 patients received 375 mg every 6 hours for 10 days) and vancomycin (266 patients received 125 mg every 10 hours for 10 days) for clinical cure of CDI (44.2%, 72.7%, and 81.1% respectively; Johnson et al., 2014). Adverse events were similar across the three treatment groups. A third phase 3 trial was terminated (NCT00466635) and it is doubtful that additional development of this compound will be pursued.

GT160-246 is a high-molecular-weight soluble anionic polymer produced by Sanofi that has been shown in hamsters to reduce mortality from CDI and in vitro data suggest GT160-246 can neutralize the activity of toxin A and B (Kurtz et al., 2001). A GT160-246 phase 1 trial showed it to be safe and well tolerated. A phase 2 trial comparing GT160-246 to vancomycin for the treatment of *Clostridium difficile* associated diarrhea was completed with results not currently posted (NCT00034294).

### 2.6.3 Host Response Modulation

One target for reducing disease severity as previously mentioned is regulating the host response to reduce host-derived mucosa damage during CDI treatment. Alanyl-glutamine is a dipeptide that has been shown to have potential therapeutic effects in reducing *C. difficile* toxin damage in intestinal epithelial cells (Figure 2.5). Rodrigues et al. showed that alanyl-glutamine reduces apoptosis and increases intestinal cell proliferation in mouse intestinal cells (IEC-6) when exposed to *C. difficile* toxin B (Rodrigues et al., 2013). Santos et al. additionally showed in IEC-6 cells that alanyl-glutamine treatment reduced TcdA toxin damage and increased RhoA expression, suggesting a potential explanation for the protective effects (Santos et al., 2013). Using intestinal loop models, Warren et al. (2012) showed that treatment with ATL 370 (an adenosine A2A receptor agonist) and alanly-glutamine reduced ileal secretions, apoptosis, mucosal injury and decreased levels of KC and IL-10 with *C. difficile* toxin A exposure (Warren et al., 2012). A phase 2 clinical trial testing the efficacy of alanyl-glutamine as a supplement (44 g orally daily for 10 days) during treatment of CDI was terminated due to low enrollment (NCT02053350). Further clinical trials are needed to test the efficacy of alanyl-glutamine in treating primary CDIs and reducing disease severity.

### 2.6.4 Antibodies

As disease is caused by the toxins produced from *C. difficile*, one potential goal during primary therapy is the neutralization of toxins with anti-toxin antibodies. While specific monocolonal and polyclonal antibodies are being studied for their effectiveness in preventing recurrence (as discussed in the recurrence section), IVIG and Serum Bovine Immunoglobulin (SBI) have been proposed to reduce disease severity by reducing toxin damage (Figure 2.5). A phase 4 trial looking at IVIG (400 mg/kg infused over 4-6 hours) effectiveness during standard therapy for severe CDI was terminated because it was unable to receive IVIG for free (NCT00177970). There is a currently active clinical trial studying the effects of giving SBI (10.0 g twice per day) on ulcerative colitis in patients who tested positive for *C. difficile* and who are on vancomycin (NCT02730325). Further study is needed to determine the efficacy of IVIG or SBI during severe CDI. Monoclonal and polyclonal antibodies directed against *C. difficile* are an alternative that requires further study for their effectiveness during therapy of CDI.

Additionally, IMM-529 is a polyclonal antibody developed by Immuron that has shown cross-reactivity with *C. difficile* vegetative cells, spores, and toxin B. IMM-529 is currently being tested in a phase 1/2 trial for the treatment of CDI (NCT03065374; Kanellos, 2017). The study aims to determine the safety and tolerability of IMM-529. Patients will receive standard of care treatment for CDI in addition to either IMM-529 (1000 mg orally three times daily) or placebo.

### 2.6.5 Bowel Prep

One method of reducing *C. difficile* cell and toxin burden is to flush the luminal content out of the intestines using bowel preps after CDI is diagnosed and before standard therapy (Figure 2.5). Nu-Lytely is a formulation of the osmotic laxative PEG 3350 and electrolytes produced by Braintree Laboratories (Braintree Laboratories, 2013). A recruiting phase 4 clinical trial will study the efficacy of oral lavage by giving Nu-Lytely after the diagnosis of CDI before antibiotics are started (NCT01630096). In this study, patients testing positive for CDI will be assigned to either a control group or the osmotic laxative group in which they will be given the PEG 3350 solution in 8oz volume every 10 minutes until 6 liters are ingested. An additional 2 liters may be ordered if necessary. Both groups will then receive standard of care antibiotic treatments. Further results are needed to determine if oral lavage before standard treatment will reduce disease severity by lowering the *C. difficile* bacterial and toxin load in the GI system.

## 2.7 Preventing and Treating Recurrent CDI

With a median recurrence of 21% and a high rate of readmissions and cost associated with recurrent CDI, developing novel therapies to prevent recurrence and treat recurrent CDI will have a substantial impact on patient morbidity, as well as on CDI healthcare burden (Olsen et al., 2015; Zilberberg et al., 2014). There are two distinct treatment goals concerning recurrent CDI that could be optimized further. The first is reducing initial recurrence in patients experiencing primary CDI, and the second is treating therapy-resistant patients that are experiencing multiple episodes of recurrent CDI. As mentioned in the primary therapy section, many of the emerging antibiotics potentially offer a reduction in recurrence risk following standard therapy, including ridinilazole,

LFF571, MCB3681, and ramoplanin. Additionally, probiotics used for prevention could potentially be also used to help recover the natural microbiota following standard therapy (Table 2.3). For the second goal, with subsequent recurrence risk increasing after each unsuccessfully treated recurrent episode, it is important to develop novel therapies that specifically treat recurrent-prone CDI. While FMT has been shown to be effective at treating recurrent CDI and preventing further recurrences, human derived fecal matter is difficult to standardize and has multiple potential risks including the transfer of infectious material and long-term consequences of inoculating the gut with a foreign fecal material. As such, research is ongoing to develop new agents for treating recurrent CDI. These agents include antibodies directed against *C. difficile* cells and toxins as well as standardized bacterial replacement cultures and mixtures (Table 2.3).

### 2.7.1 IV Antibodies

Researchers have found an inverse correlation between the development of recurrent disease and anti-toxin antibody levels (Sanchez-Hurtado et al., 2008; Kyne et al., 2001). Antibodies given intravenously enter the lumen in regions of mucosal damage and help lower the intestinal damage caused by toxins. This may help increase the recovery of the healthy mucosal layer and assist in the recovery of the natural microbiota leading to restoration of colonization resistance (Figure 2.5).

Actoxumab is a human monoclonal antibody against *C. difficile* toxin A and bezlotoxumab (MK-6072-001) is a human monoclonal antibody against toxin B developed by Merck. Two phase 3 clinical trials (MODIFY I and MODIFY II) studied the ability of these two antibodies to reduce the recurrence of CDI in 2655 patients. In these trials, it was shown that the addition of bezlotoxumab (10 mg/kg infusion) to the standard of care antibiotics for primary or recurrent *C. difficile* infections resulted in a lower rate of recurrence compared to the placebo (MODIFYI: 17% vs. 28% MODIFY II: 16% vs. 26%) and a higher sustained clinical cure compared to the placebo (64% vs. 54%). The addition of actoxumab (10 mg/kg infusion) alone did not decrease recurrence, and in combination with bezlotoxumab (10 mg/kg infusion of both bezlotoxumab and actoxumab) it did not increase efficacy compared to bezlotoxumab alone (Wilcox et al., 2017). The adverse event rates were similar among the treatment groups. These results indicate that antibodies targeted against toxin B are a potential therapy for reducing recurrence in high risk patients. A recent computer model based

| Type | Treatment Name | Admin | Current or Last Phase Completed | Mechanism | Prevention | Acute Therapy | Recurrence Prevention/ Treatment |
|---|---|---|---|---|---|---|---|
| Antitoxin Antibody | Bezlotoxumab / Actoxumab | IV | Phase 3 Ongoing | Human monoclonal antibodies against TcdA (Actoxumab) and TcdB (Bezlotoxumab) which can prevent toxin damage at the intestinal barrier. | | | X |
| Oral Bovine Antibodies | Colostrum | Oral | Phase 2/3 Withdrawn | Colostrum from *Clostridium difficile* immunized cows. | | X | X |
| Oral Bovine Antibodies | MucoMilk | Oral | Phase 2/3 Terminated | A whey protein concentrate 40% (WPC-40) enriched with polyclonal-antibodies that is produced from the milk of cows immunized with *C. difficile* cells formaldehyde-inactivated and *C. difficile* toxoid filtrate. | | | X |
| Microbial | SER-109 | Oral | Phase 3 Ongoing | A capsule consisting of bacterial spores derived from screened human donor stool. | | | X |
| Microbial | SER-262 | Oral | Phase 1 Ongoing | A manufactured microbial therapeutic. | | | X |
| Microbial | CBM588 | Oral | Phase 2 Withdrawn | A probiotic consisting of Clostridium butyricum, which lacks toxins associated with *C. difficile* infections. | | | X |
| Microbial | MET-2 | Oral | Phase 1 Recruiting | Consists of a live microbe community derived from healthy donor stools | | | X |
| Microbial | RBX2660 | Enema | Two Phase and One Phase 3 Ongoing | A stool derived standardized therapy consisting of live bacteria suspension. Suspension is given by retention enema and is derived from healthy donors | | | X |
| Microbial | RBX7455 | Oral | Phase 1 Ongoing | A lyophilized oral formulation of RBX2660, which is stable at room temperature. | | | X |
| Microbial | CP101 | Oral | Phase 2 Ongoing | Encapsulated Lyophilized fecal microbiota derived from human donors | | | X |
| Non-toxigenic *Clostridium difficile* | VP20621 | Oral | Phase 2 Ongoing | Consists of spores from the non-toxigenic *Clostridium difficile* (NTCD) strain M3. | | | X |
| Secondary Bile Acid | Ursodiol | Oral | Phase 4 Recruiting | Urodeoxycholic acid is being used as a surrogate for deoxycholic acid, a secondary bile acid. Secondary Bile acids have been shown to suppress *C. difficile* growth in vitro. | | | X |

Table 2.3 Emerging Therapies Targeted at Recurrent CDI Prevention and Management. For each emerging therapy, the administration route, current or last phase completed, and mechanism is listed. Additionally, each drug is marked for the applications it is being studied for in clinical trials, namely: Prevention, Primary Therapy, or Recurrence Prevention/Treatment. Clinical trial information was obtained from `ClinicalTrials.gov`.

analysis has predicted that bezlotoxumab will be cost-effective for the prevention of recurrence in patients receiving standard of care antibiotics for CDI (Prabhu et al., 2018; Dieterle & Young, 2017). A new Phase 3 trial studying the effects of bezlotoxumab (10 mg/kg infusion) in addition to standard antibacterial treatment in children with *C. difficile* infections is currently recruiting (NCT03182907). Bezlotoxumab (10 mg/kg infusion) is currently FDA approved for the prevention of recurrent CDI in patients currently on treatment for CDI who are at high risk for recurrence (Merck & Dohme, 2016).

### 2.7.2 Polyclonal Oral Antibodies

Unlike IV antibodies, which enter through the bloodstream, oral antibodies enter the GI lumen directly. If polycolonal antibodies are used, the oral antibodies can be designed to target not only toxins, but also spores and vegetative cells. By reducing the burden of spores and vegetative cells, these treatments can reduce the chance of recurrence. Through neutralization of toxins, these therapies can additionally protect against Clostridium toxin damage, assisting in the restoration of the mucosa and microbiota (Figure 2.5).

Colostrum is a type of milk produced during pregnancy that has high levels of antibodies to provide passive immunity to infants. Immunization of pregnant mares and cows with *C. difficile* proteins results in the production of antibodies which can be obtained from the colostrum and used therapeutically. Immunization of mares with toxin A and B binding domains resulted in colostrum that was able to block the cytotoxic activity of the *C. difficile* toxins A and B (Artiushin et al., 2013). Repeated immunization of a pregnant cow with recombinant mutants of toxin A and B produced hyperimmune bovine colostrum (HBC) that was able to reduce the disease severity of CDI in piglets (Artiushin et al., 2013). Similarly, HBC was shown to prevent, treat and reduce recurrence of CDI in mouse models (Hutton et al., 2017). A phase 2/3 clinical trial to test the efficacy and safety of colostrum derived antibodies for the prevention of CDI was withdrawn (NCT00747071). Further clinical trials are needed to assess the ability of colostrum derived antibodies to prevent, treat, and reduce recurrence of CDI. MucoMilk is a whey protein concentrate 40% (WPC-40) enriched with polyclonal-antibodies developed by MucoVax that is produced from the milk of cows immunized with formaldehyde-inactivated *C. difficile* cells and toxoid filtrate. As it is given orally, the antibodies will be available to the luminal side and have the potential to target spores, vegetative cells, and toxins. In a hamster model, hamsters infected with *C. difficile* died when untreated with WPC-40, while hamsters treated with WPC-40 had an 80% to 90% survival depending on the formulation. For preliminary data, 16 patients with CDI were given WPC-40 three times daily for two weeks following standard antibiotic therapy. The WPC-40 was well tolerated and none of the patients experienced recurrence (van Dissel et al., 2005, median follow-up 333 days, range 35 days to one year). A 60-patient phase 2/3 trial testing the efficacy and safety of MucoMilk in the prevention of recurrence of *C. difficile* was completed in 2005 and results are not posted

yet (NCT00177775). Further study is required to test the benefit of MucoMilk following standard antibiotic therapy to prevent recurrence.

### 2.7.3 Bacterial Replacement

While the exact reason for the loss of colonization is unknown, comprehensive bacterial replacement during Fecal Microbiota Transplant has shown that restoration of certain components of the microbiota is effective in treating recurrence. Decreased microbial diversity is associated with recurrent disease (Chang et al., 2008). Future research is needed to find the specific community members needed to restore colonization resistance. Emerging therapies are being developed to address this aim with the goal of standardized bacterial replacement therapeutics to restore the natural microbiota (Figure 2.5).

SER-109 is a capsule consisting of bacterial spores derived from screened human donor stool. The FDA designated SER-109 as a Breakthrough Therapy and Orphan Drug. Khanna et al. found that SER-109 potentially prevents CDI recurrence within an 8-week follow-up period in patients experiencing recurrence, presumably through diversification of the gut microbiota and recovery of natural colonization resistance (Khanna et al., 2016). A phase 2 (ECOSPOR) double-blind, placebo-controlled trial enrolled 89 patients with multiple recurrent CDI to test safety and efficacy to reduce recurrence of CDI. After patients had completed antibiotic therapy for CDI, they were split into SER-109 (59) and placebo (30) groups. The SER-109 group received one oral dose of SER-109 ($1x10^8$ bacterial spores) after completion of antibiotics, while the other received the placebo. Results from the phase 2 (ECOSPOR) trial indicate that SER-109 did not meet the primary endpoint for reducing CDI recurrence overall. However, in high-risk populations (in this case those 65 years or older) SER-109 treatment showed reduced recurrence rates (45% vs 80% recurrence risk Martin & Wilcox, 2016). Two phase 3 trials are currently recruiting (ECOSPOR III – NCT03183128, ECOSPOR IV – NCT03183141).

Seres Therapeutics is also developing another oral microbiome therapeutic called SER-262, which is a manufactured microbial therapeutic that, in contrast to SER-109, is a defined microbial community consisting of the spores of anaerobic, commensal bacteria produced by in vitro fermentation and is not an undefined consortium derived from human stool. SER-262 is currently

being tested in a phase 1 trial for adult patients to prevent recurrent *C. difficile* (NCT02830542). In this trial, patients receiving standard of care antibacterial treatment for primary CDI will be assigned to experimental groups with single doses ranging from $1x10^4$ to $1x10^8$ CFU or multiple doses ranging from $1x10^7$ to $1x10^8$ CFU. This study will examine the safety, tolerability, and the efficacy of SER-262 to prevent recurrence.

CBM588 is a probiotic consisting of Clostridium butyricum produced by Osel that is given by oral administration. Clostridium butyricum lacks toxins associated with CDI and has been shown to be safe (Isa et al., 2016). A phase 2 trial testing for the ability of CBM588 to reduce recurrence after CDI therapy, in which patients with confirmed CDI will receive standard of care antibacterial therapy in addition to either CBM588 (2g per dose) or placebo twice daily for 42 days, was suspended for lack of enrollment (NCT01077245). Future clinical studies need to be performed to test the efficacy of CBM588 in preventing CDI.

MET-2 (Microbial Ecosystem Therapeutics 2) consists of a live microbe community derived from healthy donor stool developed by NuBiyota. A small 20 patient phase 1 pilot study is currently recruiting to test the safety and efficacy in treating recurrent CDI in patients who have experienced at least two prior *C. difficile* episodes (NCT02865616). In this study, patients who are experiencing a case of recurrent CDI will be given an initial loading dose of MET-2 over two days and then a maintenance dose of MET-2 over 8 days. If the patients do not respond to the first loading dose they can be offered a second, higher dose of MET-2. Patients experiencing failure of the second dose may be offered a higher dose given by colonoscopy. Further research will be needed to test its safety and efficacy in preventing recurrence of CDI.

RBX2660 is a stool-derived standardized therapy consisting of a live bacteria suspension developed by Rebiotix. The suspension is given by retention enema and is derived from healthy donors. A phase 2 trial in patients with a least two recurrences of CDI found it to be safe and efficacious with 87.1% of patients (27/31) achieving treatment success after one or two treatments (Orenstein et al., 2016). Two phase 2 trials are currently active but not recruiting (NCT02589847, NCT02299570) and a phase 3 trial is currently recruiting (NCT03244644) to study the efficacy of RBX2660 in the treatment of recurrent CDI. Results for an open label phase 2 trial (Punch TM Open Label) were announced in April 2017 indicating a success rate of 78.8% for RBX2660 compared to the historical

control of 51.8% for the prevention of recurrent *C. difficile* infections (Dubberke E.R., 2016). The results of the active clinical trials will help address the relevant efficacy of RBX2660. Rebiotix has also made a lyophilized oral formulation of RBX2660 termed RBX7455, which is stable at room temperature. RBX7455 is currently being tested in a phase 1 study for the prevention of recurrent *C. difficile* infection, which was recently expanded (Rebiotix, 2018, 2017).

CP101 is an orally administered, capsule containing freeze-dried microbes derived from healthy human donors developed by Finch. The FDA has recently granted CP101 the Fast Track designation for the treatment of recurrent CDI. In a pragmatic cohort study, 49 patients experiencing recurrent CDI were given encapsulated lyophilized fecal microbiota (dosing ranged from $2.5\text{x}10^{12}$ bacteria in 24-27 capsules to $1.25\text{x}10^{12}$ bacteria in 2-3 capsules). This initial study observed an 88% (43/49) clinical success rate defined as no recurrent episodes over 2 months following therapy (Staley et al., 2017). CP101 is currently being tested in a recruiting phase 2 clinical trial for the treatment of recurrent CDI (NCT03110133).

### 2.7.4 Non-toxigenic *C. difficile*

Similar to bacterial replacement, one emerging therapy is utilizing non-toxigenic *C. difficile* to outcompete toxigenic *C. difficile* and prevent recurrent disease while the native microbiota recovers (Figure 2.5). This therapy may also be useful for prevention of CDI, similar to probiotics. The current clinical trials are looking at using non-toxigenic *C. difficile* for prevention or recurrence.

VP20621 (NTCD-M3) consists of spores from the non-toxigenic *C. difficile* (NTCD) strain M3. This strain has been shown to be protective in hamsters against *C. difficile* challenge. A study in healthy adults showed that VP20261 was well tolerated and resulted in colonization of the GI system following pretreatment with vancomycin (Villano et al., 2012). A phase 2 trial sponsored by Shire studied the safety and efficacy of VP20261 for the prevention of *C. difficile* in patients experiencing initial CDI or first recurrence. Patients were assigned to four groups receiving oral liquid VP20261 formulation of $10^4$ spores per day for 7 days (43 patients), $10^7$ spores per day for 7 days (44 patients), $10^7$ spores per day for 14 days (42 patients) or placebo for 14 days (44 patients). This study found that VP20261 was able to colonize the GI tract of patients following successful treatment with metronidazole or vancomycin and treatment was well tolerated.

Additionally, VP20261 treatment resulted in reduced recurrence of CDI, with 13 of 43 patients experiencing recurrence in the placebo arm and 14 of 125 experiencing recurrence in the VP20261 treatment groups. Of the patients receiving VP20261, recurrence was 2% for patients that were successfully colonized (2 out of 86) compared to a recurrence of 31% for patients not successfully colonized (12 out of 39 Gerding et al., 2015).

### 2.7.5 Bile Acid Supplementation

As particular secondary bile acids have been shown to be inhibitory to vegetative *C. difficile*, one possible therapy would be supplementation of secondary bile acids during CDI treatment (Sorg & Sonenshein, 2009; Ridlon et al., 2016; Thanissery et al., 2017). This could reduce the levels of *C. difficile* in the gut by suppressing growth and lead to a decreased rate of recurrence. A currently recruiting, phase 4 clinical trial will study the effects of urodeoxycholic acid (300 mg) supplementation for two months in total given during and following standard CDI treatment on the rates of recurrence (NCT02748616). Urodeoxycholic acid is being used as a surrogate for deoxycholic acid, a secondary bile acid.

## 2.8 Summary of Emerging CDI Therapies

*Clostridium difficile* infection causes significant morbidity and mortality while also placing a substantial burden on the healthcare system. We currently have effective therapies for primary and recurrent infections, but there is still significant improvement to be had in the areas of prevention, disease severity reduction, recurrence prevention, and treatment of recurrent CDI.

With incidence reaching nearly 500,000 cases annually in the United States, there is substantial need for directed CDI prevention therapies for susceptible patients. Already emerging therapies are being utilized in the clinic, with probiotics such as BioK and VSL#3 as examples. The main targets for preventative measures are reducing the initial microbial disruption ($\beta$-lactamases or reduced systemic antibiotics), restoring the microbiota with probiotics (BioK, VSL#3) or reducing the ability of *C. difficile* to grow and thrive by directly targeting it with vaccinations (VLA84, ACAM-CDIFF, PF-06425090, CDVAX) and growth modulators such as lactoferrin. With continued clinical

trials and development of preventative therapies, reduction of CDI incidence may soon be in reach with significant ameliorating effects on the CDI healthcare burden, patient morbidity, and patient mortality.

Once CDI has developed in a susceptible patient, the clinical goals shift to reducing severity, preventing fulminant CDI, obtaining clinical cure, and lowering recurrence risk. Development of effective toxin binders (Calcium Aluminosilicate, GT267-004, GT160-246) and immune modulators (Alanyl-glutamine), which help to reduce the toxin damage caused by *C. difficile* and restore the mucosa, may prevent severe CDI from developing and aid in the recovery of colonization resistance. Vaccinations, while seen as a method of prevention, may also act to decrease CDI severity by potentially reducing *C. difficile* organism burden and toxin activity. Bowel prep solutions that reduce luminal toxin levels and *C. difficile* organism burden may also help prevent severe CDI and increase therapeutic efficacy (Nu-Lytely). The development of antibiotics that are minimally absorbed and obtain high luminal concentrations with high activity against *C. difficile* without broad activity against other native bacteria is promising for the therapy of CDI (Cadazolid, CRS3123, LFF571, MCB3681, Nitazoxanide, Ramoplanin, Ridinilazole, Surotomycin, Tigecycline). The reduced activity against native bacteria may allow for more rapid recovery, potentially leading to lower severity and a decreased recurrence risk.

Even with effective therapy, elevated recurrence risk still persists for months after successful clinical cure. With each recurrence, the risk of subsequent recurrence increases (Olsen et al., 2015). While novel antibiotics are utilized to reduce the risk of CDI recurrence during primary treatment and may have a role in treating recurrent disease, additional therapies are needed to target the restoration of colonization resistance. The emerging use of monoclonal and polyclonal antibodies against *C. difficile* has shown success in reducing the risk of recurrence when given with standard CDI therapy. Determination of the best toxin motifs and which other *C. difficile* spore and vegetative cell molecules to target will help increase the effectiveness of toxin neutralization and *C. difficile* clearance following therapy. These treatments also could potentially be utilized during prevention and used during therapy to reduce severity, although directed clinical trials are needed to assess this possibility. Aside from lowering mucosal damage done by toxins and *C. difficile*, restoration of colonization resistance can be achieved by supplementing the microbiota with

bacterial replacement therapies (SER-109, CBM588, MET-2, RBX2600, CP101). Alternatively, or in unison, non-toxigenic *C. difficile* (VP20261) can be given to compete with toxigenic *C. difficile* cells and spores that may be remaining in the GI system or reintroduced during the high risk few months following CDI therapy.

## 2.9 Future Directions: Perspectives

While large strides are being taken towards effective prevention, primary treatment, and recurrence reduction, there are still gaps in our knowledge of CDI pathogenesis that would help direct therapy research if elucidated. For the prevention of CDI, research is currently underway in many laboratories and fields to identify the exact species and microbial interactions necessary to produce colonization resistance against *C. difficile* and what alterations to the GI microbiota lead to an increase in CDI risk. If these questions can be answered, specific bacterial replacement that is purified to only include non-pathogenic bacteria can be used to restore colonization resistance and reduce CDI rates. Additionally, if the specific alterations are known for the increased risk of CDI, such as the loss of keystone species, tests can be developed to monitor patients receiving systemic antibiotics or those at high risk demographically to determine if preventative therapies are needed, reducing the cost by targeting the use of these preventative treatments to only those requiring them.

For primary therapy, the direct mechanism leading to the development of severe and complicated CDI needs to be determined to develop targeted therapies for lowering severity. For instance, if mucosa damage is in part caused by immune response, then immune modulators can be utilized to reduce mucosa damage and protect against severe and fulminant CDI. Additionally, if the loss of specific members of the microbiota, *C. difficile* burden, or specific immune states are associated with the development of severe and fulminant CDI, these can be used to predict severity and allow physicians to treat patients prior to reaching the severe or fulminant state of infection.

In our opinion, the first class of emerging therapies that will have substantial impacts on the treatment of CDI is narrow spectrum, non-absorbable antibiotics. As research has shown that antibiotic treatments that cause large scale disruption of the microbiome are associated with

increased recurrence and other adverse outcomes such as sepsis, narrow spectrum antibiotics such as fidaxomicin and others in development (Table 2.2) have the potential to reduce microbial disruption during CDI treatment, leading to higher sustained clinical cure, lower recurrence rates, and reduced adverse outcomes (Baggs et al., 2018).

While stool products and bacterial replacements have shown considerable promise for treating recurrent CDI, longitudinal and large-scale studies are needed to examine potential long-term side-effects of altering the native bacterial community by the addition of therapeutic bacterial communities. In the meantime, the use of narrow spectrum antibiotics and other preventative measures can reduce recurrent cases without the necessity of bacterial agents such as FMT, filtered stool, or defined bacterial replacement. While the long-term effects are studied, in our opinion clinicians would prefer the development of pharmaceutical grade, FDA-approved filtered stool products and therapeutics, especially those with defined bacterial community structures. We foresee that these types of therapies will become a preferred alternative to FMT if they are shown to be efficacious and safe.

Additionally, well defined bacterial communities with single agent or limited taxa may be increasingly used as primary prophylaxis for the prevention of primary CDI (Mills et al., 2018). Through this mechanism, the number of primary and recurrent cases of CDI could be further reduced. While vaccines could be another viable preventative measure, they are currently not as effective and more clinical trials will be needed to identify an efficacious and safe vaccine.

An important limiting factor for clinical deployment of preventative and recurrence reduction therapies is the identification of patients who are at high risk of developing primary or recurrent CDI and who would benefit from these therapies. To achieve this risk stratification, large studies are starting to be performed using medical data for the development of predictive models for primary and recurrent CDI risk during a patient's treatment (Oh et al., 2018). If the risk of primary CDI or recurrence can be accurately predicted by microbial, systemic, or intestinal/colonic markers such as specific bacterial community compositions or immune cytokine levels, then the more expensive but effective therapies, such as bezlotoxumab, can be utilized to protect against future CDI. This will not only increase the effective treatment of recurrent CDI, but also lower the cost to the healthcare system by targeting only those patients predicted to experience primary or recurrent CDI.

We as a community are making immense strides towards effective prevention and management of *C. difficile*. The impact of these emerging therapies will not only affect CDI, but also other microbial illnesses that are dependent on an interaction between the host, native microbiota, and pathogen.

# CHAPTER III

# Systemic inflammatory mediators are effective biomarkers for predicting adverse outcomes in *Clostridioides difficile* infection

Results in this chapter were submitted to a journal as: *M. G. Dieterle, R. Putler, D. A. Perry, A. Menon, L. A. Close, N. S. Perlman, A. Penkevich, A. Standke, M. Keidan, K. Vendrov, I. Bergin, V. B. Young, and K. Rao. "Systemic inflammatory mediators are effective biomarkers for predicting adverse outcomes in* Clostridioides difficile *infection." 2020, accepted for publication in MBio* and are presented here with minor revisions.

## 3.1 Abstract

*Clostridioides difficile* infection (CDI) can result in severe disease and death, with no accurate models that allow for early prediction of adverse outcomes. To address this need, we sought to develop serum-based biomarker models to predict CDI outcomes. We prospectively collected sera 48 hours after diagnosis of CDI in two cohorts. Biomarkers were measured with a custom multiplex bead array assay. Patients were classified using IDSA severity criteria and the development of disease-related complications (DRCs) which were defined as ICU admission, colectomy and/or death attributed to CDI. Unadjusted and adjusted models were built using logistic and elastic net modeling. The best model for severity included procalcitonin (PCT) and hepatocyte growth factor (HGF) with an AUC of 0.74 [95% confidence interval 0.67–0.81]. The best model for 30-day mortality included IL-8, PCT, CXCL-5, IP-10, and IL-2R$\alpha$ with an AUC of 0.89 [0.84-0.95]. The best model for DRCs included IL-8, procalcitonin, HGF and IL-2R$\alpha$ with an AUC of 0.84

[0.73-0.94]. To validate our models, we employed experimental infection of mice with *C. difficile*. Antibiotic-treated mice were challenged with *C. difficile* and a similar panel of serum biomarkers were measured. Applying each model to the mouse cohort of severe and non-severe CDI revealed AUCs of 0.59 [0.44-0.74], 0.96 [0.90-1.0], and 0.89 [0.81-0.97]. In both human and murine CDI, models based on serum biomarkers predicted adverse CDI outcomes. Our results support the use of serum-based biomarker panels to inform *Clostridioides difficile* infection treatment.

## 3.2 Importance

*Clostridioides difficile* causes nearly 500,000 gastrointestinal infections that range from mild diarrhea to severe colitis and death. The ability to identify patients at increased risk for severe disease or mortality at the time of diagnosis of *C. difficile* infection (CDI) would allow clinicians to effectively allocate disease modifying therapies. In this study we developed models consisting of only a small number of serum biomarkers that are capable of predicting both 30-day all-cause mortality and adverse outcomes of patients at time of CDI diagnosis. We were able to validate these models through experimental mouse infection. This provides evidence that the biomarkers reflect the underlying pathophysiology and that our mouse model of CDI reflects the pathogenesis of human infection. Predictive models cannot only assist clinicians in identifying patients at risk for severe CDI but also be utilized for targeted enrollment in clinical trials aimed at reduction of adverse outcomes from severe CDI.

## 3.3 Introduction

*Clostridioides difficile* is a spore-forming bacillus that causes nearly 500,000 cases of toxin-mediated gastrointestinal illness yearly in the US with 29,300 deaths and a cost of 1.5 billion dollars annually (Lessa et al., 2015). The pathogenesis of *C. difficile* infection (CDI) involves local toxin production within the intestines leading to diarrhea and intestinal wall inflammation. Some patients experience severe colitis, along with a systemic inflammatory response as previously characterized (Rao et al., 2014).

We currently lack highly accurate predictive tools to assist with clinical decisions following CDI

diagnosis. The development of accurate predictive models for adverse outcomes could guide the use of emerging treatments for CDI that can ameliorate or prevent disease-related complications (DRCs) such as ICU admission, colectomy or death (Dieterle et al., 2019). For instance, fidaxomicin is costlier than vancomycin, while fecal transplants carry the risk of alterations to the host microbiome with unknown long-term effects, as well as transmission of enteric pathogens. Widespread deployment of these novel treatments in patients with CDI is impractical due to expense, invasiveness, and undetermined safety profiles, necessitating the development of tools for patient risk stratification treatment selection optimization.

The IDSA/SHEA guidelines use measurements of systemic immune response (WBC $>$ 15,000) or signs of renal dysfunction (creatinine $>$ 1.5) to define severe CDI (McDonald et al., 2018). Further signs of organ failure (shock, hypotension, ileus, or megacolon) are used to define complicated CDI. While this classification system guides management decisions, the features used are late findings and do not always allow for early identification of high-risk individuals. For instance, in a study of two cohorts consisting of 156 and 272 unique CDI cases, of the 23 all-cause mortality cases, 10 of these patients (43.5%) did not meet IDSA severity criterion at time of diagnosis. An ideal model would identify cases of CDI at the time of diagnosis that are progressing towards severe systemic disease, so that treatments to halt disease progression can be started. Models built from baseline clinical variables or standard laboratory measurements have met with limited success in accurately predicting adverse outcomes or they do not validate externally (van Beurden et al., 2017; Fujitani et al., 2011; Beauregard-Paultre et al., 2019; Archbald-Pannone et al., 2015; Butt et al., 2013; Kassam et al., 2016; Kulaylat et al., 2018). Therefore, we set out to determine if predictive models built from a panel of multiple inflammatory mediators measured at diagnosis of CDI can accurately predict adverse outcomes; specifically, 30-day all-cause mortality and disease-related complications (DRCs) defined as ICU admission, colectomy and/or death attributed to CDI. To validate these findings and provide further evidence of the utility of mouse models for CDI, we employed an experimental *C. difficile* infection in mice and tested the capability of the biomarker-based model to determine high disease severity in these mice.

## 3.4 Materials and Methods

### 3.4.1 Cohort Design

Sera was collected within 48 hours of diagnosis of CDI in two distinct cohorts of patients and frozen at 80C until analysis. The pilot cohort collections ranged from October 2010 to November 2012, contemporaneous with our prior publications on various biomarkers in CDI. The validation cohort collections ranged from January to September 2016. We felt it was important to separate these two cohorts as they were heterogeneous for several reasons: a) the four year gap in time, b) the change in testing practices (e.g. collection of stool in Cary-Blair media and no rejection of formed specimens in the pilot cohort era; use of best practice alerts and educational alerts to modify testing protocols in the validation cohort era), and c) the change in treatment practices (movement away from metronidazole and towards fidaxomicin and vancomycin per new institutional guidelines). All patients were diagnosed with CDI by the clinical microbiology laboratory using a two-step algorithm including detection of *C. difficile* glutamate dehydrogenase (GDH) and toxins A/B by enzyme immunoassay (C. DIFF QUIK CHEK COMPLETE®, Alere, Waltham, Massachusetts), with reflex to PCR for tcdB gene for discordant results (Focus Simplexa assay, DiaSorin, Saluggia, Italy [pilot cohort] and BD Geneohm assay, Becton, Dickinson and Company, Franklin Lakes, New Jersey [validation cohort]). We examined prediction tasks for three major outcomes of interest: Infectious Disease Society of America (IDSA) severity, 30-day all-cause mortality, and disease-related complications (DRCs). An IDSA severe case is defined as leukocytosis with a white blood cell count of 15000 cells/mL or a serum creatinine level >1.5 mg/dL5. DRCs included colectomy, death, or ICU admit within 30 days attributed to CDI as determined by ID physicians on our team blinded to the biomarker results (DAP and KR).

The pilot cohort was analyzed with a 14-plex assay to examine key serum biomarkers. After our preliminary results and further study, CXCL-5, IL-22, and IL-23 were added to the panel to produce the 17-plex assay that was utilized on our validation cohort (Table 3.1). A similar panel was produced for mouse with identical inflammatory mediators or the closest homologues. Our analysis is split into classification of current disease using IDSA severity as the gold standard and outcome prediction. The two outcomes of each CDI case that we set out to model were 30-day all-

cause mortality from time of diagnosis, and disease-related complications (DRCs) which included ICU admission, colectomy, or death caused by CDI specifically. Attributable CDI severity was determined through physician-based chart review.

This study was approved by the University of Michigan IRB.

### 3.4.2 Human and Mouse Luminex 17-plex Assay

Two custom, bead-based, multiplex inflammatory mediator panels were performed on samples using a Luminex® 200TM dual laser detection system. Our panel was selected from previous research cited in Table 3.1. The human multiplex panel included 17 inflammatory mediators previously identified as being potential biomarkers for CDI including: CCL-2 (MCP-1), CCL-4 (MIP-1b), CCL-5 (RANTES), CXCL-5, CXCL-9, CXCL-10 (IP-10), epidermal growth factor (EGF), hepatocyte growth factor (HGF), IL-2R$\alpha$, IL-4, IL-6, IL-8, IL-15, IL-22, IL-23, procalcitonin (PCT), and TNF-$\alpha$. The mouse 17 inflammatory mediator panel included the same cytokines for murine serum except it included KC, a mouse homologue of IL-8, instead of IL-8 and included LIX, a mouse homologue of CXCL-5, instead of CXCL-5. All resulting measurements in pg/mL were log-transformed. Demographic information and clinical variables were extracted from the electronic medical record for the human cohort.

### 3.4.3 Data Analysis Methodology

Given that measurements from Luminex assays are linear and, thus, accurate over a wide range of concentrations, generally spanning several orders of magnitude, the inflammatory mediator measurements were log-transformed prior to analysis to correct for non-normal distributions (positive skew). Principle component analysis (PCA) was performed for the panel of inflammatory mediators, independent of our outcomes of interest, using princomp in the stats package in R14. We performed redundancy analysis (RDA) for each binary variable (IDSA severity, 30-day all-cause mortality, and DRCs) as the predictor and the outcomes were the log-transformed inflammatory mediators to assess whether the biomarker profile might be different between the individuals positive for the binary metric tested (e.g. those that experienced DRCs and those that did not). This was achieved by performing analysis of variance using Euclidean distance and a permutation test

| **Inflammatory mediator** | **Alternative name(s)/ abbreviation(s)** | **Prior studies in CDI/UC** |
|---|---|---|
| Tumor necrosis factor-alpha (TNF-$\alpha$) | | Olson et al. (2014), Brito et al. (2002), Klapproth & Sasaki (2010) |
| Interleukin-2 receptor $\alpha$ (IL-2R$\alpha$) | CD25 | Rao et al. (2014) |
| Interleukin-4 (IL-4) | | Connelly et al. (2014) |
| Interleukin-6 (IL-6) | | Rao et al. (2014) |
| Interleukin-8 (IL-8) | Neutrophil chemotactic factor | Rao et al. (2014), Steiner et al. (1997), Jiang et al. (2007) |
| Interleukin-15 (IL-15) | | Rao et al. (2014) |
| Interleukin-22 (IL-22) | | Sadighi Akha et al. (2015) |
| Interleukin-23 (IL-23) | | Cowardin et al. (2015), Buonomo et al. (2013) |
| Chemokine (C-C motif) ligand 2 (CCL2) | Monocyte chemotactic protein-1 (MCP-1) or small inducible cytokine A2 (SCYA2) | Rao et al. (2014) |
| CCL5 | RANTES | Rao et al. (2014) |
| Chemokine (C-C motif) ligand 4 (CCL4) | Macrophage inflammatory protein-1beta (MIP-1b) | Rao et al. (2014) |
| Chemokine (C-X-C motif) ligand 5 (CXCL5) | | El Feghaly et al. (2013) |
| Chemokine (C-X-C motif) ligand 9 (CXCL9) | Monokine induced by gamma interferon (MIG) | Rao et al. (2014) |
| Hepatocyte growth factor (HGF) | | Rao et al. (2014) |
| Epidermal growth factor (EGF) | | Rao et al. (2014) |
| Chemokine (C-X-C motif) ligand 10 (CXCL10) | Interferon gamma-induced protein 10 (IP-10) or small inducible cytokine B10 (SCYB10) | Rao et al. (2014) |
| Procalcitonin (PCT) | | Rao et al. (2013) |

Table 3.1 Support for inclusion of inflammatory mediators previously shown to be associated with CDI severity and adverse outcomes. Reproduced with permission from Limsrivilai et al. (2018).

to find P values. This was performed using the vegan package in R (Oksanen et al., 2018). We assessed the impact of individual inflammatory mediators on the outcomes by performing unadjusted logistic regression for each inflammatory mediator.

We first attempted to model our outcomes using multivariable logistic regression with binomial deviance as our error measure. However, our overall goal was to identify important inflammatory mediators and construct models in a manner that avoided overfitting and would be more likely to generalize to an external cohort. With this in mind, we utilized five-fold cross-validated elastic net multivariable logistic regression with the goal of testing the impact of adjusting the stringency of inclusion criterion and tuning parameters. A lambda value was selected where deviance was within one standard error of the minimum (1se, more stringent) or at the minimum (min). Additionally, we swept through alpha values range from pure ridge regression (alpha = 0) to pure lasso regression (alpha = 1) to identify which biomarkers would be included in each condition. For each value of alpha tested, 100 iterations across different seeds were performed. All of these methods (regularized regression, cross validation, evaluating different lambda values, and sweeping the alpha tuning parameter) were aimed at avoiding overfitting, and even though this results in models that do not perform as well, the resulting claims about model performance are more conservative and more likely to validate externally. This was performed using the glmnet package in R (Friedman et al., 2010). Comparison of elastic net models was performed by creating receiver operating characteristic (ROC) curves and calculating the area under the ROC (AUC) using the pROC package in R (Robin et al., 2011). Net reclassification improvement index analysis was done using the PredictABEL package in R (Kundu et al., 2014). All analysis was performed using R and RStudio (R Core Team, 2009; Team, 2015).

### 3.4.4 Mouse Experimental Methods

8–12 week-old, Specific Pathogen Free (SPF) C57BL/6 mice were treated with 10 days of cefoperazone (0.5g/L) delivered in their drinking water to render them sensitive to *Clostridioides difficile* infection. The C57BL/6 mice used were produced by the Young Lab breeding colony at the University of Michigan established from mice purchased from the Jackson laboratory. After two days off antibiotics, mice were given an oral gavage of water, 630g *C. difficile* spores, or VPI

10463 *C. difficile* spores. Inoculum was estimated between 103 and 104 spores. While Mock mice gain weight over the course of the observational time, 630g infected mice remain at the same weight while VPI 10463 mice lose significant weight over two days. In our model, VPI 10463 infection following cefoperazone will result in a high proportion of death if allowed to progress beyond 48 hours. To obtain serum samples, VPI 10463 mice were sacrificed 2 days after infection, while half of the 630g infected mice were sacrificed at day 2 as time controls and the rest were sacrificed at 4 days post infection when they reach their maximum disease. Cecum and colon histopathology were scored from 0-12 by a blinded pathologist for edema, epithelial damage, and inflammatory cell infiltration. Each mouse was given a clinical score from 0-20 at euthanization based on posture, coat, activity, diarrheal signs, and weight change from day 0 (D0). Further description of the model can be found in Leslie et al. (2015).

## 3.5 Results

### 3.5.1 Serum markers of epithelial damage, inflammation, and neutrophilic migration are significantly associated with mortality and disease related complications

We studied an initial pilot cohort of 156 CDI cases, of which 58 (37.2%) met IDSA severity criteria, 4 (2.6%) died within 30 days, and 10 (6.4%) had disease related complications. Of the 4 patients with CDI who died within 30 days, 2 did not meet IDSA severity criterion at time of diagnosis. Serum collected near time of diagnosis was tested with a custom panel for serum biomarkers ranging from inflammatory markers to epithelial growth factors (Table 3.1). Biomarker profiles of serum from severe and non-severe cases showed separation by principal component analysis (Figure 3.1), while redundancy analysis (RDA) of biomarkers differentiated severe and non-severe episodes by permutational MANOVA (P=.005) and differentiated cases that developed DRCs (P=.025) (Figure 3.1). These biomarkers did not distinguish between patients who died within 30-days of diagnosis, most likely due to the limited number of 30-day mortality cases in our pilot study (n = 4). Unadjusted logistic regression revealed that IL-6, procalcitonin, IL-8, IL-2R$\alpha$, and HGF were significantly associated with severity (P<.001, <.01, <.05, <.05, <.05 respectively). All of

these biomarkers except procalcitonin were also significantly associated with DRCs but not overall 30-day mortality (Table 3.2).

| Individual Log Transformed Biomarker Population Statistics | | | | Unadjusted Analysis for IDSA Severity | | | Unadjusted Analysis for 30 Day Mortality | | | Unadjusted Analysis for DRC | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bio-marker | Median | Mean | Std. | Bio-marker | Odds Ratio | Sign. | Bio-marker | Odds Ratio | Sign. | Bio-marker | Odds Ratio | Sign. |
| IL-6 | 3.18 | 3.24 | 1.42 | IL-6 | 1.71 [1.30-2.24] | *** | IP-10 | 2.33 [1.09-4.96] | * | HGF | 2.07 [1.22-3.52] | ** |
| PCT | 0.11 | 0.46 | 0.9 | PCT | 2.95 [1.53-5.70] | ** | IL-6 | 2.05 [0.98-4.27] | - | IL-8 | 2.07 [1.16-3.68] | * |
| IL-8 | 3.47 | 3.44 | 1.02 | IL-8 | 1.45 [1.04-2.04] | * | IL-4 | 0.58 [0.31-1.09] | - | IL-2R | 1.84 [1.12-3.02] | * |
| IL-2R | 6.07 | 5.78 | 1.75 | IL-2R | 1.27 [1.02-1.58] | * | MCP-1 | 0.63 [0.28-1.41] | - | IL-6 | 1.76 [1.09-2.84] | * |
| HGF | 6.23 | 6.03 | 1.71 | HGF | 1.25 [1.00-1.57] | * | CXCL-9 | 0.57 [0.21-1.58] | - | TNFa | 0.52 [0.27-0.98] | * |
| EGF | 2.8 | 2.99 | 1.19 | EGF | 1.25 [0.91-1.70] | - | IL-8 | 1.56 [0.67-3.65] | - | MCP-1 | 2.06 [1.02-4.15] | * |
| RANTES | 8.58 | 8.46 | 1 | RANTES | 0.85 [0.60-1.19] | - | IL-2R | 0.90 [0.52-1.56] | - | MIP-1b | 1.44 [0.73-2.82] | - |
| IL-15 | 3.5 | 3.28 | 1.44 | IL-15 | 1.09 [0.85-1.40] | - | TNFa | 0.85 [0.33-2.21] | - | EGF | 1.32 [0.76-2.30] | - |
| MIP-1b | 4.7 | 4.7 | 0.98 | MIP-1b | 1.11 [0.77-1.9] | - | MIP-1b | 0.85 [0.31-2.33] | - | CXCL-9 | 1.22 [0.70-2.13] | - |
| IP-10 | 4.89 | 5.15 | 1.35 | IP-10 | 1.05 [0.81-1.36] | - | RANTES | 0.87 [0.32-2.35] | - | IL-4 | 0.87 [0.54-1.40] | - |
| IL-4 | 3.66 | 3 | 1.25 | IL-4 | 0.96 [0.74-1.24] | - | HGF | 0.93 [0.53-1.64] | - | PCT | 1.09 [0.53-2.24] | - |
| MCP-1 | 5.57 | 5.24 | 1.17 | MCP-1 | 0.99 [0.73-1.34] | - | PCT | 1.17 [0.33-4.12] | - | IL-15 | 1.02 [0.65-1.61] | - |
| CXCL-9 | 4.25 | 4.2 | 1.13 | CXCL-9 | 1.01 [0.74-1.39] | - | IL-15 | 0.94 [0.48-1.84] | - | IP-10 | 0.98 [0.61-1.58] | - |
| TNFa | 1.79 | 2.06 | 1.06 | TNFa | 1.00 [0.73-1.36] | - | EGF | 1.04 [0.45-2.43] | - | RANTES | 1.01 [0.53-1.92] | - |

Table 3.2 Pilot Cohort for Biomarker Analysis. Biomarker Population Statistics and Simple Unadjusted Logistic Regression Analysis for IDSA Severity and the Disease Outcomes 30-Day-All-Cause Mortality and Disease Related Complications (DRCs).

We employed a validation cohort of 272 unique CDI cases among 253 patients, of which 71 (26.1%) met IDSA severity criteria, 19 (7.0%) died within 30 days, and 18 (6.6%) had DRCs (Table 3.3). 8 of 19 cases experiencing 30-day all-cause-mortality did not meet IDSA severity criteria at time of diagnosis. There were 14 patients that experienced 30-day all-cause mortality and developed DRCs. Similar to the pilot, biomarker-based RDA of the validation cohort differentiated severe and non-severe CDI cases by permutational MANOVA (P=.001) and DRCs (P=.002). With the increase in the number of patients who died, biomarker profiles from patients with 30-day mortality were also differentiated by RDA (P=.001) (Figure 3.2). Characterization of biomarker associations with each outcome was performed with unadjusted logistic regression and showed that 12 of the 17 inflammatory markers were individually associated with at least one outcome, with 6 biomarkers (HGF, Procalcitonin, IL-6, IL-2R$\alpha$, IL-8, and TNF-$\alpha$) significantly associated with all three outcomes. With unadjusted inflammatory mediators, the most significant positive associated biomarkers (P<.001) with IDSA severity were HGF, PCT, IL-6 and IL-2R$\alpha$, with 30-day mortality were IL-2R$\alpha$, PCT, IL-8, and IP-10, and with DRCs were PCT, IL-8, and IL-2R$\alpha$ (Table 3.5). Table of all associations are shown in Table 3.6. These findings validate the associations between biomarkers and adverse outcomes seen in the pilot cohort.

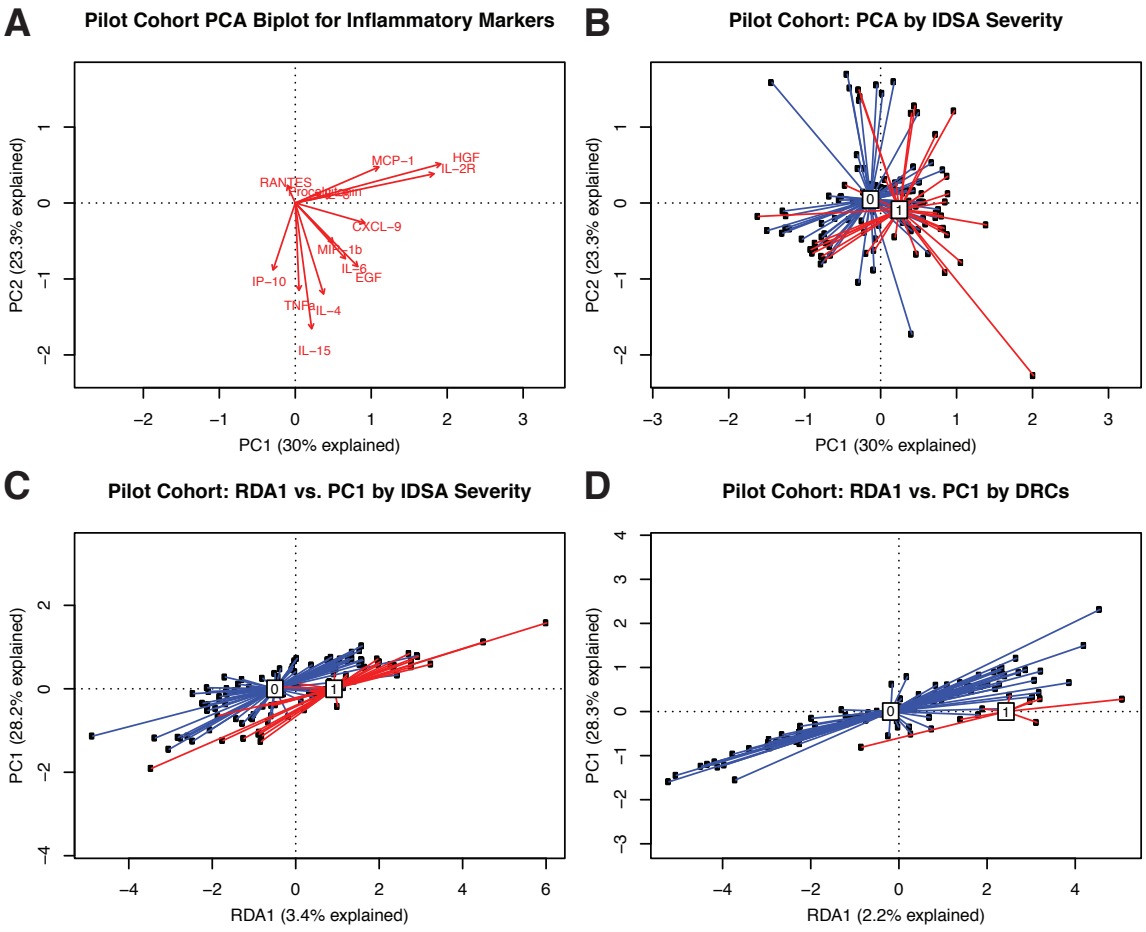



Figure 3.1 Pilot cohort analysis – PCA and RDA for predicting adverse outcomes with inflammatory mediators. (A) Biplot showing the direction of each inflammatory mediator with respect to PCA axes 1 and 2 in the pilot cohort. Of note, IL-6, IL-2R, and HGF are strong drivers in the same direction as the separation in the PCA plot colored by IDSA severity (B). PCA showing separation between severe (1) and non-severe (0) CDI cases, with PC1 explaining 30% of the variance and PC2 explaining 23.3% of the variance. RDA axis 1 vs PC1 plots for (C) IDSA severity and (D) DRCs. RDA differentiated the biomarker profile of the positive (1) and negative (0) cases for IDSA severity (C) and (D) DRCs by PERMANOVA ($P<.01$ and $P<.05$, respectively).

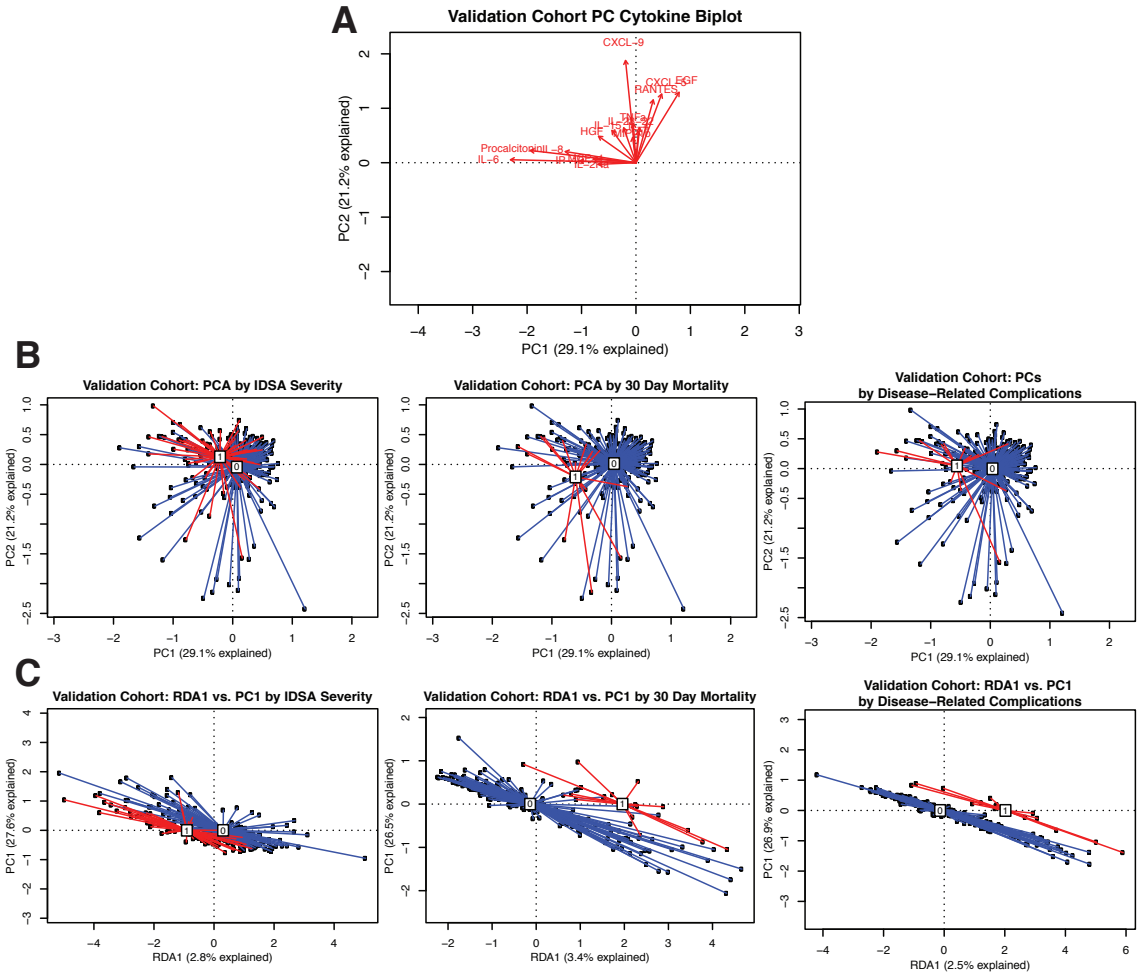



Figure 3.2 Validation cohort analysis – PCA and RDA for predicting adverse outcomes with inflammatory mediators. (A) Biplot showing the direction of each inflammatory mediator with respect to PCA axes 1 and 2 in the validation cohort. (B) PC1 and PC2 of the validation cohort inflammatory mediator profiles colored by IDSA severity (left), 30-day mortality (center) and DRCs (right), with PC1 explaining 29.1% and PC2 explaining 21.2% of the variance. Cases positive for severity, 30-day mortality or DRCs are colored in red (1) and those cases that are negative for those outcomes are blue (0). (C) RDA axis 1 vs PC1 plots for IDSA severity (left), 30-day mortality (center), and DRCs (right), all of which RDA differentiated biomarker profiles of positive (red) and negative (blue) cases by PERMANOVA ($P<.001$, $P< .001$ and $P<.01$, respectively).

| Demographic Category | Subcategory | Pilot | Validation |
|---|---|---|---|
| Number of Cases | N/A | 156 | 272 |
| Age | N/A | 56 ± 18 | 55 ± 21 |
| Sex | Male | 67 (43.0%) | 131 (48.2%) |
| | Female | 89 (57.0%) | 141 (51.8%) |
| Race | Caucasian | 137 (87.8%) | 236 (86.8%) |
| | Black or African American | 10 (6.4%) | 18 (6.6%) |
| | Asian | 0 (0%) | 4 (1.5%) |
| | American Indian or Alaska Native | 2 (1.3%) | 3 (1.1%) |
| | Native Hawaiian and Pacific Islander | 1 (0.6%) | 0 (0%) |
| | Other or Unknown | 6 (3.9%) | 11 (4.0%) |
| Ribotypes | 027 Ribotype | 15 (9.6%) | 25 (9.2%) |
| | 014-020 Ribotype | 30 (19.2%) | 47 (17.3%) |
| Method of CDI Diagnosis | Toxins A/B Enzyme Immunoassay | 71 (46%) | 69 (25%) |
| | Reflex to PCR for tcdB gene | 85 (54%) | 203 (75%) |
| Disease Measures | IDSA Severity | 58 (37.2%) | 71 (26.1%) |
| | 30 Day Mortality | 4 (2.6%) | 19 (7.0%) |
| | DRCs | 10 (6.4%) | 18 (6.6%) |
| | Subset with 30-Day All-Cause Mortality and DRCs | 2 (1.3%) | 14 (5.2%) |
| Pertinent Medical History | Elixhauser Score | N/A | 4.6 ± 3.3 |
| | Concurrent Abx | 112 (71.8%) | 87 (32.0%) |
| | History of C. difficile Infection | 40 (25.6%) | 52 (19.6%) |
| | IBD | N/A | 41 (15.1%) |

Table 3.3 Cohort Demographics and Pertinent Patient Information.

### 3.5.2 Development of high performance, multivariable models to estimate CDI severity and predict adverse outcomes

While logistic regression models were initially produced to test feasibility of predicting 30-day all-cause mortality and DRCs from serum biomarkers at diagnosis (Table 3.6), these models are often not useful outside the particular cohort in which they were built. To produce more refined

| Individual Log Transformed Biomarker Population Statistics | | | | Unadjusted Analysis for IDSA Severity | | | | Unadjusted Analysis for 30 Day Mortality | | | | Unadjusted Analysis for DRC | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Bio-marker | Median | Mean | Std. | Bio-marker | Odds Ratio | OR Sig. | AUC | Bio-marker | Odds Ratio | OR Sig. | AUC | Bio-marker | Odds Ratio | OR Sig. | AUC |
| HGF | 5.85 | 5.95 | 1.06 | HGF | 1.97 [1.49-2.60] | *** | 0.71 [0.64-0.78] | IL-2Ra | 8.28 [3.41-20.11] | *** | 0.85 [0.77-0.92] | PCT | 1.94 [1.43-2.64] | *** | 0.82 [0.75-0.90] |
| PCT | 4.83 | 5.35 | 1.43 | PCT | 1.57 [1.3-1.89] | *** | 0.69 [0.62-0.76] | PCT | 1.92 [1.42-2.58] | *** | 0.82 [0.73-0.9] | IL-8 | 2.03 [1.44-2.86] | *** | 0.78 [0.67-0.88] |
| IL-6 | 2.68 | 3.02 | 1.57 | IL-6 | 1.39 [1.17-1.65] | *** | 0.68 [0.62-0.75] | IL-8 | 2.03 [1.45-2.86] | *** | 0.80 [0.71-0.90] | IL-2Ra | 4.86 [2.16-10.94] | *** | 0.79 [0.69-0.88] |
| IL-2Ra | 7.6 | 7.61 | 0.65 | IL-2Ra | 2.29 [1.47-3.57] | *** | 0.65 [0.58-0.72] | IP-10 | 1.76 [1.31-2.36] | *** | 0.67 [0.54-0.79] | IL-6 | 1.49 [1.17-1.90] | ** | 0.69 [0.54-0.84] |
| IL-8 | 3.65 | 3.88 | 1.16 | IL-8 | 1.44 [1.14-1.82] | ** | 0.64 [0.57-0.71] | EGF | 0.59 [0.43-0.8] | *** | 0.70 [0.58-0.83] | HGF | 1.94 [1.3-2.89] | ** | 0.71 [0.59-0.84] |
| TNFa | 4.01 | 3.89 | 0.51 | TNFa | 2.87 [1.29-6.39] | ** | 0.61 [0.53-0.69] | CXCL-5 | 0.53 [0.36-0.8] | ** | 0.75 [0.65-0.85] | IP-10 | 1.42 [1.05-1.94] | * | 0.62 [0.49-0.75] |
| MIP-1b | 6.16 | 6.1 | 0.4 | MIP-1b | 2.48 [1.16-5.30] | * | 0.61 [0.53-0.68] | IL-6 | 1.44 [1.13-1.83] | ** | 0.70 [0.55-0.84] | MCP-1 | 1.58 [0.98-2.56] | - | 0.62 [0.45-0.79] |
| IL-22 | 6.07 | 5.96 | 0.51 | IL-22 | 1.93 [1.03-3.63] | * | 0.59 [0.51-0.67] | RANTES | 0.7 [0.51-0.97] | * | 0.73 [0.61-0.85] | CXCL-5 | 0.71 [0.47-1.06] | - | 0.63 [0.51-0.75] |
| IL-15 | 3.06 | 2.79 | 1.14 | IL-15 | 1.23 [0.95-1.60] | - | 0.57 [0.49-0.65] | IL-22 | 0.45 [0.22-0.93] | * | 0.61 [0.45-0.76] | EGF | 0.77 [0.56-1.07] | - | 0.62 [0.48-0.76] |
| RANTES | 9.61 | 9.34 | 0.96 | RANTES | 1.3 [0.88-1.93] | - | 0.55 [0.48-0.63] | HGF | 1.53 [1.03-2.27] | * | 0.63 [0.47-0.78] | IL-15 | 1.39 [0.82-2.35] | - | 0.65 [0.50-0.80] |
| EGF | 4.14 | 3.89 | 1.32 | EGF | 1.14 [0.92-1.41] | - | 0.54 [0.47-0.62] | MCP-1 | 1.58 [0.98-2.53] | - | 0.62 [0.47-0.78] | RANTES | 0.84 [0.59-1.20] | - | 0.61 [0.48-0.75] |
| IL-4 | 5.25 | 5.1 | 0.48 | IL-4 | 1.3 [0.70-2.38] | - | 0.49 [0.41-0.57] | IL-4 | 0.52 [0.25-1.06] | - | 0.66 [0.52-0.80] | IL-4 | 0.78 [0.33-1.83] | - | 0.57 [0.42-0.72] |
| MCP-1 | 5.75 | 5.89 | 0.85 | MCP-1 | 1.14 [0.84-1.56] | - | 0.52 [0.44-0.60] | IL-15 | 1.7 [0.95-3.07] | - | 0.66 [0.52-0.80] | MIP-1b | 1.29 [0.38-4.43] | - | 0.56 [0.40-0.72] |
| CXCL-5 | 6.79 | 6.7 | 1.1 | CXCL-5 | 1.1 [0.85-1.41] | - | 0.52 [0.45-0.60] | MIP-1b | 0.41 [0.14-1.17] | - | 0.57 [0.41-0.74] | TNFa | 1.20 [0.42-3.40] | - | 0.51 [0.37-0.65] |
| CXCL-9 | 6 | 5.82 | 1.39 | CXCL-9 | 1.08 [0.87-1.33] | - | 0.58 [0.50-0.65] | CXCL-9 | 0.87 [0.66-1.13] | - | 0.52 [0.35-0.70] | IL-23 | 0.96 [0.65-1.42] | - | 0.58 [0.45-0.72] |
| IP-10 | 2.65 | 2.99 | 1.18 | IP-10 | 0.97 [0.77-1.23] | - | 0.55 [0.47-0.63] | TNFa | 0.71 [0.34-1.48] | - | 0.57 [0.42-0.72] | CXCL-9 | 1.01 [0.71-1.45] | - | 0.49 [0.34-0.65] |
| IL-23 | 3.75 | 3.48 | 1.25 | IL-23 | 0.98 [0.78-1.22] | - | 0.51 [0.43-0.59] | IL-23 | 0.87 [0.61-1.24] | - | 0.58 [0.43-0.72] | IL-22 | 0.97 [0.38-2.47] | - | 0.53 [0.39-0.66] |

Table 3.4 Validation Cohort for Biomarker Analysis. Validation Cohort – Biomarker Population Statistics and Simple Unadjusted Logistic Regression for IDSA Severity and the Disease Outcomes 30 Day Mortality and Disease Related Complications (DRCs).

| Unadjusted Analysis for IDSA Severity | | | | Unadjusted Analysis for 30 Day All-Cause Mortality | | | | Unadjusted Analysis for DRC | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Bio-marker | Odds Ratio | OR Sig. | AUC | Bio-marker | Odds Ratio | OR Sig. | AUC | Bio-marker | Odds Ratio | OR Sig. | AUC |
| HGF | 1.97 [1.49-2.60] | *** | 0.71 [0.64-0.78] | IL-2Ra | 8.28 [3.41-20.11] | *** | 0.85 [0.77-0.92] | PCT | 1.94 [1.43-2.64] | *** | 0.82 [0.75-0.90] |
| PCT | 1.57 [1.3-1.89] | *** | 0.69 [0.62-0.76] | PCT | 1.92 [1.42-2.58] | *** | 0.82 [0.73-0.9] | IL-8 | 2.03 [1.44-2.86] | *** | 0.78 [0.67-0.88] |
| IL-6 | 1.39 [1.17-1.65] | *** | 0.68 [0.62-0.75] | IL-8 | 2.03 [1.45-2.86] | *** | 0.80 [0.71-0.90] | IL-2Ra | 4.86 [2.16-10.94] | *** | 0.79 [0.69-0.88] |
| IL-2Ra | 2.29 [1.47-3.57] | *** | 0.65 [0.58-0.72] | IP-10 | 1.76 [1.31-2.36] | *** | 0.67 [0.54-0.79] | IL-6 | 1.49 [1.17-1.90] | ** | 0.69 [0.54-0.84] |
| IL-8 | 1.44 [1.14-1.82] | ** | 0.64 [0.57-0.71] | EGF | 0.59 [0.43-0.8] | *** | 0.70 [0.58-0.83] | HGF | 1.94 [1.3-2.89] | ** | 0.71 [0.59-0.84] |
| TNF- | 2.87 [1.29-6.39] | ** | 0.61 [0.53-0.69] | CXCL-5 | 0.53 [0.36-0.8] | ** | 0.75 [0.65-0.85] | IP-10 | 1.42 [1.05-1.94] | * | 0.62 [0.49-0.75] |

Table 3.5 Top Six Inflammatory Mediators by Simple Unadjusted Logistic Regression. Factors sorted by outcome of interest for IDSA Severity, 30-Day-All-Cause Mortality, and Disease Related Complications (DRCs). (P-value: * $\leq$.05, ** $\leq$.01, *** $\leq$.001)

and generalizable models, we used five-fold cross validated elastic net regression modeling. As our goal is not to produce necessarily the best model, but to describe which biomarkers have the potential to predict adverse outcomes in a generalizable way that would be most likely to validate in external cohorts, we show the modeling results for a range of tuning parameters. Alpha values were tested from pure ridge regression (alpha = 0) to pure lasso regression (alpha = 1), allowing the visualization of which biomarkers are retained in the model as the inclusion criterion becomes more stringent (towards lasso regression). Additionally, biomarker inclusion is impacted by the selection of lambda, where deviance was within one standard error of the minimum (1se) or at the minimum (min).

As smaller models are more useful for clinical applications and performance did not differ drastically between min (higher potential for overfitting) and 1se (higher potential for being generalizable) models, biomarker inclusion for each model and the AUC performance for the 1se models are shown

**Models Estimating IDSA Severity Models**

| Model | Included Biomarkers | AIC | BIC | Log-Likelihood | AUC |
|---|---|---|---|---|---|
| mAIC | HGF, Procalcitonin, RANTES, and CXCL-9 | 254.41 | 272.12 | -122.21 | 0.75 [0.68-0.82] |
| m25AIC | HGF, Procalcitonin, and EGF | 277.8 | 292.17 | -134.9 | 0.74 [0.68-0.81] |
| mLRT | HGF, Procalcitonin, and RANTES | 255.78 | 269.95 | -123.89 | 0.75 [0.68-0.82] |
| m25LRT | HGF and Procalcitonin | 279.51 | 290.28 | -136.75 | 0.73 [0.67-0.8] |

**Models Predicting 30-Day All-Cause Mortality**

| Model | Included Biomarkers | AIC | BIC | Log-Likelihood | AUC |
|---|---|---|---|---|---|
| mAIC | IL-2Ra, IL-8, CXCL-5, HGF, IP-10, IL-6, CXCL-9 and TNFa | 94.58 | 126.52 | -38.29 | 0.9 [0.82-0.98] |
| m25AIC | IL-2Ra, IL-8, CXCL-5, HGF, IP-10, IL-6 and IL-15 | 105.83 | 134.61 | -44.91 | 0.9 [0.83-0.97] |
| mLRT | IL-2Ra, IL-8, CXCL-5, HGF, IP-10 and IL-6 | 94.4 | 119.24 | -40.2 | 0.9 [0.82-0.97] |
| m25LRT | IL-2Ra, IL-8 and CXCL-5 | 108.23 | 122.62 | -50.11 | 0.89 [0.84-0.95] |

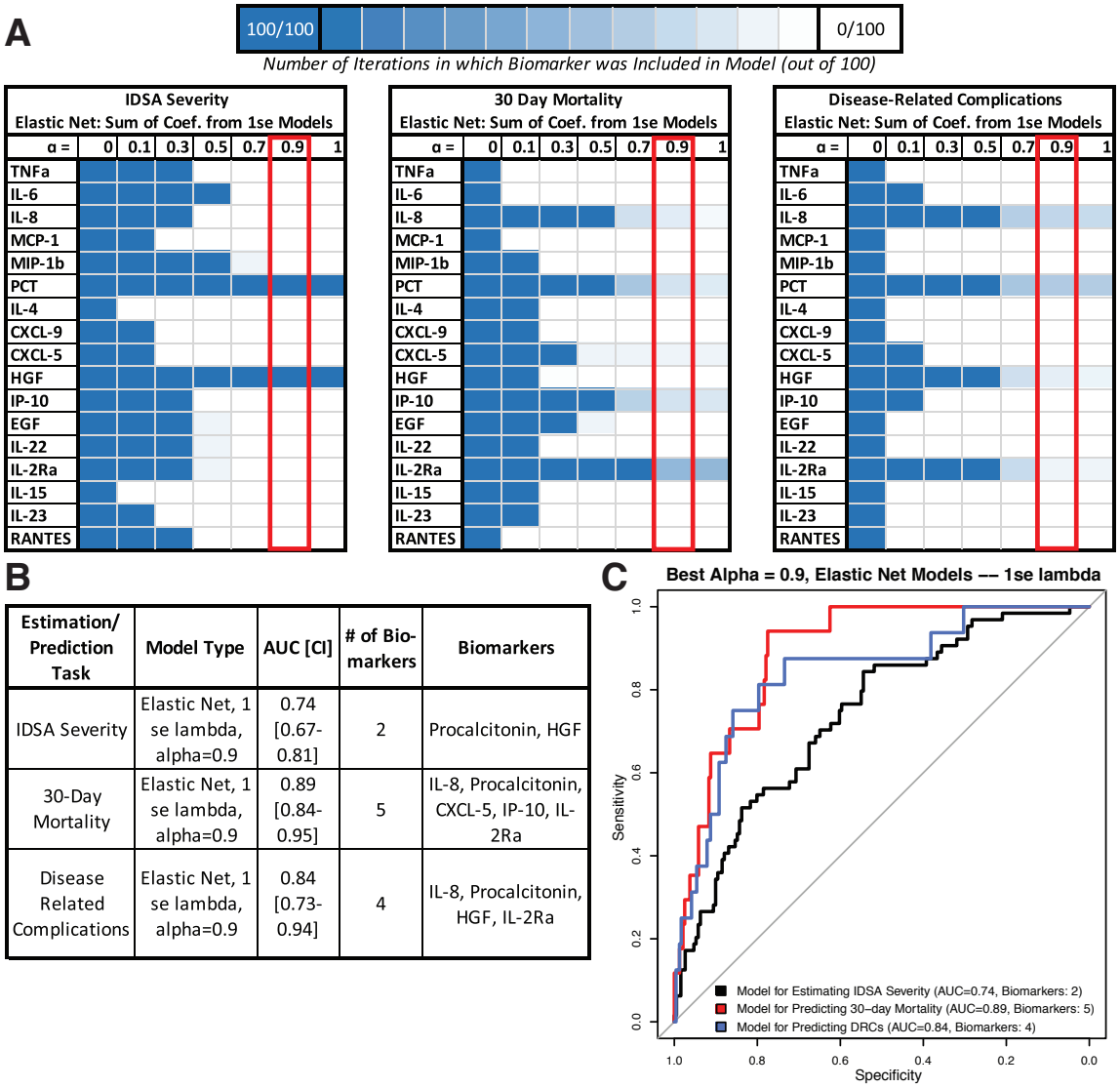
**Models Predicting Disease Related Complications (DRCs)**

| Model | Included Biomarkers | AIC | BIC | Log-Likelihood | AUC |
|---|---|---|---|---|---|
| mAIC | IL-8, HGF, and IL-2Ra | 100.84 | 115.04 | -46.42 | 0.85 [0.74-0.96] |
| m25AIC | IL-8, HGF, and IL-2Ra | 111.78 | 126.21 | -51.89 | 0.85 [0.75-0.94] |
| mLRT | IL-8, HGF, and IL-2Ra | 100.84 | 115.04 | -46.42 | 0.85 [0.74-0.96] |
| m25LRT | IL-8, HGF, and IL-2Ra | 111.78 | 126.21 | -51.89 | 0.85 [0.75-0.94] |

Table 3.6 Logistic Regression with backward selection for estimating IDSA Severity and predicting adverse outcomes (30 Day Mortality and DRCs). The resulting AUC is stable for each outcome across biomarker inclusion and selection processes. mAIC = Stepwise Regression, Backward Selection with all biomarkers. m25AIC = Stepwise Regression, Backward Selection including only biomarkers with $P < 0.25$ from unadjusted logistic regression. mLRT = Stepwise Regression, Drop One Selection with all biomarkers. m25LRT = Stepwise Regression, Drop One Selection including only biomarkers with $P < 0.25$ from unadjusted logistic regression.

in Figure 3.3 while the results for the min models are shown in Figure 3.4. To create the most parsimonious model, the 0.9 models are strongly weighted to reduce unnecessary biomarkers and are the chosen highlighted models, although similar performance is seen across lambda and alpha values. ROCs and AUCs for the best elastic net models at each alpha value are shown in Figure 3.5, highlighting the stability of the model performance with decreasing biomarker inclusion.

For IDSA severity estimation, elastic net modeling shows that PCT and HGF are included in all models and are the only biomarkers in 1se models with alpha >0.5. The 1se (alpha=0.9) model includes 2 biomarkers and produces an AUC of 0.74 [0.67–0.81] (Figure 3.3), while the min




| Estimation/ Prediction Task | Model Type | AUC [CI] | # of Bio-markers | Biomarkers |
|---|---|---|---|---|
| IDSA Severity | Elastic Net, 1 se lambda, alpha=0.9 | 0.74 [0.67-0.81] | 2 | Procalcitonin, HGF |
| 30-Day Mortality | Elastic Net, 1 se lambda, alpha=0.9 | 0.89 [0.84-0.95] | 5 | IL-8, Procalcitonin, CXCL-5, IP-10, IL-2Ra |
| Disease Related Complications | Elastic Net, 1 se lambda, alpha=0.9 | 0.84 [0.73-0.94] | 4 | IL-8, Procalcitonin, HGF, IL-2Ra |

Figure 3.3 Biomarker inclusion and AUCs for 1 se lambda Glmnet models across 100x iterations for estimating IDSA severity or predicting adverse outcomes. (A) Table showing which biomarkers are included in each Glmnet model. Inclusion was determined by on 1) classification task (estimating IDSA severity or predicting adverse outcomes) and 2) the penalty for including additional low yield variables. Each model was performed across 100 iterations with different initial seeds for each value of alpha. An alpha value closer to 0 weights towards ridge regression and a value closer to 1 weights towards lasso regression. Lasso regression places a higher penalty on including additional biomarkers, resulting in fewer biomarkers included in the final model for higher alpha values. Color of each square indicates out of the 100 iterations how many times that individual biomarker was included in the produced models for the given alpha value. (B) Table showing the performance of the best alpha = 0.9 model and biomarkers included. (C) ROCs and AUCs for best alpha = 0.9 models.

(alpha=0.9) model includes 13 biomarkers and produces an AUC of 0.78 [0.71–0.84] (Figure 3.4).

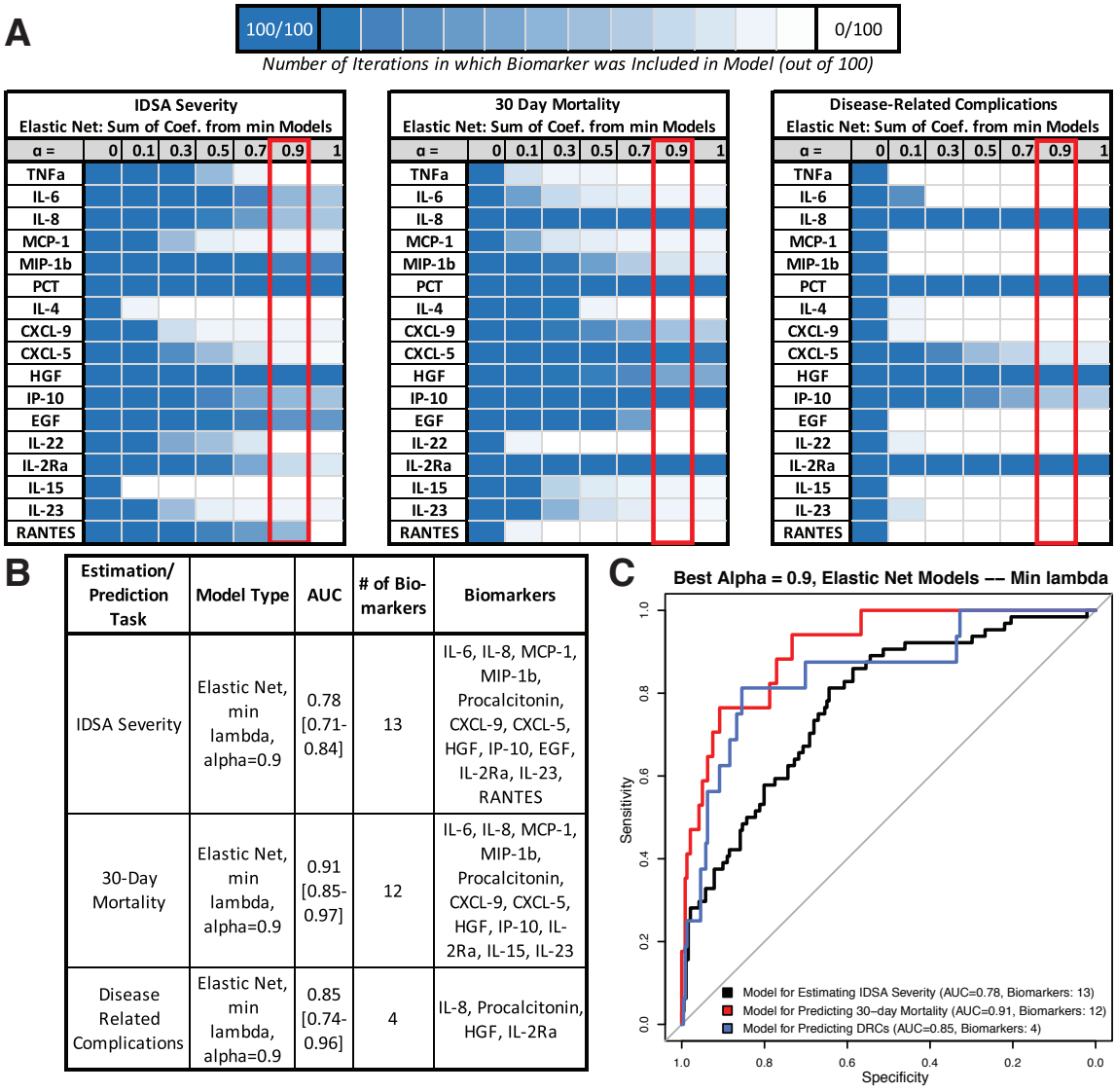
For 30-day mortality prediction, elastic net modeling shows that IL-8, PCT, IP-10, and IL-2R$\alpha$ are the most included biomarkers for 1se models and are included in all min models along with CXCL-5. The 1se (alpha=0.9) model includes 5 biomarkers and produces an AUC of 0.89 [0.84-0.95] (Figure 3.3), while the min (alpha=0.9) model includes 12 biomarkers and produces an AUC of 0.91 [0.85-0.97] (Figure 3.4).

For DRCs prediction, elastic net modeling shows that IL-8, PCT, HGF and IL-2R$\alpha$ were included in most 1se models and all min models. The 1se (alpha=0.9) model includes 4 biomarkers and produces an AUC of 0.84 [0.73-0.94] (Figure 3.3), while the min (alpha=0.9) model includes 4 biomarkers and produces an AUC of 0.85 [0.74-0.96] (Figure 3.4). The same biomarkers were included in both models, indicating that determining DRCs is highly dependent on these four markers.

Regardless of model parameters, performance was similar across the largest and smallest models for each outcome and had AUCs higher than the highest among individual biomarker regression models. PCT was the only shared biomarker between 30-day mortality and IDSA severity models. DRCs models included the two most significant biomarkers for IDSA severity (HGF and PCT) as well as two others found in 30-day mortality models (IL-2R$\alpha$ and IL-8). This indicates that the task of predicting DRCs has a solution that overlaps at least in part with estimating IDSA severity and predicting 30-day mortality. Similar to results from logistic regression modeling, the best performing models were for 30-day mortality, followed closely by DRC, and the worst performance was seen in models for estimating IDSA severity.

### 3.5.3 Biomarker-based models outperform basic clinical models for predicting 30-day mortality and DRCs

IDSA severity is used clinically to assess the severity of CDI and inform treatment, while the Elixhauser comorbidity index (Elixhauser), which was developed in order to predict mortality, is used as an aggregate measure of the burden of comorbid disease at baseline. We used IDSA severity and Elixhauser to estimate adverse outcomes and compare to our biomarker-based models. Simple logistic regression models showed that IDSA severity was significantly associated with 30-day all-




| Estimation/ Prediction Task | Model Type | AUC | # of Bio-markers | Biomarkers |
|---|---|---|---|---|
| IDSA Severity | Elastic Net, min lambda, alpha=0.9 | 0.78 [0.71-0.84] | 13 | IL-6, IL-8, MCP-1, MIP-1b, Procalcitonin, CXCL-9, CXCL-5, HGF, IP-10, EGF, IL-2Ra, IL-23, RANTES |
| 30-Day Mortality | Elastic Net, min lambda, alpha=0.9 | 0.91 [0.85-0.97] | 12 | IL-6, IL-8, MCP-1, MIP-1b, Procalcitonin, CXCL-9, CXCL-5, HGF, IP-10, IL-2Ra, IL-15, IL-23 |
| Disease Related Complications | Elastic Net, min lambda, alpha=0.9 | 0.85 [0.74-0.96] | 4 | IL-8, Procalcitonin, HGF, IL-2Ra |

Figure 3.4 Biomarker inclusion and AUCs for 1 min lambda Glmnet models across 100x iterations for estimating IDSA severity or predicting adverse outcomes. (A) Table showing which biomarkers are included in each Glmnet model. Inclusion was determined by on 1) classification task (estimating IDSA severity or predicting adverse outcomes) and 2) the penalty for including additional low yield variables. Each model was performed across 100 iterations with different initial seeds for each value of alpha. An alpha value closer to 0 weights towards ridge regression and a value closer to 1 weights towards lasso regression. Lasso regression places a higher penalty on including additional biomarkers, resulting in fewer biomarkers included in the final model for higher alpha values. Color of each square indicates out of the 100 iterations how many times that individual biomarker was included in the produced models for the given alpha value. (B) Table showing the performance of the best alpha = 0.9 model and biomarkers included. (C) ROCs and AUCs for best alpha = 0.9 models.

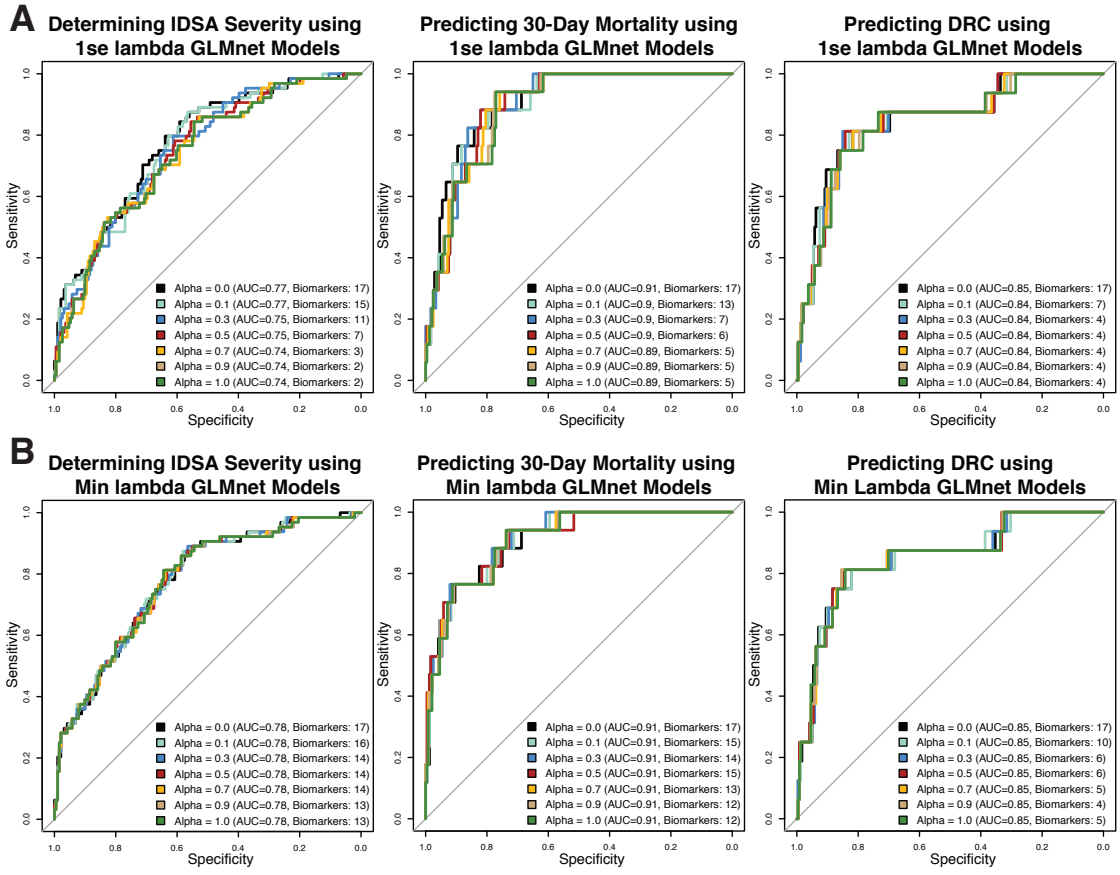



Figure 3.5 Stability of elastic net modeling for predicting adverse CDI outcomes and estimating IDSA severity with adjustment of lambda and alpha values. An alpha value closer to 1 weights towards lasso regression which increase the penalty of including more variables in the model. Each value of alpha was utilized in 100 model iterations across different initial seeds, with the best model by highest AUC chosen and plotted for (A) 1se lambda models and (B) min lambda for each outcome or estimation task. Min lambda models select the minimum value resulting in a more overfit model with increased number of biomarkers while the 1se lambda models select a value 1 standard deviation away resulting in a more generalizable model with fewer biomarkers. Comparing the same column in A and B, we see that while the AUC is higher in the min lambda models, there is a substantial increase in biomarkers included. As such, 1se models with an alpha value of 0.9 retain high AUC performance while limiting biomarker inclusion for improved clinical application.

cause mortality (P=.003, AUC= 0.67 [0.55-0.79]) and DRCs (P=.002, AUC= 0.69 [0.57-0.80], but performed substantially worse than our biomarker models (Figure 3.6a). Simple logistic regression models showed that Elixhauser index was significantly associated with 30-day all-cause mortality (P<.001, AUC= 0.77 [0.69-0.84]) and DRCs (P=.018, AUC= 0.71 [0.63-0.80]), but not with IDSA severity (P= .51, AUC= 0.53 [0.45-0.61]) and similarly performed worse than our biomarker-based models (Figure 3.6b).

The best biomarker-based elastic net model is able to improve the correct classification of 30-day-all-cause mortality cases at time of diagnosis when compared to the IDSA severity model for predicting 30-day all-cause mortality. This is demonstrated by a positive continuous Net Reclassification Improvement (NRI) (P=.022, NRI= 0.53 [ 0.078 - 0.98]) when comparing the two models. NRI ranges from -2 (100 percent of positives and 100% of negatives incorrectly reclassified) to plus 2 (100 percent of positives and 100% of negatives correctly reclassified), thus an NRI of 0.53 is a moderate improvement in classification of individuals with 30-day all-cause mortality by the biomarker-model over the baseline IDSA severity model.

To test if Elixhauser and IDSA severity would add additional information to the models, we incorporated Elixhauser and IDSA severity into the best elastic net biomarker-based models (Figure 2c-d) and into the best logistic regression models (Figure 3.7) for 30-day mortality and DRC. For alpha of 0.9, the AUC for the 1se model for 30-day mortality increased from 0.89 [0.84-0.95] to 0.91 [0.84-0.97], while the AUC for the 1se model for DRCs increased from 0.84 [0.74-0.95] to 0.87 [0.78-0.97] with the addition of IDSA severity and Elixhauser. In the 1se model for DRC did not include Elixhauser as a coefficient indicating poor predictive capability of that variable, while the addition of Elixhauser and IDSA severity resulted in procalcitonin not being included in the 1se model for 30-day all-cause mortality.

### 3.5.4 Multivariable, predictive models for 30-day mortality and DRCs do predict outcomes in a murine model of severe and non-severe CDI

We and others have developed murine models of CDI where experimentally infected animals will develop disease ranging from mild diarrhea to severe colitis. These murine models of CDI allow us to test our predictive biomarker models in a model organism that can develop similar disease but

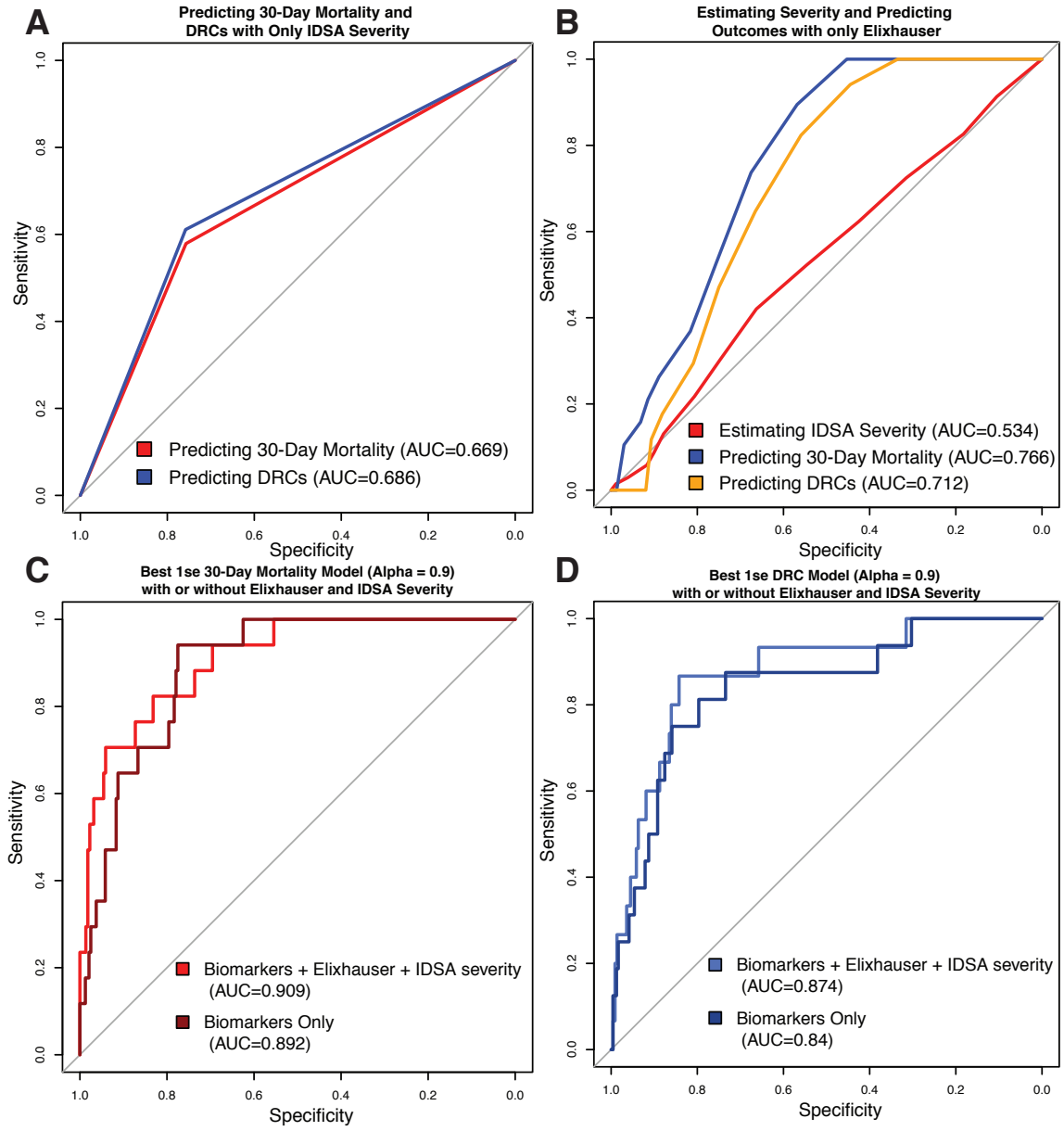



Figure 3.6 IDSA severity and Elixhauser perform worse than biomarker models, but slightly improve performance when added to biomarker models directly. (A) ROCs and AUCs for logistic regression using only IDSA severity to predict 30-day mortality and DRCs. (B) ROCs and AUCs for best 1se models using only Elixhauser score to predict 30-day mortality, DRCs, and IDSA severity. (C-D) ROCs and AUCs for predicting 30-day mortality and DRCs with best 1se elastic net biomarker model alone or with Elixhauser score and IDSA severity.

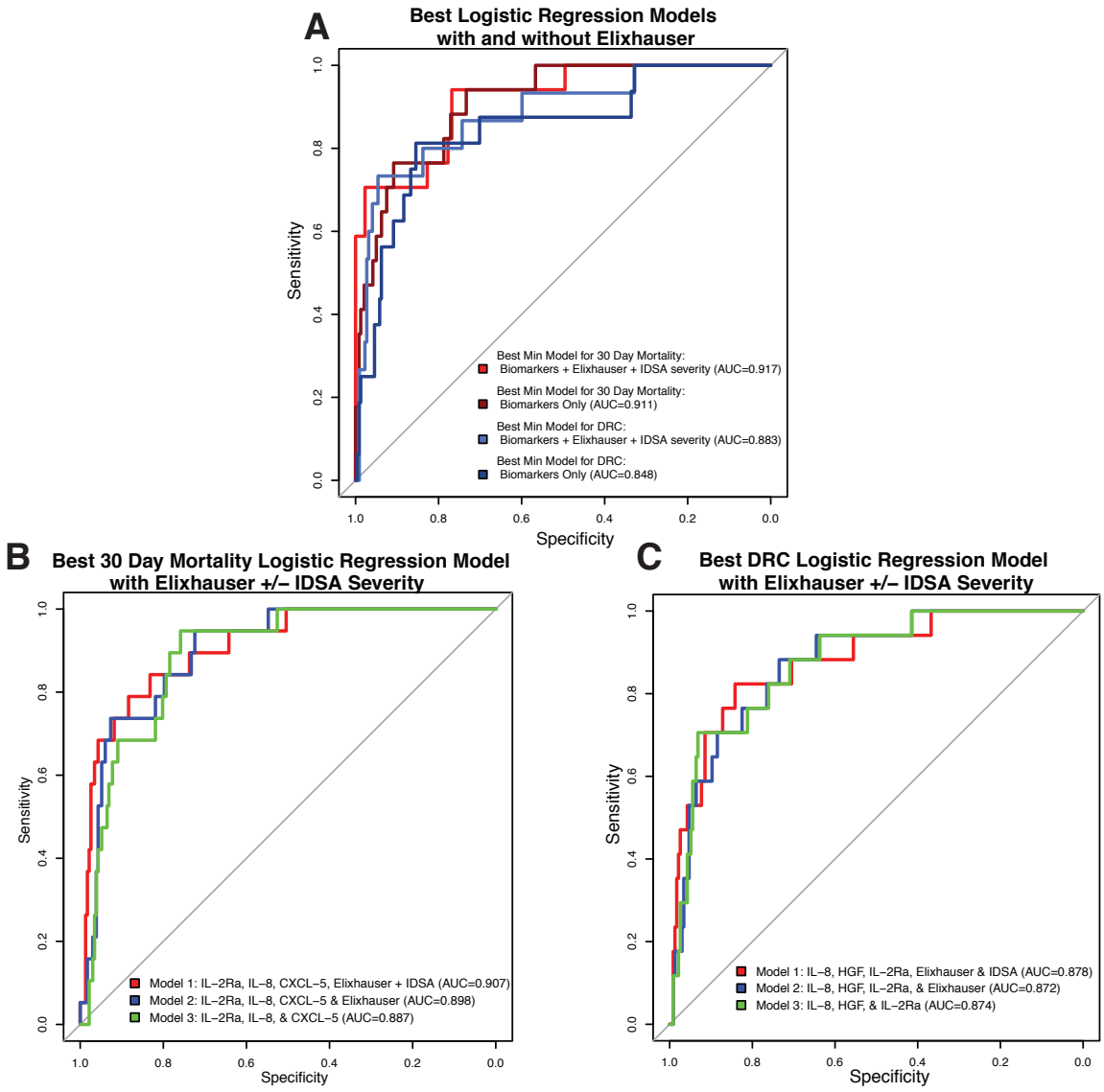



Figure 3.7 Inclusion of Elixhauser Score and IDSA Severity gives only slight improvements to best elastic net biomarker models. (A) ROC for min lambda elastic net models with alpha value of 0.9 for predicting 30-day mortality and DRC with and without the inclusion of Elixhauser and IDSA severity in addition to serum biomarkers. ROCs of the best biomarker-based logistic regression models with the addition of Elixhauser or Elixhauser + IDSA severity for (B) 30-day mortality and (C) DRC. While the addition of Elixhauser and IDSA severity increases AUC for the DRC model, it is not by a substantial margin. For 30-day mortality, the inclusion of Elixhauser and IDSA severity makes minimal improvement to the AUC.

lacks the potential comorbidities of human patients. We felt this was important to assess, since if our models perform well in an animal system, this gives support to the notion that our models are being fit towards biologically relevant biomarkers for CDI rather than comorbid disease or other confounding features that would not be present in an animal system.

For this validation, we used a CDI model employing antibiotic pretreatment followed by experimental infection with *C. difficile* spores. We have previously demonstrated that murine infection with VPI 10463 results in severe, rapidly fatal disease within 48 hours while infection with strain 630 results in a more indolent course (Theriot et al., 2011). For these experiments we employed 78 antibiotic-treated mice that were challenged with either strain 630g (37 mice), strain VPI 10463 (30 mice), or water (11 mice) and assessed serum responses with a murine version of our multiplex panel. VPI 10463 infected mice exhibited higher weight loss, more histopathologic intestinal damage, and higher clinical severity (Figure 3.8a-c and Figure 3.9). As such, we classify mice infected with VPI 10463 to have severe and fatal CDI, while those infected with 630g are classified to have mild and non-fatal CDI. The best models from our human cohort were applied to the mouse cohort (best 1se and min lambda models with alpha of 0.9 for IDSA severity, 30-day mortality, and DRCs). Descriptions of which biomarkers are included in each model are found in Panel B of Figure3.3 and Panel B of Figure 3.4. To apply the models to the mice serum data, each outcome (severity/mortality/DRCs) was defined as positive for VPI 10463 infected and negative for 630g infected mice (Figure 3.8d) or by a cutoff of weight loss, cecum histopathology score, or colon histopathology score as higher weight loss or histopathology represents more severe disease in mice CDI (Figure 3.10).

The 1se models for prediction of 30-day all-cause mortality and DRCs accurately identified mice infected with high virulence *C. difficile*. Specifically, applying each 1se model to the mouse cohort for high vs. low virulence infections revealed AUCs of 0.59 [0.44-0.74] for the models built for IDSA severity, 0.96 [0.91-1.0] for the models built for 30-day Mortality, and 0.85 [0.75-0.94] for the models built for DRCs.

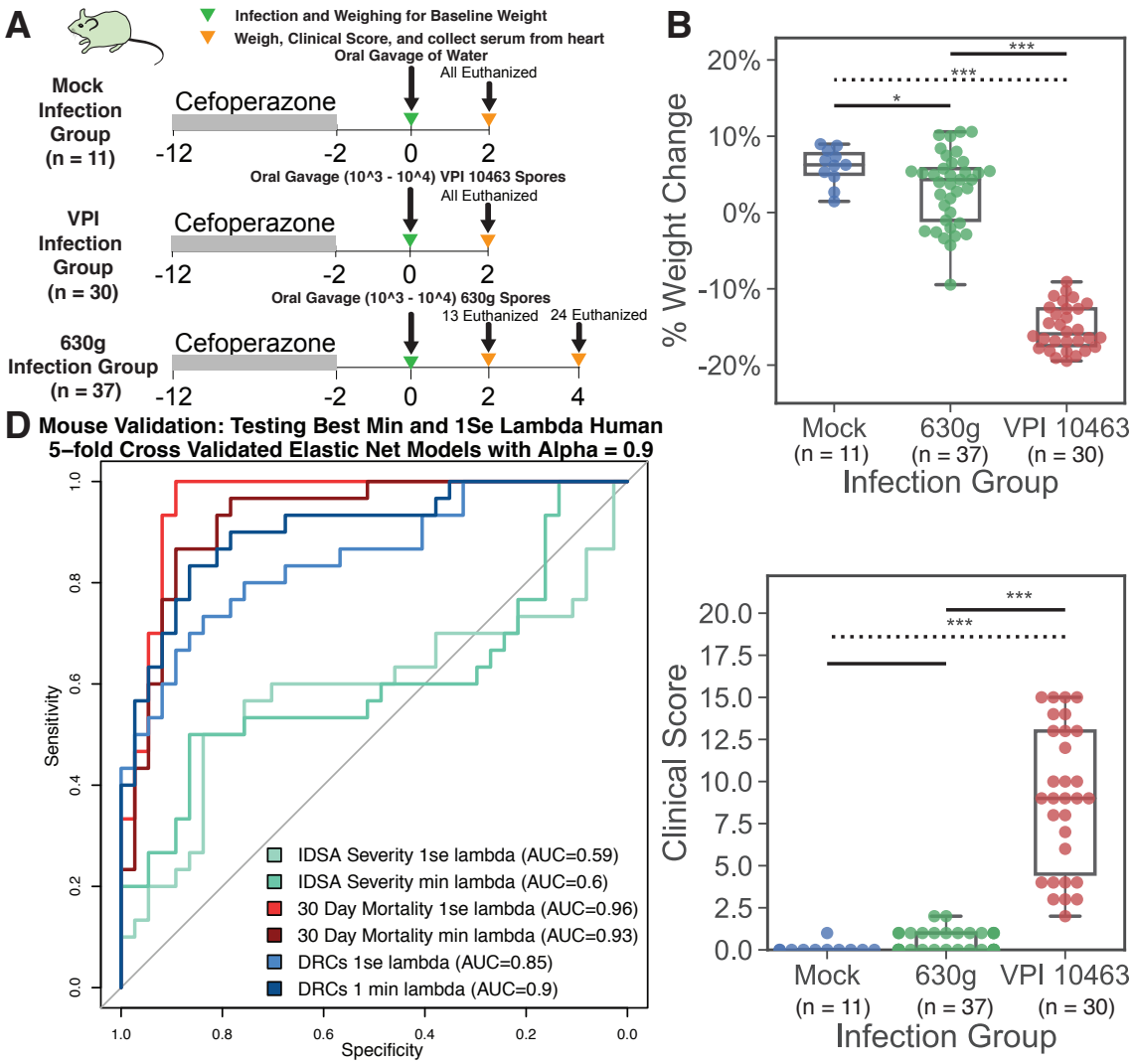



Figure 3.8 Mouse model of CDI to validates human CDI biomarker models. (A) Diagram showing method for mouse model of CDI. (B) Scatter plot showing weight change at day of euthanization compared to weight at day 0 for Mock, 630g infected, and VPI 10463 infected mice. (C) Scatter plot showing clinical score (Based on activity, coat, posture, diarrhea, and eyes/nose) at euthanization for Mock, 630g infected, and VPI 10463 infected mice. VPI 10463 infected mice have more weight loss and higher clinical scores compared to 630g infected mice (D) Mice infected with VPI were categorized as severe and those infected with 630g were categorized as non-severe CDI cases. The 1se and min lambda elastic net models with alpha = 0.9 for IDSA, 30-day mortality, and DRCs were applied to the mouse cohort with the resulting ROCs and AUCs. Weight change was analyzed using a t-test with a Bonferroni post-hoc adjustment and clinical scores were analyzed using a Mann–Whitney U test with a Bonferroni post-hoc adjustment, *p $<$ 0.05; **p $<$ 0.01, ***p $<$0.001.

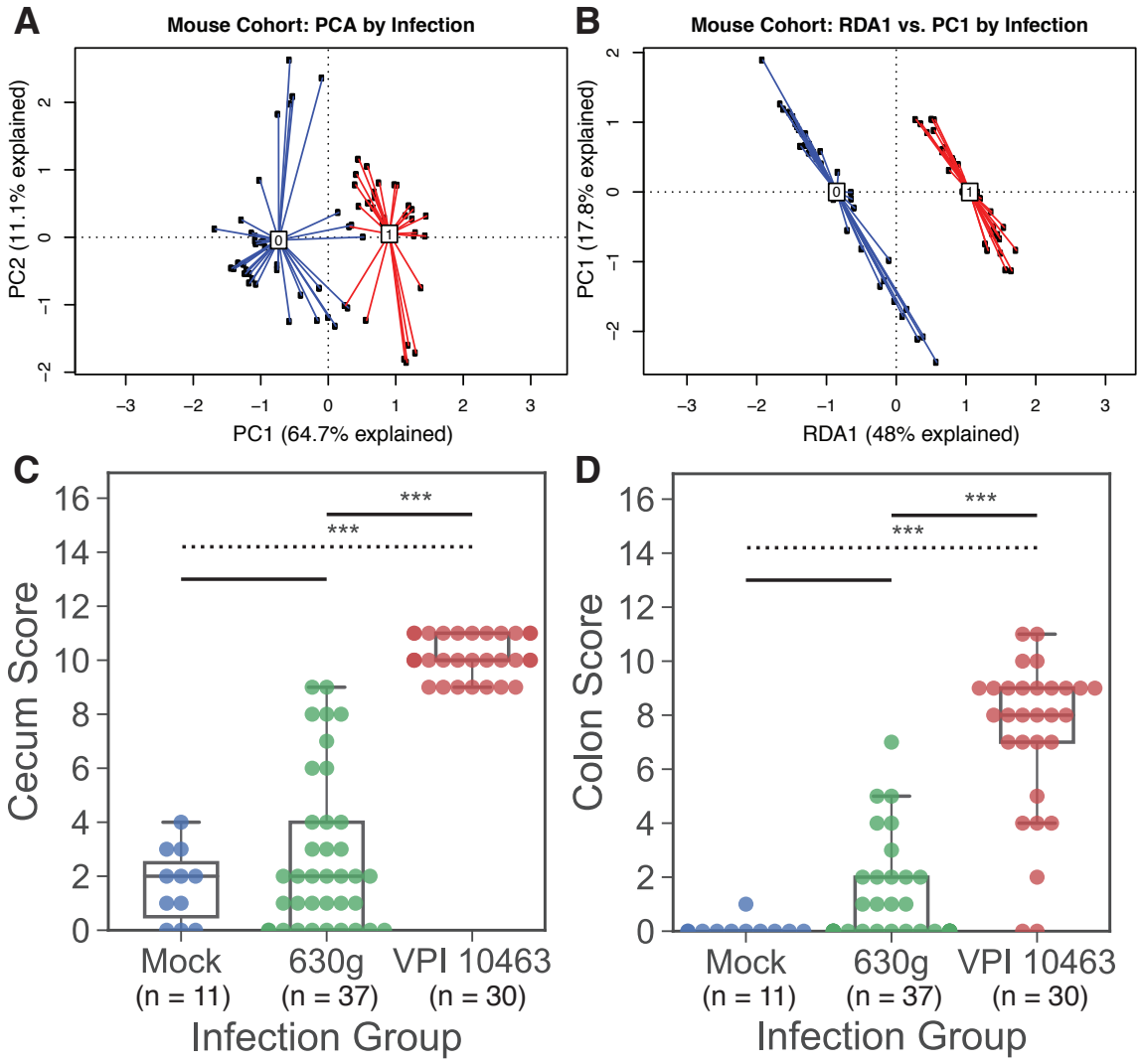



Figure 3.9 Unadjusted PCA analysis and histological damage in mouse model of CDI. (A) Inflammatory and Disease Severity PCA plot and (B) RDA analysis for 630g infected mice (0), categorized as having non-severe CDI infection, and VPI 10463 infected mice (1), categorized as having sever CDI infection. (C-D) Scatter plots showing (C) cecum score and (D) colon score based on the total score of edema (0-4), inflammatory cell infiltration (0-4) and epithelial damage (0-4) at euthanization for Mock, 630g infected, and VPI 10463 infected mice. VPI 10463 infected mice have higher cecum and colon damage compared to 630g infected mice. Cecum and colon scores were analyzed using a Mann–Whitney U test with a Bonferroni post-hoc adjustment, *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$.

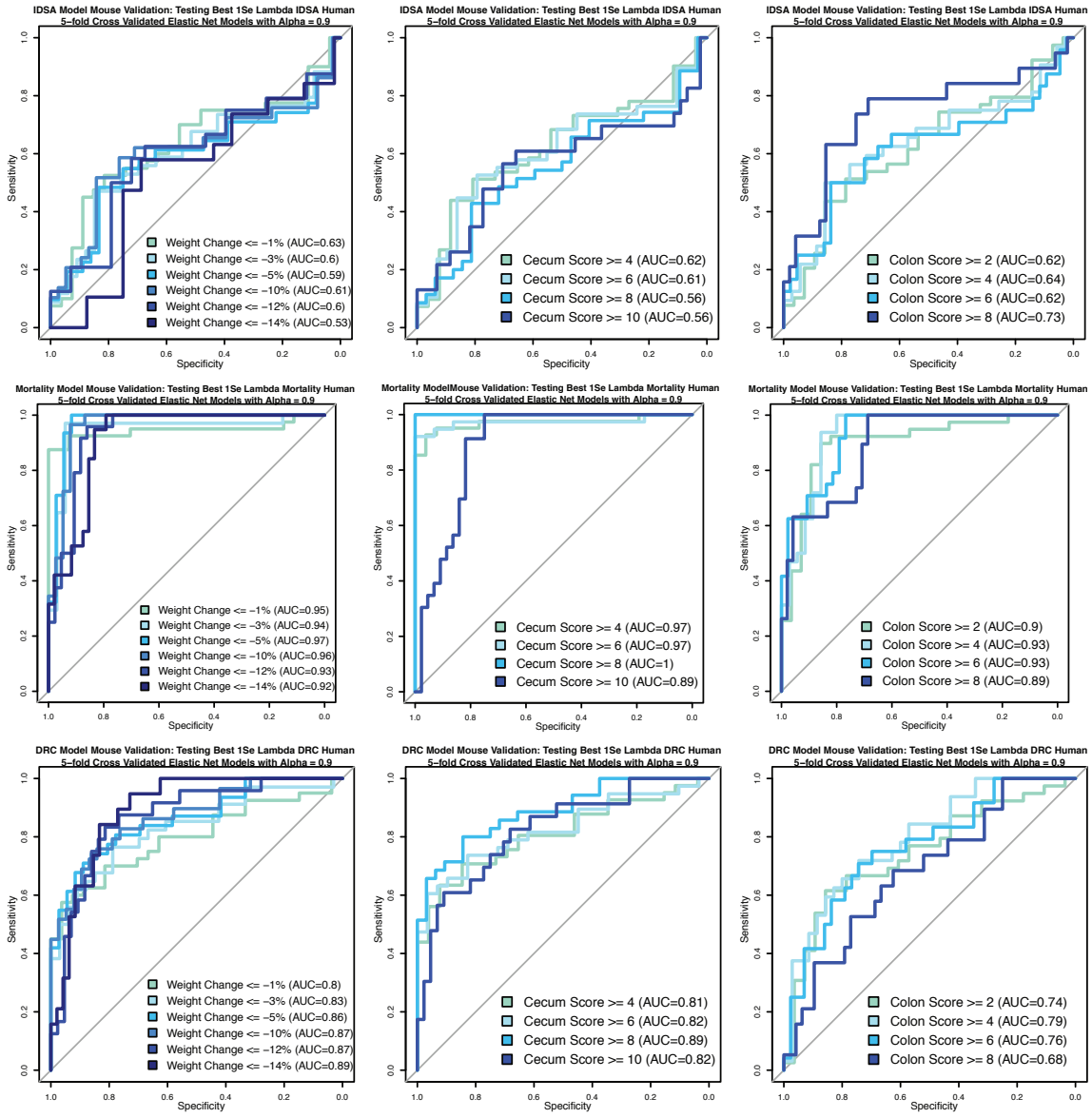



Figure 3.10 ROCs for best 1se lambda, alpha 0.9 models produced on human validation cohort applied to mouse cohort. Severity for each model was defined by a cutoff in weight loss, cecum score, or colon score. Top panels show results for IDSA model, middle panels show results from 30-day mortality model, and bottom panels show DRC model results. (Left) ROC for defining severity by weight change from day 0 to euthanization ranging from <1% to <14%. (Middle) ROC for severity defined as cecum score >=4,6,8, or 10. (Right) ROC for severity defined as colon score >=2,4,6, or 8. Cutoffs were chosen to range around median and mean of each metric. Overall, AUCs are stable across severity definitions.

## 3.6 Discussion

CDI is associated with an increased risk of mortality and, at present, we are inadequately determining who will experience adverse outcomes. Multiple models have been produced to address this problem including those utilizing electronic medical records, standard laboratory tests, and past medical history (van Beurden et al., 2017; Fujitani et al., 2011; Beauregard-Paultre et al., 2019; Archbald-Pannone et al., 2015; Butt et al., 2013; Kassam et al., 2016; Kulaylat et al., 2018). However, these models have met with limited success in external validation and there is room for improvement in predictive ability of CDI adverse outcomes. Additional studies have examined specific biomarkers in serum that could be associated with severe CDI, but no study to date has looked across a wide spectrum of serum-based biomarkers to determine their effectiveness of predicting cases of mortality or DRCs. Our results support the hypothesis that models built from a panel of multiple inflammatory mediators measured early in the course of CDI can accurately predict adverse outcomes and can do so better than current measures commonly used to predict adverse outcomes upon CDI diagnosis.

Our panel and model could be utilized at time of diagnosis to evaluate the risk of mortality for an individual patient. A negative result reduces the risk of 30-day-all-cause mortality, while a positive result increases mortality risk from 10% at baseline to 25%. Currently, the therapeutic options are limited in scope, but identifying a high-risk patient could tip the scale towards using more aggressive therapy such as colectomy. A secondary use of the panel could be to enable the study of therapies targeted specifically at reducing mortality in CDI, which otherwise are infeasible due to lack of statistical power. For example, if a study was being performed for a therapy against standard of care with a theoretical 30% reduction of mortality in the standard population with baseline 10% mortality risk with a targeted power of 80% and an alpha (i.e. type I error) of 0.05, 2700 patients would be required for the study. However, if our panel and model were used to identify only high-risk individuals that would be considered for enrollment, the population mortality risk would be increased to 25%, reducing the needed number of patients to 928 patients. This would decrease the number of required subjects by three-fold, substantially reducing cost and improving feasibility.

Validation is an important step in determining if a model is overfit to the particular cohort and/or confounding factors rather than the disease process itself. Utilizing murine CDI allowed us to test the models in a separate system without potentially confounding factors such as age, treatments, and co-morbidities. Our results show that the risk model of 30-day all-cause mortality and DRCs are related to the underlying biology of the infection as the models are also predictive of severe outcomes in murine CDI. Additionally, this provides additional support for the observation that murine CDI has a similar immune response to human CDI, supporting continued use of the animal model in the study of the biology of CDI.

Overall, our results confirm our hypothesis that a serum-based biomarker panel predicts adverse outcomes from CDI. Additionally, we show that models constructed from serum biomarkers outperform both IDSA severity criteria and Elixhauser comorbidity index for predicting adverse outcomes. As such, serum biomarker-based models could be used to inform medical decision-making for patients with CDI and this study has explored models from a range of modeling algorithms to inform which biomarkers are the most promising. Specific interest should be placed on continuing to study HGF, procalcitonin, IL-8, IL-2R$\alpha$, IP-10, and CXCL-5 as they were the most prevalent biomarkers selected in models of adverse outcomes from CDI in this study.

**Acknowledgements**

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# CHAPTER IV

# Aging dampens the intestinal innate immune response during severe *Clostridioides difficile* infection and is associated with altered cytokine levels and granulocyte mobilization

Results in this chapter were published in: *Lisa Abernathy-Close*, Michael G. Dieterle*, Kimberly C. Vendrov, Ingrid L. Bergin, Krishna Rao, Vincent B. Young. "Aging dampens the intestinal innate immune response during* Clostridioides *difficile infection and is associated with altered intestinal eosinophil mobilization." 2020, accepted at Infection and Immunity.* and are reproduced here with minor revisions. *Lisa Abernathy-Close and Michael G. Dieterle contributed equally to this work. Authors are in order of seniority.

## 4.1 Abstract

*Clostridioides* (formerly *Clostridium*) difficile is the most common cause of hospital-acquired infection, and advanced age is a risk factor for *C. difficile* infection. Disruption of the intestinal microbiota and immune responses contribute to host susceptibility and severity of *C. difficile* infection. However, the specific impact of aging on immune responses during *C. difficile* infection remains to be well described. This study explores the effect of age on cellular and cytokine immune responses during *C. difficile* infection. Young mice (2-3 months old) and aged mice (22-28 months old) were rendered susceptible to *C. difficile* infection with the antibiotic cefoperazone and then infected with *C. difficile* strains of varying disease-causing potential. We resolve that host age

and infecting *C. difficile* strain influenced the severity of disease associated with infection. Tissue-specific CD45+ immune cell responses occurred at the time of peak disease severity in the cecum and colon of all mice infected with a high-virulence strain of *C. difficile*; however, significant deficits in intestinal neutrophils and eosinophils were detected in aged mice, with a corresponding decrease in circulating CXCL1, an important neutrophil recruiter and activator. Interestingly, this lack of intestinal granulocyte response in aged mice during severe *C. difficile* infection was accompanied by a simultaneous increase in circulating white blood cells, granulocytes, and IL-17A. These findings demonstrate that age-related alterations in neutrophils and eosinophils and systemic cytokine and chemokine responses are associated with severe *C. difficile* infection and support a key role for intestinal eosinophils in mitigating *C. difficile*-mediated disease severity.

## 4.2 Introduction

In the last two decades, the frequency of *C. difficile* infection (CDI) among hospitalized patients has steadily increased, particularly among those 65 years of age and older (Leffler & Lamont, 2015). Several studies have demonstrated that as an individual's age increases, so does their risk of *C. difficile* infection and the severity of CDI-associated disease (Carignan et al., 2008; Lessa et al., 2012). While the connection between advanced age and severe CDI disease outcomes has been well established, the contribution of the aging host's immune responses during acute CDI disease development and pathogenicity of *C. difficile* strain remains to be clarified. Eosinophils are innate immune cells that predominantly reside in close proximity to microbes that colonize mucosal surfaces under non-inflammatory homeostasis (Travers & Rothenberg, 2015). The biological function of eosinophils in health and disease are most well studied and described in the protection against helminth infections (Huang & Appleton, 2016) and in the pathogenesis of allergy (Martin et al., 1996). There is now growing evidence supporting a previously under-appreciated role for eosinophils as important mediators of intestinal immune responses (Jung & Rothenberg, 2014), and the expression of a broad range of pattern-recognition receptors in eosinophils suggest a potential role in bacterial infection (Kvarnhammar & Cardell, 2012). Recent efforts by multiple research groups have indicated a role for eosinophils in CDI disease (Buonomo et al., 2016; Cowardin et al.,

2016; Kulaylat et al., 2018). However, the specific role for eosinophils in CDI disease severity has yet to be completely elucidated, and few studies characterize the innate immune responses to *C. difficile* strains with a range of virulence potential in animals of advanced age. CDI disease severity is influenced by host factors and the virulence of the *C. difficile* strain (Theriot et al., 2011). Aging is known to cause immune dysfunction and negatively impacts patients in the setting of infectious diseases in the intestine (Mabbott et al., 2015). While immunosenescence likely plays a role in modulating CDI outcomes (van Opstal et al., 2016; Shin et al., 2018), dysregulation of particular cytokine responses and immune cell subsets associated with advanced age may differentially contribute to CDI disease severity. In the present study, we characterize the effect of *C. difficile* strain virulence and host age on the cellular immune response using a murine model of CDI utilizing *C. difficile* strain 630 (low-virulence) and strain VPI 10463 (high-virulence), as well as a young cohort and an aged cohort of adult mice reared in the same animal facility.

## 4.3 Materials and Methods

### 4.3.1 Mice

Male and female specific pathogen-free (SPF) C57BL/6 wild-type adult mice that were young (2-3 months old) or aged (22-28 months old) were used in these studies. These mice were from a breeding colony at the University of Michigan that was originally founded with breeding stock obtained from the Jackson Laboratories in 2002. Euthanasia was carried out via CO2 inhalation at the conclusion of the experiment. Animal studies were approved by the Institutional Animal Care Use Committee (IACUC) at the University of Michigan and animal husbandry was performed in an AAALAC-accredited facility.

### 4.3.2 *C. difficile* strains and experimental murine infection

The *C. difficile* strains used in this study include reference strain VPI 10463 (ATCC 43255) and strain 630 (ATCC BAA-1382). The differential virulence of these two strains, with VPI 10463 causing rapidly fatal infection and strain 630 causing a more limited disease, has been previously described in a murine model of CDI by Theriot et al. (2011). Mice were rendered susceptible to

*C. difficile* infection by placing mice on 0.5 mg/mL cefoperazone (MP Pharmaceuticals) in sterile distilled drinking water (Gibco) ad libitum. The antibiotic-supplemented water was provided for 10 days, followed by 2 days of drinking water without antibiotics. Animals were then inoculated by oral gavage with $10^3$-$10^3$ colony-forming units (CFU) of *C. difficile* spores suspended in 20-100 $\mu$l of distilled water (Gibco) or mock-infected with vehicle alone. Viable spores in each inoculum was enumerated by plating for CFU per mL on pre-reduced taurocholate cycloserine cefoxitin fructose agar (TCCFA). TCCFA was prepared as originally described (George et al., 1979) with modifications. Briefly, the agar base consisted of 40 g of Proteose Peptone No. 3 (BD Biosciences), 5 g of Na2HPO4 (Sigma-Aldrich), 1 g of KH2PO4 (Fisher), 2 g NaCl (J.T. Baker), 0.1 g MgSO 4 (Sigma), 6 g fructose (Fisher), and 20 g of agar (Life Technologies) dissolved in 1L of Milli-Q water. The prepared medium was autoclaved and supplemented with a final concentration of 250 $\mu$g/mL D-cycloserine (Sigma-Aldrich), 16 $\mu$g/mL cefoxitin (Sigma-Aldrich), and 0.1% taurocholate (Sigma). Over the course of the experiment, mice were regularly weighed and cecal contents were collected for quantitative culture.

### 4.3.3 *C. difficile* quantification

Cecal contents were collected in a pre-weighed sterile tube from each mouse at time of euthanasia. Immediately following collection, the tubes were re-weighed to determine fecal weight and passed into an anaerobic chamber (Coy Laboratories). Each sample was then diluted 10% (w/v) with pre-reduced sterile PBS and serially diluted onto pre-reduced TCCFA plates with or without erythromycin supplementation. *C. difficile* strain 630 is erythromycin-resistant, whereas *C. difficile* strain VPI 10463 is sensitive to erythromycin. The plates were incubated anaerobically at 37 degrees Celsius, and colonies were enumerated after 18 to 24 hours of incubation.

### 4.3.4 Clinical disease severity scoring

Mice were monitored for clinical signs of disease. Disease scores were averaged based on scoring of the following features for signs of disease: weight loss, activity, posture, coat, diarrhea, eyes/nose. A 4-point scale was assigned to weight loss and activity, and a 3-point score was applied to each other feature and the sum of these scores (with 0 representing no signs of disease and 20 representing

signs of the most severe disease) determined the clinical disease severity score (Warren et al., 2012). Formalin-fixed tissue sections prepared from cecum and colon were HE stained and evaluated by a blinded animal pathologist. Histopathologic damage in each tissue was scored using epithelial destruction, immune cell infiltration, and edema on a 4-point scale for each category and the sum of these scores determined the histological score (Theriot et al., 2011; Reeves et al., 2011).

### 4.3.5 Lamina propria cell isolation

Cecum and colon were excised, separated, and the lumen was flushed. Residual fat was removed and tissues were opened longitudinally. Tissue was placed in pre-warmed RPMI medium containing 0.5M EDTA, dithiothreitol, and fetal bovine serum (FBS) and incubated at 37C on an orbital shaker at 150 rpm for 15 min. After incubation, a steel strainer was used to separate tissue pieces from the epithelium-containing supernatant. Tissue was minced in RPMI medium containing dispase, collagenase II, DNase I, and FBS and incubated at 37 degrees Celsius on an orbital shaker at 150 rpm for 30 min. Digested tissue was filtered through a 100 $\mu$m cell strainer followed by a 40 $\mu$m cell strainer. The resultant single cell suspensions were counted on a hemocytometer using trypan blue exclusion test.

### 4.3.6 Flow cytometry

Lamina propria single-cell suspensions from colon or cecum were incubated with anti-CD16/32 to reduce non-specific binding. Cells were incubated on ice for 30 mins in the dark, with a cocktail of fluorescent antibodies consisting of anti-CD45.2 PerCP-Cy5 (clone: 104), CD3 PE (clone: 145-2C11), CD11b PE-eFluor 610 (clone: M1/70), CD11c Alexa Fluor 700 (clone: N418), Ly6G PE-Cy7 (clone: 1A8), and Siglec-F Alexa Fluor 647 (clone: E50-2440). All antibodies were purchased from eBioscience, Biolegend, or BD Biosciences. Stained cells were incubated with an eFluor 450 fixable viability dye (eBioscience) and fixed with 0.5% paraformaldehyde. Cells were acquired using a BD LSRFortessa X-20 flow cytometer (BD Biosciences, San Jose, CA) and analyzed on FlowJo v10 software (Tree Star Inc., Ashland, OR).

### 4.3.7 White blood cell enumeration in blood

Blood was collected via cardiac puncture in microtainer tubes with K2 EDTA (Sarstedt, Nümbrecht, Germany) at the experimental endpoint. Blood samples were taken immediately to the Unit for Laboratory Animal Medicine In-Vivo Animal Core and processed for a complete blood count on an automated hematology analyzer (Hemavet 950, Drew Scientific, Miami Lakes, FL).

### 4.3.8 Serum preparation and cytokine analysis

Blood was collected via cardiac puncture utilizing a polymer gel-based separator tube (BD Microtainer SST) at the experimental endpoint. Tubes were centrifuged according to the manufacturer's instructions and serum was collected and stored at -80 degrees Celsius until use. Cytokine levels in the serum were measured using a Luminex Multiplex System (Invitrogen).

### 4.3.9 Statistics

One-way analysis of variance (ANOVA) with Tukey's or Sidak's post-hoc test for *C. difficile* burden, cell population, and serum cytokine analyses was performed using R or GraphPad Prism 8. Clinical, cecum, and colon summary scores were analyzed using a Kruskal-Wallis test and Dunn's post-hoc test using the R or GraphPad Prism 8. Clinical score as an outcome was modeled using multivariable linear regression modeling to minimize the sum of squares of the residuals using the R function lm. Clinical score was log-transformed and a significant interaction term was found between age and infecting strain as input variables. Standard errors for the mean clinical change for each condition were calculated using the deltamethod in the r package msm. A p-value less than 0.05 was considered statistically significant.

## 4.4 Results

### 4.4.1 The severity of clinical disease and intestinal histopathology associated with *C. difficile* infection as influenced by host age and infecting strain

Young mice (2-3 months old) and aged mice (22-28 months old) were rendered susceptible to *C. difficile* infection (CDI) by treatment with the antibiotic cefoparazone prior to oral challenge

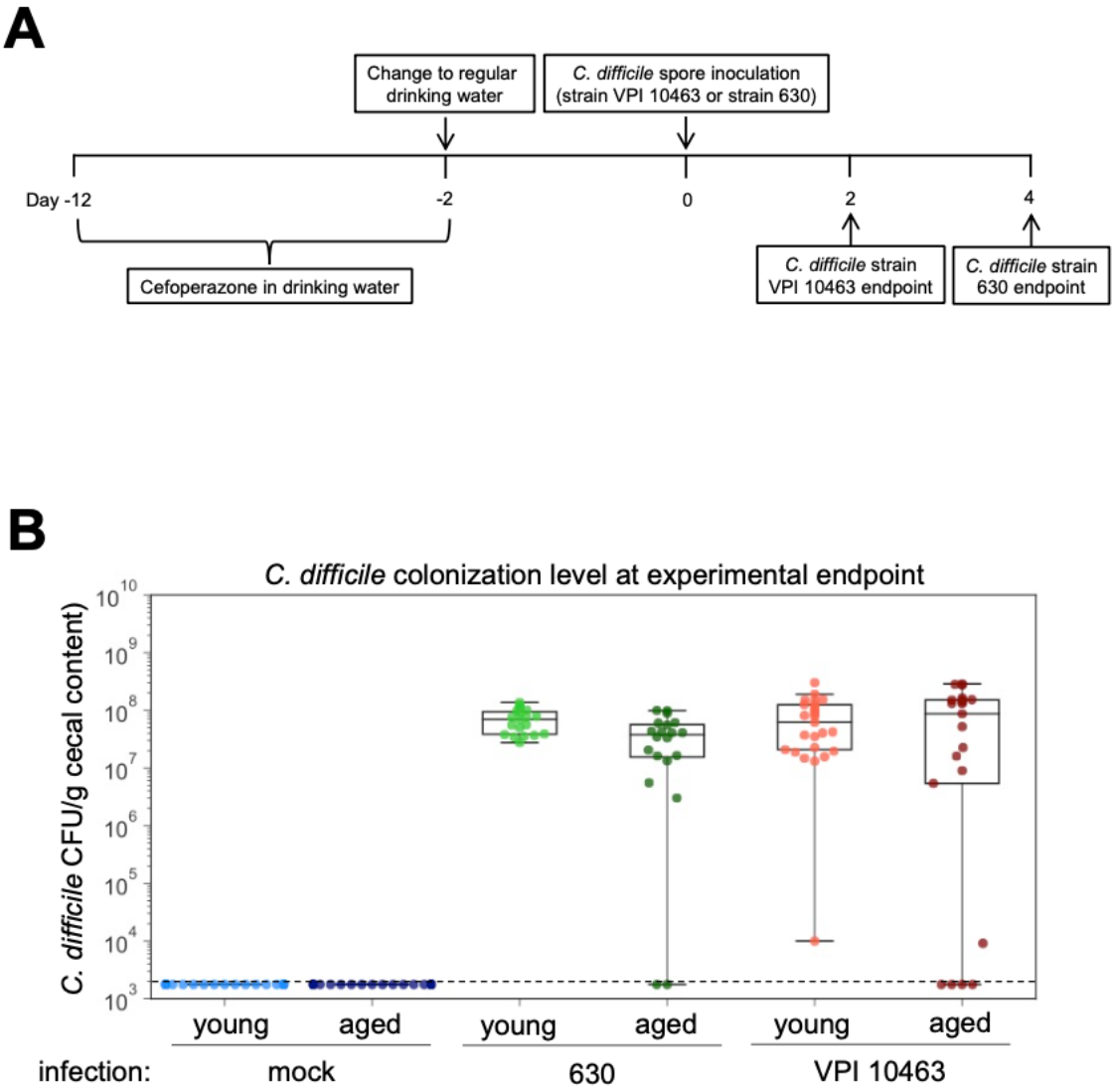


Figure 4.1 Host age does not significantly impact *C. difficile* colonization. Panel A) Mouse model of *C. difficile* infection with a low-virulence strain (630) or a high-virulence strain (VPI 10463) in young and aged animals. Panel B) Cecal contents were collected from young mice and aged mice at the time of peak disease severity (day 2 post-infection with strain VPI 10463 or day 4 post-infection with strain 630) or after mock infection with vehicle and plated anaerobically on selective agar plates to quantify *C. difficile* burden. Dotted line indicates limit of detection for *C. difficile* quantification ($10^3$ CFU).

with spores derived from *C. difficile* strain 630 (low virulence) or strain VPI 10463 (high virulence) (Figure 4.1, Panel A). Samples utilized in this study were obtained from animals at the time of peak clinical disease severity; day 2 post-infection with *C. difficile* strain VPI 10463 and day 4 post-infection with *C. difficile* strain 630 (Theriot et al., 2011). Day 2 post-infection was chosen for infection with VPI 10463 as infected mice succumb to disease shortly afterwards, hindering collection of viable tissue for subsequent cellular analyses. There was no age-associated difference in *C. difficile* colonization in mice infected with strain VPI 10463 or strain 630 (Figure 4.1, Panel B). We confirmed that *C. difficile* strain VPI 10463 causes significantly worse disease, compared to strain 630, regardless of age (Figure 4.2, Panel A; $p < 0.0001$ for young mice and $< 0.01$ for aged mice). At the time of peak clinical disease, two factors significantly contributed to developing severe disease: infecting strain of *C. difficile* and age. Linear regression modeling demonstrated that both advanced age and infection with *C. difficile* strain VPI 10463 interacted to increase clinical score. Therefore, the largest difference in infected mice was seen between young mice infected with strain 630 compared to aged mice infected with strain VPI 10463 (mean change in clinical score of 7, 95% CI [5 – 10]) (Figure 4.2, Panel B; p-values for regression modeling individual effects reported in Table 4.1). In addition to assessing overt clinical disease, we explored whether aging modifies the degree of intestinal tissue pathology during *C. difficile* infection. Histopathology in the cecum (Figure 4.2, Panel C) and colon (Figure 2D) of young and aged mice was quantified by a veterinary pathologist that scored tissues for the amount of edema, infiltration of leukocytes, and epithelial damage. Cecum and colon histopathology scores were similar between young and old at baseline as well as during CDI with strain 630 (Figure 4.2, Panels C and D), corresponding with the minimal clinical disease observed with this strain of *C. difficile* (Figure 4.2, Panel A). In contrast, infection with *C. difficile* strain VPI 10463 was associated with a significantly higher degree of cecum ($p < 0.0001$) and colon ($p < 0.0001$ for young mice and $p < 0.01$ for aged mice) histopathology, regardless of age (Figure 2C and 2D). Interestingly, mice of advanced age responded with significantly less colonic histopathology during infection with *C. difficile* strain VPI 10463 when compared to young mice (Figure 4.2, Panel D; $p < 0.01$).

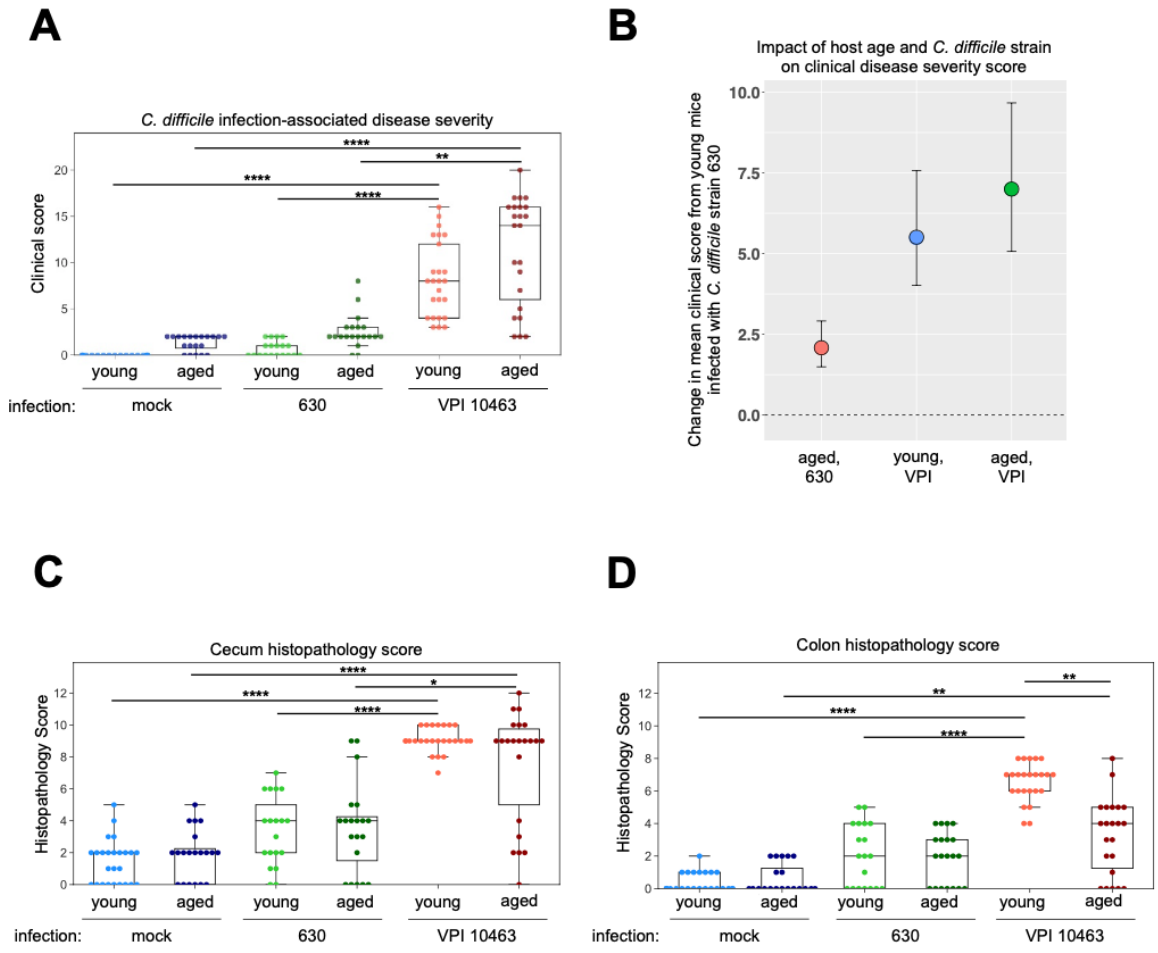


Figure 4.2 *C. difficile* strain VPI 10463 causes more severe CDI compared to strain 630, regardless of age. A) Clinical scores of young and aged mice infected with *C. difficile* strain VPI 10463 or strain 630 at peak clinical disease severity (day 2 and 4 post-infection, respectively). B) Mean change in clinical score with the 95% confidence interval compared to young mice infected with 630. Data are arranged by magnitude of clinical score change and modeling coefficients are shown in Table 4.1. Histopathology scores for C) cecum and D) colon tissues collected from young and aged mice. Epithelial destruction, immune cell infiltration, and edema were scored on a 4-point scale for each category and the sum of these scores determined the histological score in each tissue. Kruskal-Wallis one-way ANONA with Dunn's post-hoc test, *p $<$ 0.05; ***p $<$0.001.

### 4.4.2 Age and infecting *C. difficile* strain influence the cellular immune response in the intestine of mice

Total immune cells and myeloid cells subsets in the lamina propria of cecum and colon from young and aged mice were analyzed by flow cytometry at the time of peak disease severity (representative plots in Figure 4.3, Panels A and C). *C. difficile* strain 630 did not elicit an early cellular intestinal immune response in the cecum (Figure 4.3, Panels B and C) or colon (Figure 4.3, Panels E and F), independent of age. Due to the absence of a local intestinal cellular immune response during infection with the low-virulence *C. difficile* strain 630, we focused on further characterizing the nature of the immune cells infiltrating the distal intestinal tract during infection with the more virulent *C. difficile* strain VPI 10463.

Of the CD45+ immune cells in the cecum and colon young and aged mice infected with *C. difficile* strain VPI 10463 at the time of peak disease severity, the majority are CD11b+ cells (Figure 4.4 panels A and B). While there is a significant increase in CD11b+ cell numbers in the cecum of young ($p < 0.0001$) and aged mice ($p < 0.01$) infected with *C. difficile* VPI 10463, this response is significantly blunted in aged mice (Figure 4.4 panels C and D; $p < 0.001$). Interestingly, we observed local differences in intestinal CD11b+ cell responses in aged mice. There was a lack of response by CD11b+ cells in the colon of aged mice infected with *C. difficile* VPI 10463, in contrast to a significant increase in colonic CD11b+ cells in young counterparts (Figure 4.4 panels E and F; $p < 0.0001$). Similarly, subsets of CD11b+ myeloid cells including CD11b+Ly6G+Siglec-F- neutrophils and CD11b+Ly6G-Siglec-F+ eosinophils showed an age-dependent difference in intestinal response to *C. difficile* infection (Figure4.5). While aged mice indeed mount a cecal neutrophil response during severe CDI ($p < 0.01$), it is significantly dampened compared to their young counterparts (Figure 4.5, Panel C; $p < 0.01$). Furthermore, colonic neutrophil infiltration during CDI is not observed in aged mice during severe CDI (Figure 4.5, Panel C.

**Figure 3**

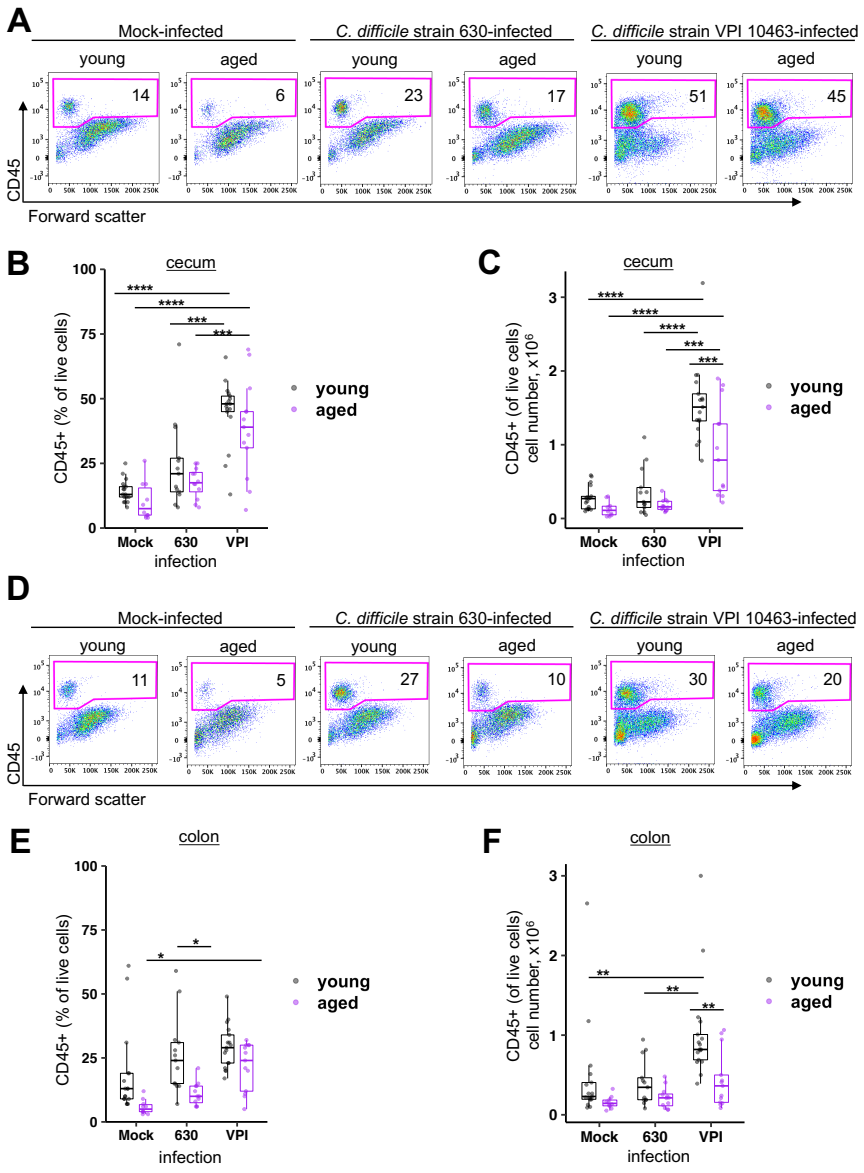


Figure 4.3 CD45+ leukocyte behavior in cecum and colon. CD45+ leukocytes are preferentially increased in the cecum and colon lamina propria of young and aged mice infected with *C. difficile* strain VPI 10463, compared to infection with *C. difficile* strain 630. Mice were mock-infected with vehicle or infected with *C. difficile* strain VPI 10463 or strain 630 and total immune cells were quantified in the cecum and colon at the time of peak disease severity. A) Representative flow cytometry plots indicating the percentage of CD45+ leukocytes in cecum lamina propria. B) Percentage and C) absolute number of CD45+ leukocytes in total live lamina propria cells harvested from cecum. D) Representative flow cytometry plots indicating the percentage of CD45+ leukocytes in colon lamina propria. E) Percentage and F) absolute number of CD45+ leukocytes in total live lamina propria cells harvested from colon. ANOVA and Tukey's test *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

**Figure 4**

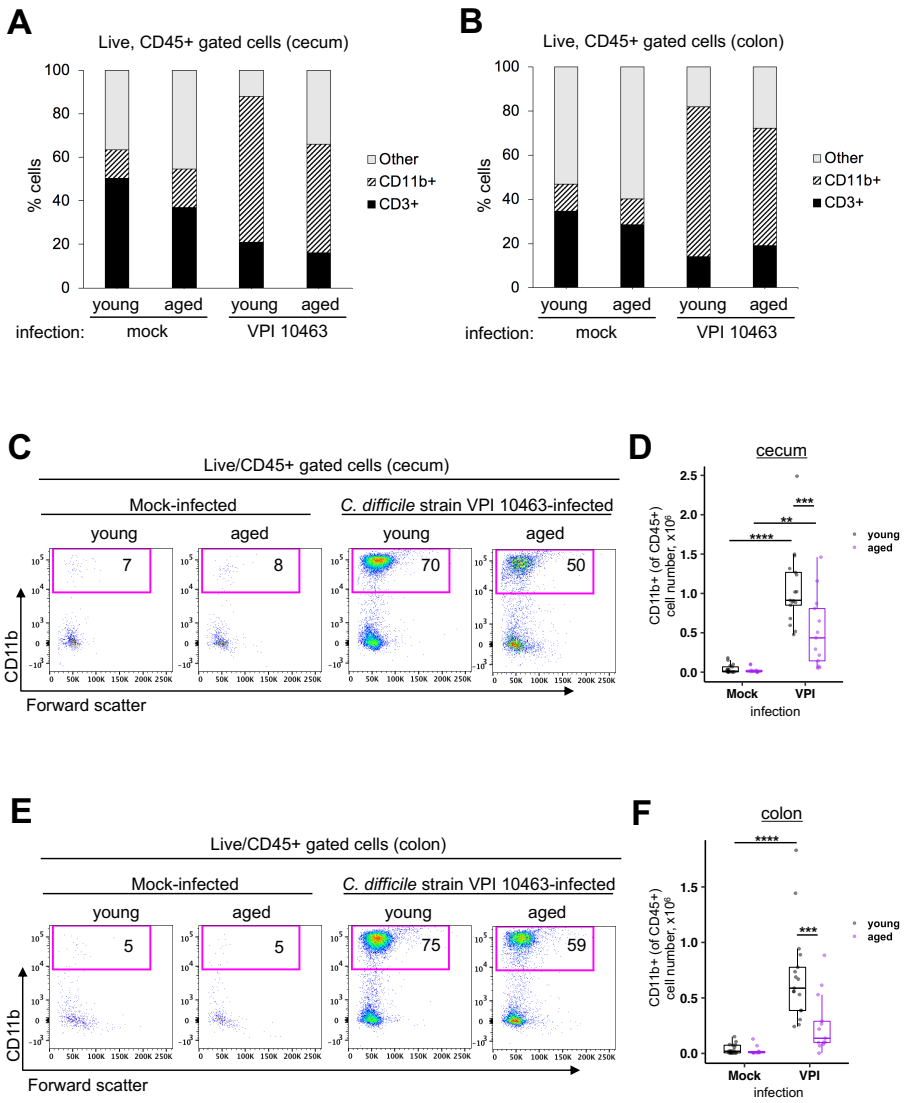


Figure 4.4 The dominant immune cell type in the cecum and colon lamina propria of young and aged mice. CD11b+ cells are the dominant immune cell type in the cecum and colon lamina propria of young and aged with severe CDI. Mock-infected or *C. difficile* strain VPI 10463-infected young and aged mice 2 days post-infection analyzed by flow cytometry for immune cell subsets. The ratio of CD11b+ cells, CD3+ lymphocytes, and "other" CD11b- CD3- cells of the total CD45+ immune cells in A) cecum and B) colon of mock or *C. difficile* strain VPI 10463-infected young and aged mice 2 days post-infection. C) Representative flow cytometry plots indicating the percentage of CD11b+ myeloid cells in colon lamina propria. D) Absolute number of CD11b+ cells determined by flow cytometry in cecum. E) Representative flow cytometry plots indicating the percentage of CD11b+ cells in colon lamina propria. F) Absolute number of CD11b+ cells determined by flow cytometry in colon. ANOVA and Tukey's test *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

**Figure 5**

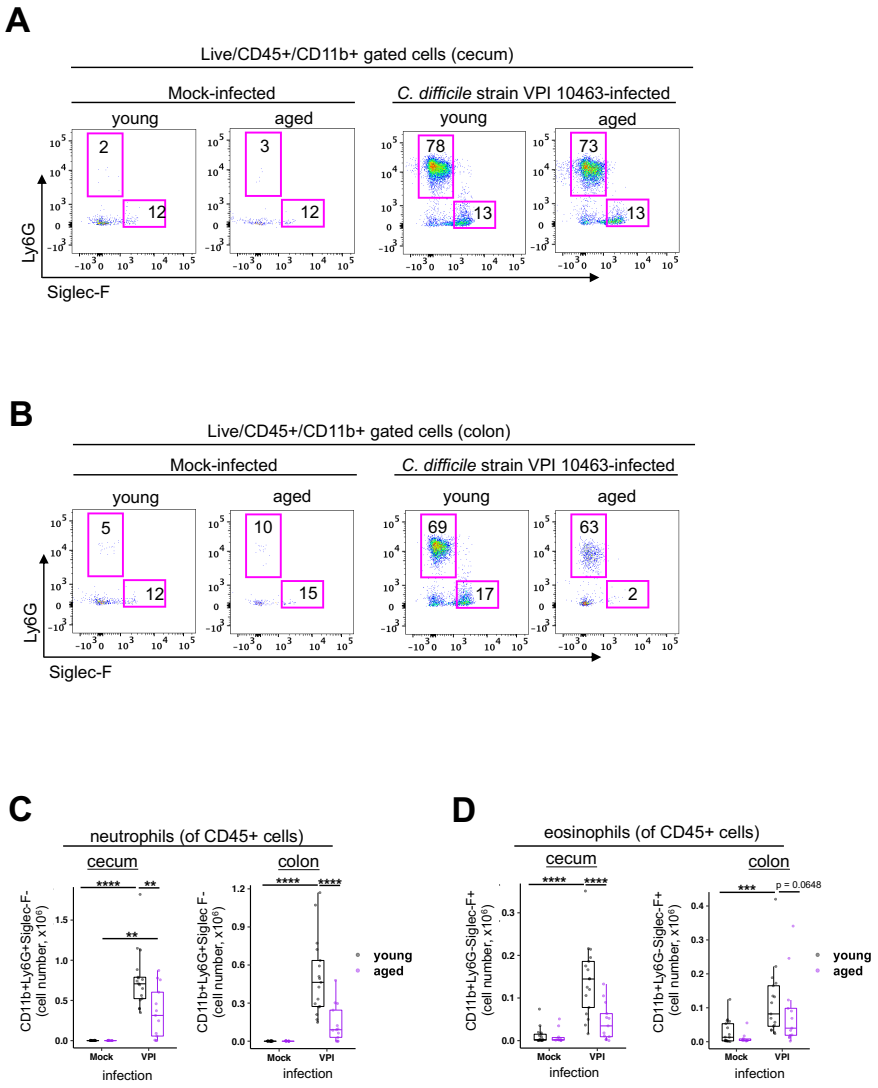


Figure 4.5 Neutrophil and eosinophil infiltration in the distal intestine is significantly decreased in aged mice with severe CDI, compared to young counterparts. Mock-infected or *C. difficile* strain VPI 10463-infected young and aged mice 2 days post-infection analyzed by flow cytometry for CD11b+Ly6G+Siglec-F- neutrophils and CD11b+Ly6G-Siglec-F+ eosinophils. Representative flow cytometry plots indicating the percentage of CD11b+Ly6G+Siglec-F- neutrophils and CD11b+Ly6G-Siglec-F+ eosinophils in A) cecum and B) colon lamina propria. C) Absolute numbers of neutrophils in the cecum and colon lamina propria. D) Absolute eosinophil number in cecum and colon lamina propria. ANOVA and Tukey's test *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

### 4.4.3 Differential intestinal and systemic neutrophil response and eosinophil cellular response during severe *C. difficile* infection in aged mice

Granulocytes, including neutrophils and eosinophils, are important cellular mediators of innate immunity. Since it has been suggested that eosinophils play a protective role in CDI using a mouse model of *C. difficile* infection (Buonomo et al., 2016), we hypothesized that eosinophil responses in older animals during *C. difficile* infection would differ significantly compared to their relatively young counterparts. Young mice at the time of peak CDI disease severity mounted a robust cecal and colonic eosinophil cellular response; however, intestinal eosinophil infiltration during peak CDI disease severity is absent in aged mice infected with *C. difficile* strain VPI 10463 (Figure 4.5, Panel D). We sought to determine if there was an age-related difference in peripheral white blood cells, with a focus on neutrophil and eosinophil responses during *C. difficile* infection (Figure 4.6). We found that aged mice infected with the high-virulence *C. difficile* strain VPI 10463 respond with increased eosinophil to total leukocyte ratio (Figure 4.6, Panel C; $p < 0.01$) compared to young mice infected with the same strain of *C. difficile*. Although there is a complete lack of local eosinophil infiltration in the distal intestine of aged mice with severe CDI, there is a concomitant significant increase in the absolute number of peripheral eosinophils in the blood of aged mice infected with *C. difficile* strain VPI 10463 compared to their young counterparts (Figure 4.6, Panel C; $p < 0.01$). In contrast, young mice do not demonstrate a change in peripheral blood eosinophil levels at the peak of CDI severity, regardless of infecting *C. difficile* strain (Figure 4.6, Panel C).

### 4.4.4 Age alters systemic cytokines and chemokine levels during severe *C. difficile* infection

We sought to determine if age affected particular circulating cytokine and chemokine responses associated with granulocytes during severe *C. difficile* infection (Figure 4.7). Severe CDI induced significant increases of serum CCL11 ($p < 0.0001$), IL-17A ($p < 0.0001$), and CXCL1 ($p < 0.0001$) in both young and aged mice. Interestingly, in the setting of severe CDI, IL-17A levels were significantly elevated ($p < 0.01$) and CXCL1 levels were significantly reduced ($p < 0.0001$) in aged mice compared to young mice. In addition, while serum levels of IL-5 were significantly elevated

**Figure 6**

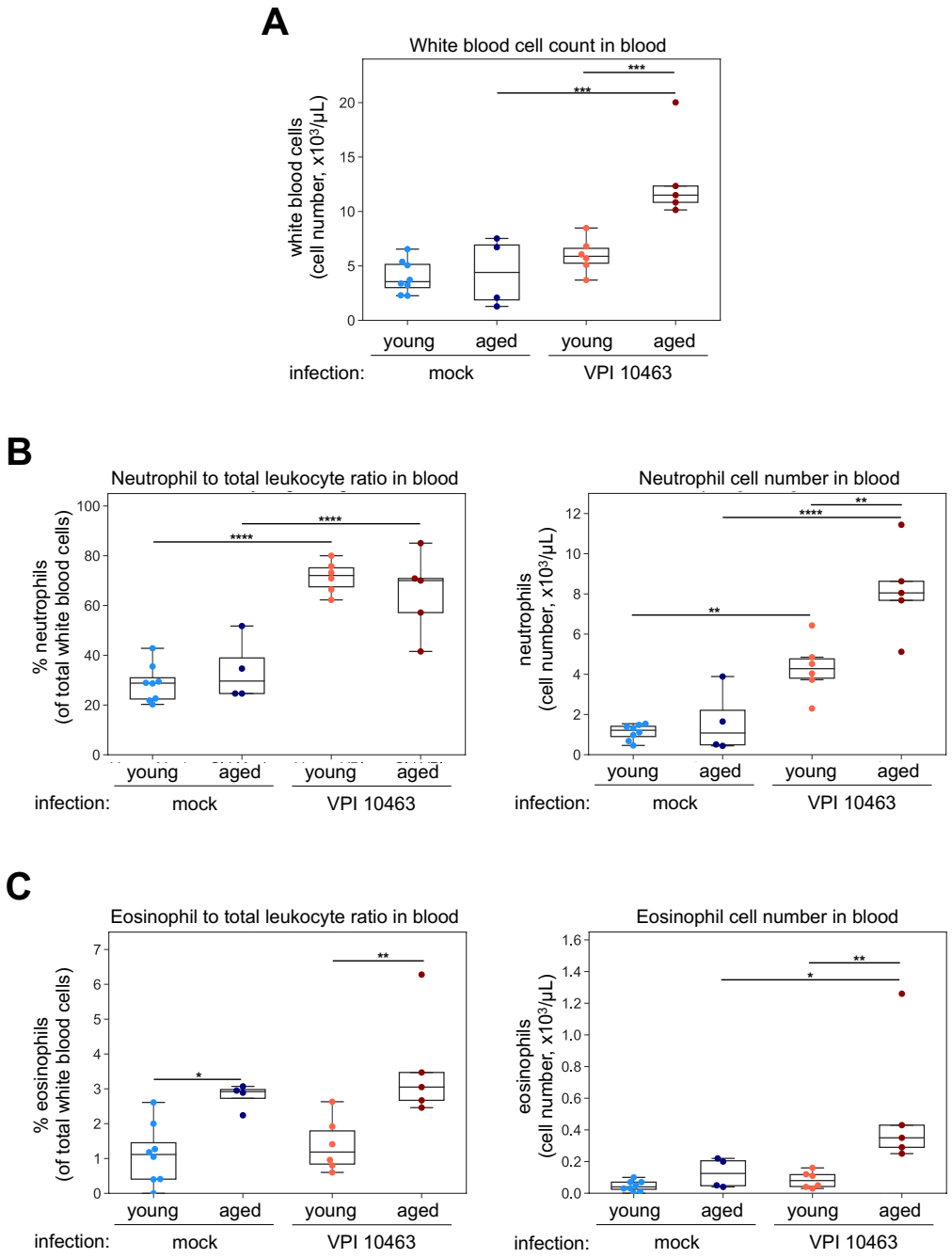


Figure 4.6 Severe CDI results in differential systemic neutrophil and eosinophil responses in aged mice, compared to young mice. A) Number of white blood cells in mock-infected or *C. difficile* strain VPI 10463-infected young and aged mice 2 days post-infection young and aged mice. Frequency and absolute numbers of B) neutrophils and C) eosinophils in young and aged mice at baseline or during peak CDI disease severity. ANOVA and Sidak's test *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

in young mice during severe CDI ($p < 0.0001$), the amount of IL-5 in aged mice with severe CDI was not statistically noticeable when compared to age-matched controls (Figure 4.7). We found no measurable age-related difference in CDI-induced IL-13 in the serum of young or aged mice.

## 4.5 Discussion

Although advanced age is a risk factor for *C. difficile* infection (CDI) (Leffler & Lamont, 2015), the relationship between the aging immune system and *C. difficile* infection is not well known. This study demonstrates an overall decrease in intestinal innate immune responses during acute *C. difficile* infection in mice with advanced age, and identifies specific aging-related alterations in neutrophil and eosinophil responses during CDI. We show that mice of advanced age (22 – 24 months old) and infection with a highly virulent strain of *C. difficile* (VPI 10463) develop more severe CDI disease and mount a significantly blunted intestinal cellular immune response compared to young mice (2 – 3 months old), with a notable absence of an intestinal eosinophil response. Young animals have a robust cellular intestinal immune response during acute CDI, whereas aged mice have a blunted intestinal neutrophil and eosinophil cellular response to *C. difficile* infection with concomitant peripheral neutrophilia and eosinophilia. Recently, peripheral eosinophil counts were found to be predictive of CDI disease severity and mortality in patients (Kulaylat et al., 2018), and eosinophils were shown to potentially be protective in mouse models of CDI (Buonomo et al., 2016; Cowardin et al., 2016). Interestingly, aged mice had a significant peripheral eosinophil response whereas young mice lacked a detectable increase in circulating eosinophils during severe CDI. Our results also suggest that eosinophils may play differential roles, whether that be protective or pathogenic. Additionally, eosinophil counts may predict different disease outcomes during *C. difficile* infection depending on their location in intestinal tissue or circulation in the periphery. In the present study, we characterized the intestinal cellular immune response to *C. difficile* infection during peak disease severity in young and aged mice, with a focus on myeloid cell subsets mobilized during the innate immune response. While the aging immune system is known to increase risk for CDI, the protective or pathogenic contributions of particular immune responses to disease outcomes are currently being explored in more detail. Peniche et al. showed that middle-aged mice (12 – 14

**Figure 7**

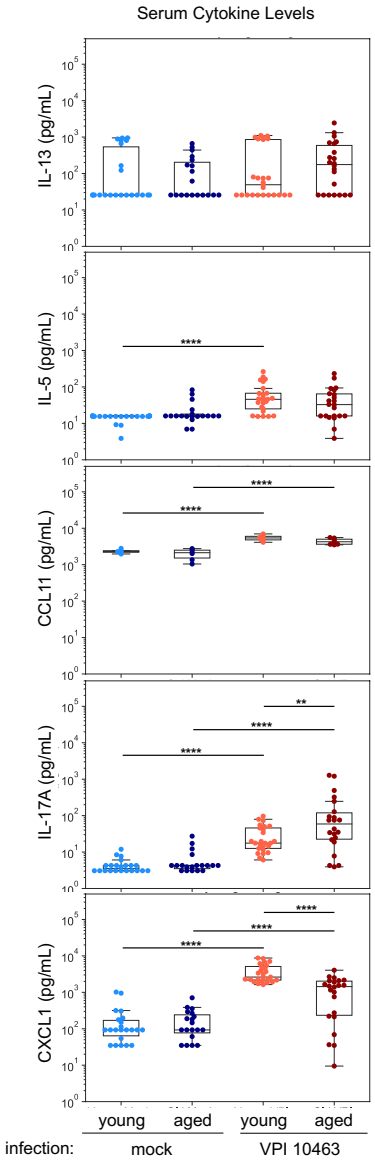


Figure 4.7 Aged mice mount altered systemic cytokine responses during severe CDI compared to young mice. Select cytokine and chemokine levels in the serum of young and aged mice mock-infected or 2 days post-infection with *C. difficile* strain VPI 10463 were quantified using a Luminex Multiplex System. ANOVA and Sidak's test *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

months old) have increased susceptibility to *C. difficile* infection and worse disease, compared to young controls (Peniche et al., 2018). They report that this observation was driven by impaired innate immune responses; however, eosinophils were not evaluated in this study. Our data showing a decreased intestinal neutrophil response in aged mice infected with *C. difficile* agrees with a recent study that examined the effect of age on *C. difficile* infection in a mouse model (Shin et al., 2018). Type 2 immune responses include IL-5, IL-13, and C-C motif chemokine 11 (CCL11), also known as eosinophil chemotactic protein and eotaxin-1, while CXCL1 and IL-17A are associated with Type 3 immune responses. Our results demonstrate an age-related defect in IL-5 and CCL11, which are responses associated with eosinophil recruitment. Although IL-17 is known to induce CXCL1, we measured significantly lower levels of CXCL1 in the serum of aged mice with severe CDI compared to young mice. The contribution of Type 2 and Type 3 immune responses to *C. difficile* pathogenesis and infection is an active area of research (Donlan & Petri, 2020; Saleh & Petri, 2019). Our results add to the growing evidence that eosinophils play a critical role in the pathogenesis of CDI. One study demonstrated that *C. difficile* binary toxin suppresses a host protective colonic eosinophil responses in a toll-like receptor 2 (TLR2) dependent manner (Cowardin et al., 2016). However, our study explores responses to infection with *C. difficile* strain VPI 10463, which does not express binary toxin produced by *C. difficile* strain typically associated with severe CDI (Hammond & Johnson, 1995). Our data indicate that severe disease associated with *C. difficile* infection also occurs via binary toxin-independent modulation of eosinophils. Recently, Buonomo et al. reported that an increase in intestinal eosinophils was associated with reduced host mortality during *C. difficile* infection (Buonomo et al., 2016). In the aforementioned study, cytokine, IgA and IgG, and muc2 analysis were assessed in the cecum while eosinophils were enumerated in the colon. While we detected robust eosinophil infiltration in the cecum of young mice, we did not observe this in the colon of young or aged animals. Another group found that peripheral loss of eosinophils in patients with *C. difficile* infection was predictive of severe disease (Kulaylat et al., 2018). We report that young mice had significantly increased numbers of intestinal eosinophils during *C. difficile* infection, compared to mock-infected young controls, while aged mice did not have intestinal eosinophil infiltration and significantly worse CDI disease, compared to young mice. However, eosinophils in the blood of aged mice infected with the more virulent strain of *C. difficile* were significantly

increased at the time of peak CDI disease severity, compared to young counterparts. It is possible that eosinopenia at the time of symptom onset is predictive of increased CDI disease severity and mortality, but eosinophil levels may increase in the blood as CDI disease progresses over time. Additionally, eosinophil responses may be altered in older patient populations and may not be predictive of CDI outcomes, an important consideration for studies utilizing immune response biomarkers for CDI outcome prediction (Walker et al., 2013; Yu et al., 2017). Our results also suggest tissue-specific eosinophil responses in the distal intestinal tract during *C. difficile* infection, warranting further examination of local tissue responses associated with CDI. Clinical heterogeneity of disease associated with *C. difficile* infection is a major medical challenge in the management of CDI, particularly in vulnerable patients at risk for increased morbidity and mortality. Focus on precision medicine-based therapeutic approaches adapted to fit the specific characteristics of a particular patient population with CDI should be explored to advance current CDI treatment methods. Understanding the age-related and site-specific differences in immune responses during CDI is critical for the appropriate care of inherently diverse adult patient populations. The general blunted cellular immune response observed in the intestine of aged mice with CDI appears to lead to worse clinical disease, yet intestine-specific changes in immune responses would not be accurately captured by routine blood testing in patients. Our data support further exploration of innate immune mediators in age-associated CDI outcomes, including neutrophils and eosinophils and accompanying cytokine/chemokine responses. Knowledge of the relationship between local and systemic immune responses and CDI-associated disease in high-risk patient populations is required to effectively monitor disease progression and to tailor patient-specific treatment.

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**Baseline**

| **Age** | **Infection** | **Mean Clinical Score** *[95% Confidence Interval]* | **P-value** |
|---|---|---|---|
| Young | 630 | 1.51 [1.19-1.92] | 0.0011 |

**Comparison to Baseline**

| **Age** | **Infection** | **Mean Change in Clinical Score from Young+630 Baseline** *[95% Confidence Interval]* | **P-value** |
|---|---|---|---|
| Aged | 630 | 2.09 [1.49-2.91] | <0.0001 |
| Young | VPI 10463 | 5.51 [4.01-7.57] | <0.0001 |
| Aged | VPI 10463 | 7.00 [5.07-9.67] | 0.029 |

Table 4.1 The impact of age and infecting strain on clinical score determined by linear regression modeling. Baseline was set as young mice infected with 630 strain of *C. difficile*. The mean clinical score and associated p-value is shown, then the mean change in clinical score from this baseline due to age and infecting strain. Both being older and infected with VPI 10463 strain of *C. difficile* increase mean clinical score from baseline.

# CHAPTER V

# Microbial Community Types in the mouse intestine, factors associated with community transitions, and their association with *Clostridioides difficile* infection severity

## 5.1 Abstract

*Clostridioides difficile* is an anaerobic, gram positive bacterium that causes ∼500,000 gastrointestinal infections each year in the United States, with clinical manifestations ranging from diarrhea to life-threatening colitis. There are over 15,000 deaths attributable to CDI in the U.S., 80% of which occur in patients 65 years or older, which suggests that aging impacts CDI pathogenesis and leads to worse outcomes. We employ a system level approach to determine how the community state of the intestinal microbiota influences colonization and disease by profiling the gut microbiota in young mice (2-3 months old) and old mice (18-28 months old) from a single colony before antibiotics, after antibiotics (susceptible to *C. difficile* infection), and during *C. difficile* colonization and subsequent disease. At baseline there were two distinct enterotypes, the first was associated with younger mice having higher relative abundance of *Porphyromonadaceae*, *Verrucomicrobiaceae*, *Erysipelotrichaceae*, and *Bifidobacteriaceae*. After antibiotic administration, there is a shift in community types where age, sex, and specific antibiotic treatment are associated with differences in microbial community structure. The first enterotype was associated with no or low dose antibiotics

and was similar to baseline community structure and was resistant to *C. difficile* colonization. The second enterotype was associated with high dose clindamycin, highest levels of *Enterobacteriaceae*, and low disease severity with highly virulent *C. difficile*. Enterotype four developed after high dose cefoperazone (0.5mg/ml) and was almost exclusively composed of *Lactobacillaceae*. Mice with this enterotype became highly colonized and exhibited the highest disease severity when infected with high virulence *C. difficile*. These findings demonstrate that age contributes to different baseline intestinal microbiota, which transition to different enterotypes dependent on antibiotic treatments seen in clinical settings. The post-antibiotic enterotypes are associated with subsequent *C. difficile* infection severity, with communities high in Enterobacteriacea associated with development of mild disease and those with high levels of *Lactobacillaceae* associated with the development of severe disease.

## 5.2 Introduction

*Clostridioides difficile* is an anaerobic, gram positive bacterium that produces disease by colonizing the intestine of susceptible individuals and producing a variety of toxins including TcdA, TcdB, and binary toxin, all of which disrupt intestinal barrier integrity. *C. difficile* causes $\sim$ 500,000 gastrointestinal infections each year in the United States, with clinical manifestations ranging from diarrhea to life-threatening colitis (Lessa et al., 2015). This wide range of disease severities makes treatment difficult, as aggressive therapies need to be appropriately targeted towards high risk patients as they carry higher potential side-effects and healthcare costs. Tremendous strides have been taken in developing novel therapies for preventing and treating *C. difficile* infections (CDI), as well as developing risk models to determine which individuals are at the highest risk for developing the most severe outcomes (Fujitani et al., 2011; Butt et al., 2013; Rao et al., 2013; Archbald-Pannone et al., 2015; Kassam et al., 2016; van Beurden et al., 2017; Limsrivilai et al., 2018; Kulaylat et al., 2018; Dieterle et al., 2019; Beauregard-Paultre et al., 2019).

In terms of the pathogenesis of CDI, multiple researchers have discovered strong correlations between the microbial community within the intestines and susceptibility to *C. difficile* spore germination and colonization. It has been shown that alterations of the intestinal microbial community

through antibiotic administration or specific gastrointestinal diseases can lead to an increase in susceptibility. Another key feature of CDI is that upwards of 25% of individuals will experience recurrence of their CDI following successful treatment. The recovery of the native microbial community after such perturbations is vital to the prevention of recurrent CDI, a finding which has led to the use of fecal microbial transplant (FMT) and specific microbial administration therapy for the prevention of recurrence (McDonald et al., 2018).

There are over 15,000 deaths attributable to CDI in the U.S., 80% of which occur in patients 65 years or older, which suggests that aging impacts CDI pathogenesis and leads to worse outcomes (Riaz Rajoka et al., 2018; Clements & R Carding, 2018; Salazar et al., 2019; Gao et al., 2019). Studies have found that age impacts bile acid synthesis, which plays a key role in *C. difficile* germination, and cross-sectional studies have shown changes in the microbial community in individuals as they age (Bertolotti et al., 2007; Odamaki et al., 2016; Frommherz et al., 2016; Aleman & Valenzano, 2019).

We have shown that aged mice experienced altered systemic inflammatory marker profiles during severe CDI, which occurred with reduced localization of neutrophils and eosinophils to the intestines compared to young mice (Abernathy-Close, Dieterle, et al. 2020). Another important host factor is sex, as women have also been shown to have an increased risk of CDI in clinical studies. Past studies have shown gender differences in bile acid production and the impact of specific antibiotic exposures to gut microbial community (Sheng et al., 2017; Gao et al., 2019). Additional studies have found patterns of the intestinal microbial diversity associated with both age and sex in human adults (de la Cuesta-Zuluaga et al., 2019).

The microbial community is a key player in the development of CDI and the progression of the CDI as it sits at the junction of initial antibiotic perturbation, infecting strain, and host intestinal barrier interaction and immune response. Previous research has shown stable microbial communities in young mice from the same colony and that subsequently, the intestinal microbial community shifts during aging in mice (Langille et al., 2014; Schloss et al., 2012). Additionally, previous research has studied the impact of varying antibiotics and doses on colonization resistance to *C. difficile* in young mice, as well as the changes in microbial composition following antibiotic administration and subsequent infection (Reeves et al., 2011; Theriot et al., 2014; Schubert et al.,

2015; Koenigsknecht et al., 2015; Theriot et al., 2016).

In this study, we utilize a systems biology approach to examine *C. difficile* infection pathogenesis in order to identify the host and microbial drivers of variation in clinical outcome. We have constructed a coherent cohort of mice that vary host features (sex and age), antibiotic treatment rendering them susceptible to CDI, and infecting strain of *C. difficile* with differing virulence (mild – 630g, moderate – R20291, and severe – VPI 10463). We choose these strains of *C. difficile* to cover the range of CDI disease (no signs of disease to severe colitis and death) seen in humans and utilize different antibiotic regimens to give additional variation into the cohort before infection and mimic the wide variety of initial microbial perturbations seen in healthcare settings. We use this cohort to examine the microbial community types that exist before and after antibiotics, during initial colonization with *C. difficile*, and at peak disease severity in mice.

## 5.3 Materials and Methods

### 5.3.1 Murine *C. difficile* infection model

8–12 week-old (young) or 18-28 month old (old), Specific Pathogen Free (SPF) C57BL/6 mice were treated with either 10 days of cefoperazone (0.5mg/ml or 0.1mg/ml) delivered in their drinking water or a single intraperitoneal injection of clindamycin (10mg/kg, 2mg/kg, or 0.2mg/kg). After a two-day washout period for cefoperazone treated mice or a single day following clindamycin injection, mice were given an oral gavage of water, 630g *C. difficile* spores, R20291 *C. difficile* spores, or VPI 10463 *C. difficile* spores (Figure 5.1). Inoculum was estimated between $10^3$ and $10^4$ spores. VPI 10463 infected mice were majority euthanized at two days post infection at peak disease, a portion that were given clindamycin or lose dose cefoperazone were monitored and euthanized at clinical end point (20% weight loss) or at day post 9. R20291 infected mice were euthanized at two days post infection. 630g infected mice were euthanized at 4 days post infection at peak disease. Cecum and colon histopathology were scored by a pathologist blinded to the samples for edema, epithelial damage, and inflammatory cell infiltration on a 4-point scale and then summed (total ranging from 0-12). At time of euthanization, each mouse was given a clinical score from 0-20 at euthanization based on posture (0-3), coat (0-3), activity (0-3), diarrheal signs

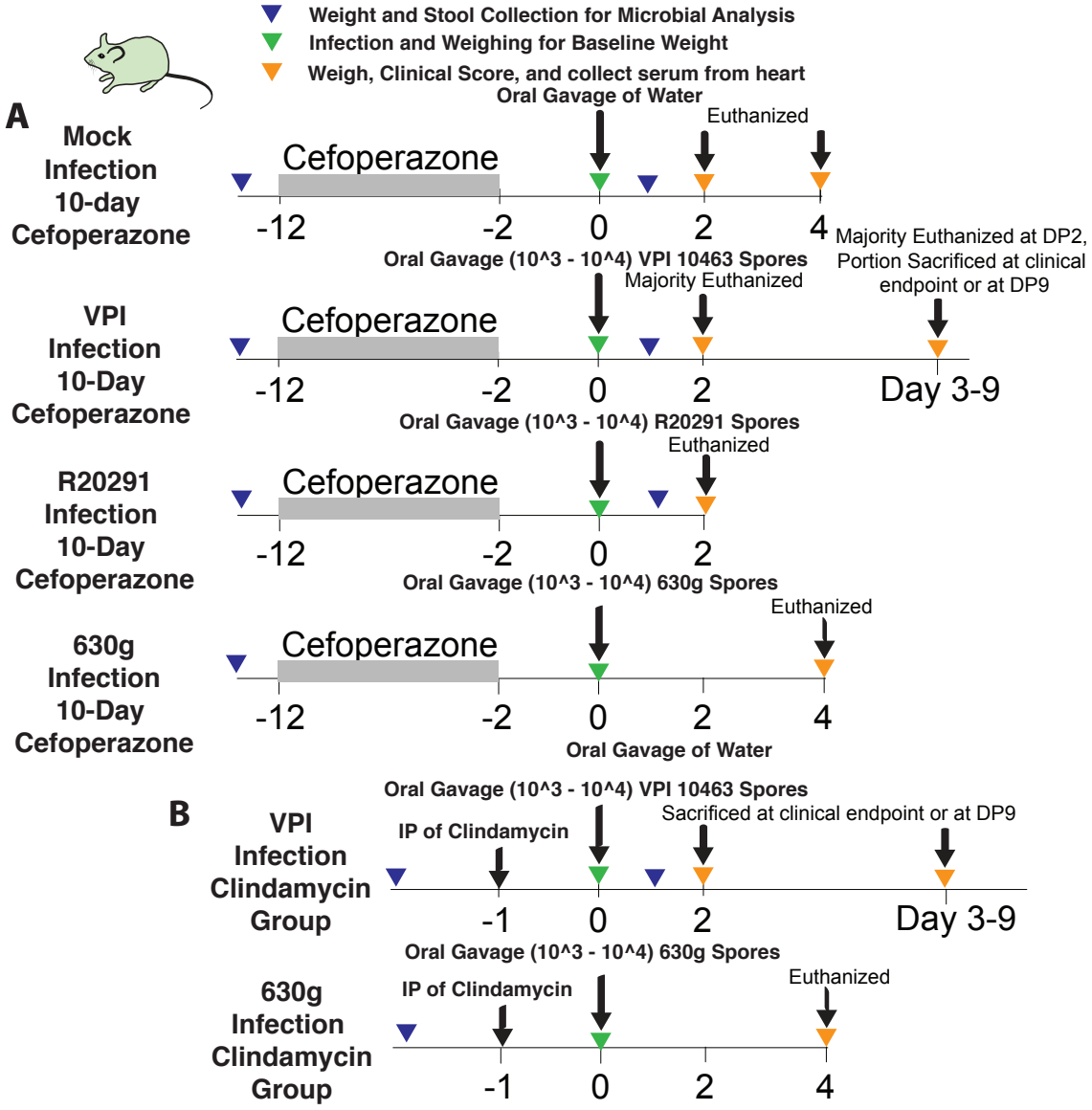


Figure 5.1 Experimental design for mouse model of *Clostridioides difficile* infection. Experimental group A shows timeline for 10-day cefoperazone treatment with day of infection marked as day zero. Experimental group B shows timeline for single dose IP injection of clindamycin.

(0-3), and weight change (0-4) from day 0 (D0). Further description of the mouse model can be found in Leslie et al. (2019).

### 5.3.2 Mice

We utilized specific pathogen-free (SPF) C57BL/6 wild-type adult mice that were young (2-3 months old) or aged (18-28 months old) with roughly a 1:1 male to female ratio. These mice were from a breeding colony at the University of Michigan that was originally founded with breeding stock obtained from the Jackson Laboratories in 2002. Euthanasia was performed utilizing CO2 inhalation at the experimental endpoint. Animal studies were approved by the Institutional Animal Care  Use Committee (IACUC) at the University of Michigan and animal husbandry was performed in an AAALAC-accredited facility.

### 5.3.3 *C. difficile* quantification

Fecal contents at day post 1 were collected in a pre-weighed sterile tube from each mouse and then re-weighed to determine fecal weight. Each fecal sample was diluted 10% with pre-reduced sterile PBS and serially diluted onto pre-reduced TCCFA plates with or without erythromycin supplementation. *C. difficile* strain 630 is erythromycin-resistant, whereas *C. difficile* strain VPI 10463 and R20291 are sensitive to erythromycin. The plates were incubated anaerobically at 37 degrees C, and colonies were counted after 18 to 24 hours of incubation.

### 5.3.4 DNA extraction and Illumina MiSeq sequencing

DNA extraction was performed according to the previously detailed approach found in Leslie et al. (2019). 200-300 microliters of feces diluted 10-fold in sterile PBS were added in a random fashion to DNA extraction plates and then was submitted for DNA extraction using a MagAttract PowerMicrobiome DNA isolation kit (Qiagen, Germantown, MD). Each plate contained positive and negative controls. DNA amplification was performed using barcoded dual-index primers specific to the V4 region of the 16S rRNA gene. A 500-cycle MiSeq V2 reagent kit was utilized for library preparation and sequencing.

### 5.3.5 Microbial data processing and analysis

Bacterial 16S rRNA gene sequencing was performed with the V4 variable region and then analyzed using the publicly available Mothur program (Schloss et al., 2009) according to the available SOP. Sequences were assembled, filtered, trimmed and aligned using the Silva 16S rRNA database. Chimeras were removed and contigs binned by 97% similarity to produce OTUs and then classified using the Silva 16S rRNA database. Dirichlet-multinomial mixture modeling was performed using the get.community function in Mothur and the best fit was determined by Laplace approximation (Holmes et al., 2012). Relative abundance of bacterial families was calculated by summation of the associated OTUs in a sample and then division by the total number of sequences for each sample. Data handling and analysis was performed in Python, R, and GraphPad Prism 8.

### 5.3.6 Statistics

Sex and age composition statistical analysis was performed with a Fisher's exact test in GraphPad Prism. One-way analysis of variance (ANOVA) with Tukey's post-hoc test for *C. difficile* burden using GraphPad Prism 8. Clinical, cecum, and colon summary scores were analyzed using a Kruskal-Wallis test followed by a two-stage step-up method (Benjamini, Krieger, and Yekutieli) false discovery rate adjustment for multiple comparisons using GraphPad Prism 8. P-value assignment on figures follows: *p0.05; **p0.01; ***p0.001; ****p0.0001.

## 5.4 Results

### 5.4.1 Cohort design using three strains of *C. difficile* captures the wide range of disease severity from no signs of disease to severe colitis

We utilized a murine model of *C. difficile* where mice are rendered susceptible to infection by administration of either a 5 or 10-day course of oral cefoperazone or a single intraperitoneal injection of clindamycin followed by infection (Figure 5.1). Feces and weights were taken before antibiotics, at time of infection (D0), and one day post infection. Varying doses of cefoperazone and clindamycin were used to test the impact of dose on colonization and severity.

Three-hundred and sixty-five young mice (2-3 months old) and one-hundred and thirty-six old

| Experimental Cohort | | | Experimental Infection Group | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Category** | **Sub-category** | **Dose** | **Mock** | | **630g** | | **R20291** | | **VPI 10463** | |
| Sex | Female | | 54 | 43% | 63 | 49% | 13 | 45% | 92 | 48% |
| | Male | | 73 | 57% | 66 | 51% | 16 | 55% | 100 | 52% |
| Age | Young | | 106 | 78% | 87 | 67% | 13 | 45% | 159 | 77% |
| | Old | | 30 | 22% | 42 | 33% | 16 | 55% | 48 | 23% |
| Treatment | No Antibiotics | | 14 | 10% | 16 | 12% | 0 | 0% | 24 | 12% |
| | 10 Day Cefoperazone | 0.5 mg/mL | 113 | 83% | 96 | 74% | 29 | 100% | 120 | 58% |
| | | 0.1 mg/mL | 0 | 0% | 0 | 0% | 0 | 0% | 12 | 6% |
| | 5 Day Cefoperazone | 0.5 mg/mL | 9 | 7% | 0 | 0% | 0 | 0% | 15 | 7% |
| | Single IP Clindamycin | 10 mg/kg | 0 | 0% | 17 | 13% | 0 | 0% | 11 | 5% |
| | | 2 mg/kg | 0 | 0% | 0 | 0% | 0 | 0% | 13 | 6% |
| | | 0.2 mg/kg | 0 | 0% | 0 | 0% | 0 | 0% | 12 | 6% |
| **Total** | 477 Mice | | 127 | 25% | 129 | 26% | 29 | 6% | 192 | 38% |

Table 5.1 Demographic Table. The demographics of the mice in this experiment by their experimental infection group.

mice (18-28 months old) were used in the experiments summarized in Table 5.1. Of these mice, two-hundred and thirty-two were females and two-hundred and sixty-nine were males (approximately 1:1). We utilize three strains of *C. difficile* with varying virulence caused by differences in toxin production: strain 630g (mild virulence, peak disease obtained at four days infection), R20291 (moderate virulence, peak disease obtained at four days infection), and VPI 10463 (high virulence, mice succumb to disease shortly after 48 hours of infection).

To visualize the range of colonization, we first look only at mice receiving the full course of 10-day cefoperazone at 0.5 mg/mL in their drinking water, or a single intraperotineal injection of clindamycin at 10 mg/kg and then subsequently orally gavaged with 103-104 spores of *C. difficile* (630g, R20291, or VPI 10463). Higher colonization is seen in females, and old mice (Figure 5.2).

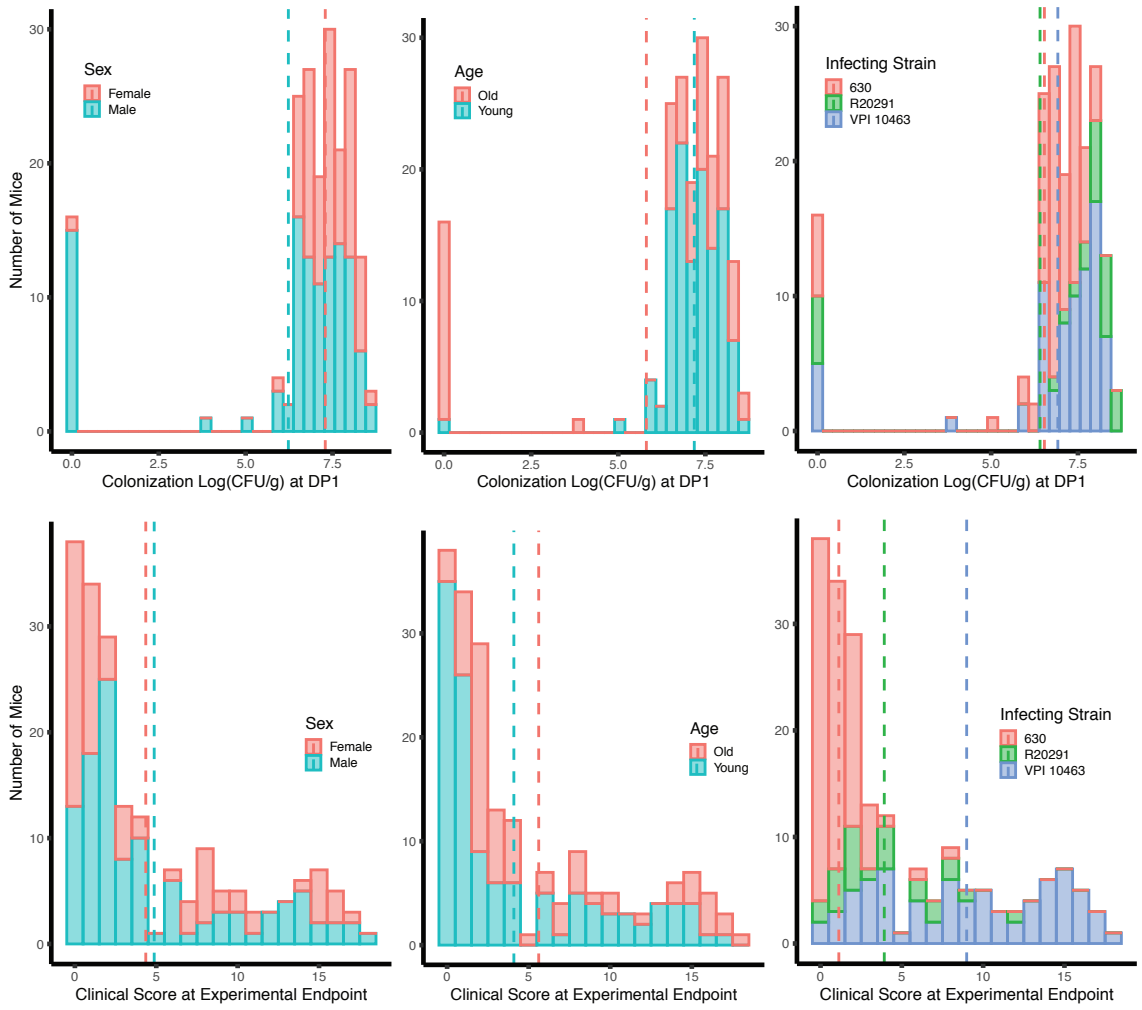


Figure 5.2 *C. difficile* colonization one day post infection and clinical score at peak disease in mice receiving full dose cefoperazone or clindamycin shown by sex, age and infecting strain. Only mice receiving either 10-day course of 0.5 mg/mL cefoperazone in their drinking water or a single intraperitoneal injection of 10 mg/kg clindamycin and then infected with *C. difficile* are shown. The top row shows histograms of the colonization level of *C. difficile* at one day post infection (DP1), while the bottom row shows histograms of clinical score at experimental end point (two days post infection for all VPI 10463 and a time-matched group of 630g infected mice, four days post infection for the remaining 630g and all R20291 infected mice). The same histogram is colored by sex (first column), age (second column) and finally infecting strain (third column). Lines represent the mean of the corresponding colored data.

To measure clinical disease, mice were scored for posture, coat, activity, diarrheal signs, and weight change with 0 representing no clinical signs of disease and 20 representing severe signs of disease. The use of the three *C. difficile* strains captures the range of clinical scores from no signs of disease to severe clinical disease. Higher mean clinical score is seen in old mice compared to young mice. In terms of strain virulence, we found that infection with the 630 strain causes the least amount of clinical disease, the R20291 strain causes moderate levels, and the VPI 10463 strain causes the highest clinical disease.

Cecum and colon tissues were taken at time of euthanization and scored for edema, epithelial damage, and inflammatory cell infiltration (Figure 5.3). Again, we see that the three strains of *C. difficile* cause a range of intestinal damage from no signs to severe colitis. We also witness higher mean cecum and colon score in females and young mice. VPI 10463 and R20291 cause similar cecal and colon damage, and both cause much higher mean intestinal than 630g infection.

### 5.4.2 Baseline microbiota show different enterotypes associated with young and old mice, but no differences between females and males

The first step of examining our mouse cohort is to determine if there are baseline enterotypes before the administration of antibiotics and infection with *C. difficile*. Feces was taken from each mouse prior to antibiotic administration, and 16S ribosomal RNA gene sequencing was utilized followed by microbial community analysis and enterotype assignment utilizing Dirichlet multinomial mixture (DMM) modeling (Holmes et al., 2012; Koren et al., 2013; Ding & Schloss, 2014; Schloss & Westcott, 2011).

The baseline microbial communities fall into two enterotypes, visualized by principal coordinates analysis (PCoA) utilizing the operational taxonomic units (OTUs) in Figure 4A. Breaking down the PCoA into sex, we see no distinction between sexes on the PCoA (Figure 5.4 B), but do see a difference in the distribution of young and old mice between the enterotypes (Figure 5.4 C). There are no differences between the distribution of male and female between the enterotypes using a Fisher's two-sided exact test ($p = 0.11$, Figure 5.5 A-C), but there is a statistically significant difference in age distribution between the two enterotypes (p 0.001) with baseline enterotype 2 composed of 93% old mice (Figure 5.5 D-E).

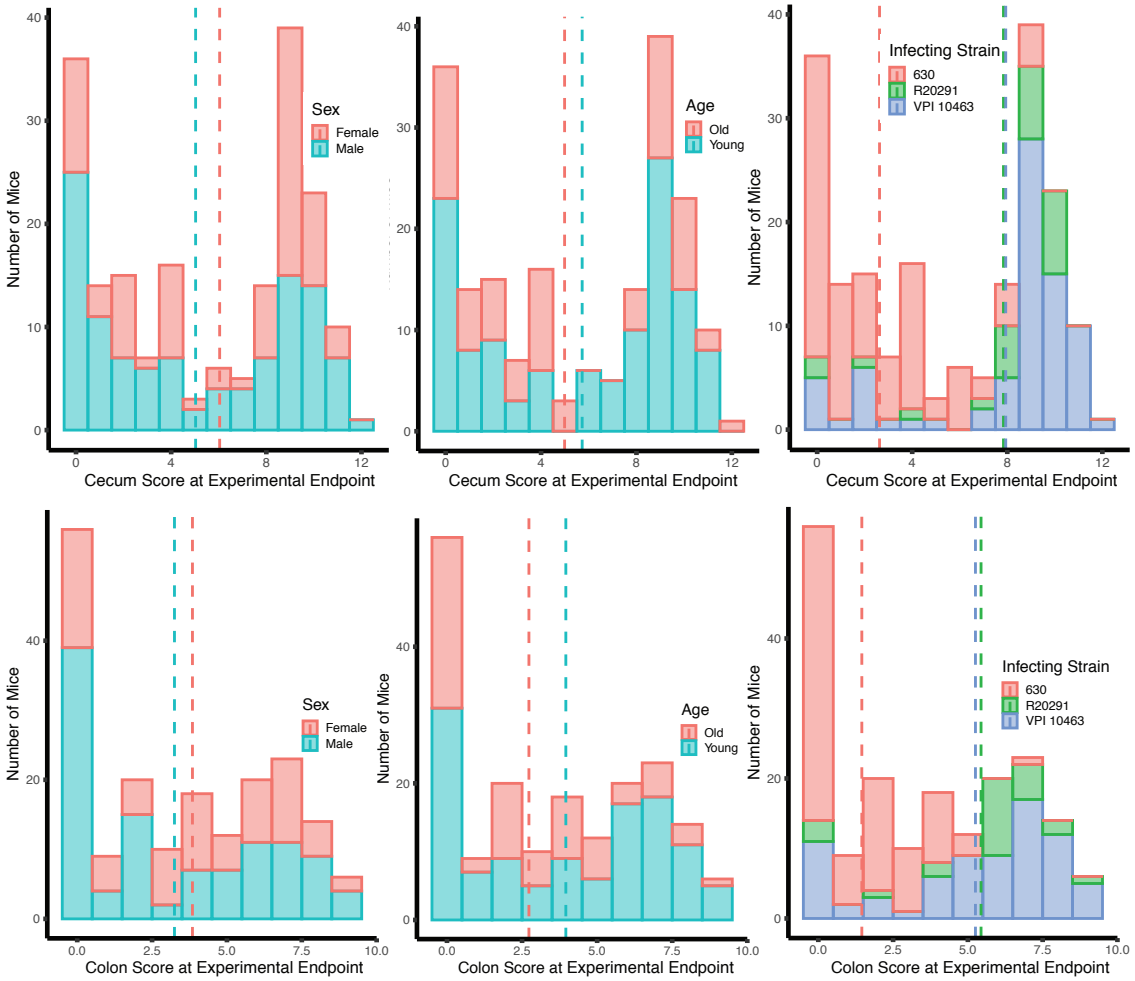


Figure 5.3 Cecum and colon histopathology scores at peak disease in mice receiving full dose cefoperazone or clindamycin shown by sex, age and infecting strain. Only mice receiving either 10-day course of 0.5 mg/mL cefoperazone in their drinking water or a single intraperitoneal injection of 10 mg/kg clindamycin and then infected with *C. difficile* are shown. The top row shows histograms of the top row shows histograms of cecum score at peak disease (two days post infection for all VPI 10463 and a time-matched group of 630g infected mice, four days post infection for the remaining 630g and all R20291 infected mice) and the bottom row shows histograms of colon score at peak disease. Cecum and colon sections were scored (0-12) for edema, epithelial damage, and inflammatory cell infiltration. The same histogram is colored by sex (first column), age (second column) and finally infecting strain (third column). Lines represent the mean of the corresponding colored data. Higher colonization is seen in females, and old mice.

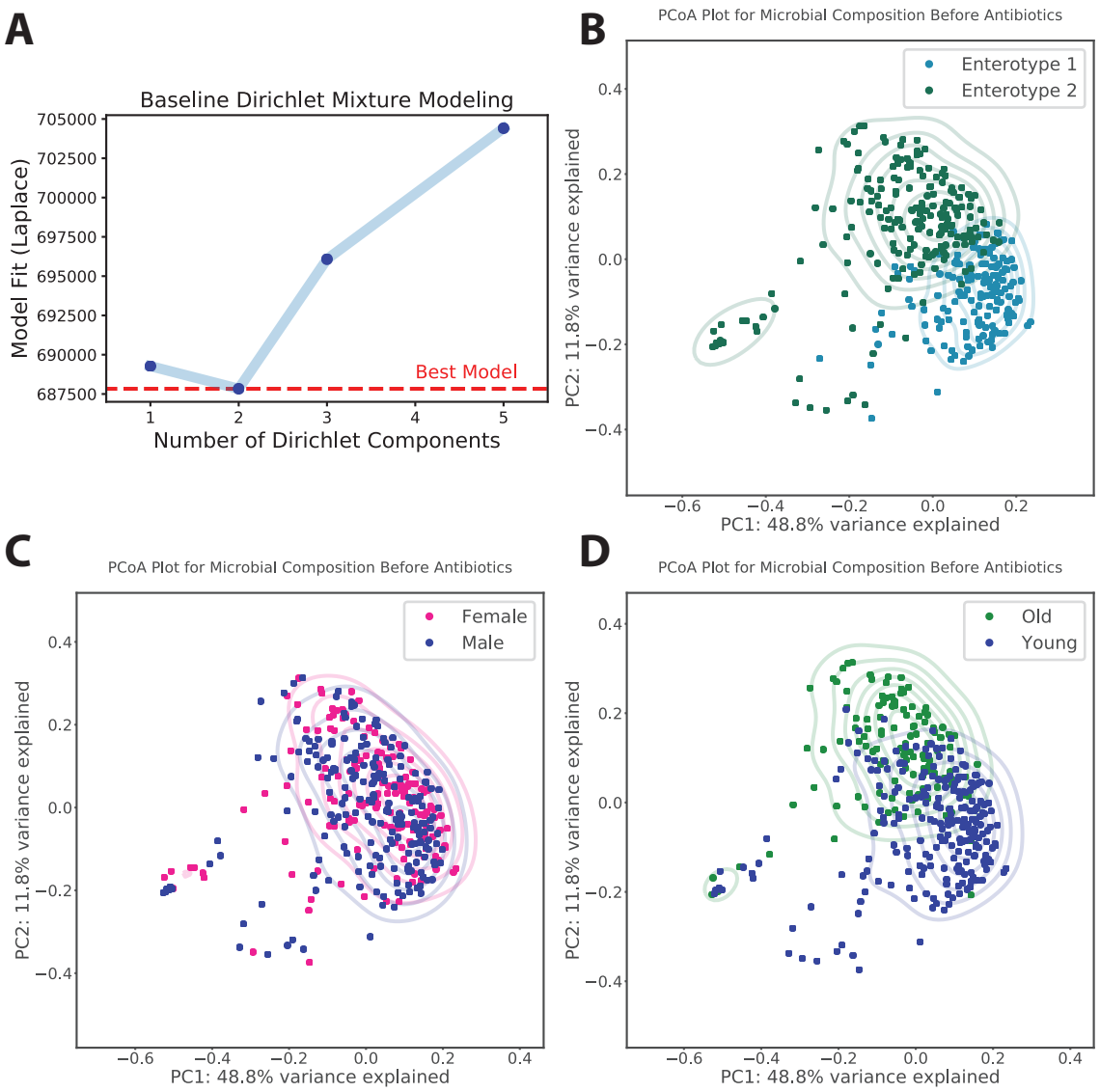


Figure 5.4 Baseline microbial community analysis using Dirichlet mixture modeling and Principal Coordinate Analysis. A) Dirichlet mixture modeling Laplace fitting results using feces microbial OTUs from baseline. The best model is represented by the red, dashed line indicating the model with 2 enterotypes is the best fit. B-D) Principal coordinate plot using baseline microbial OTUs for each mouse. Principal coordinate one explains 48.8% of the variance while principal coordinate two explains 11.8% of the variance. The plot is colored by DMM enterotype (B), sex (C) and age (D).

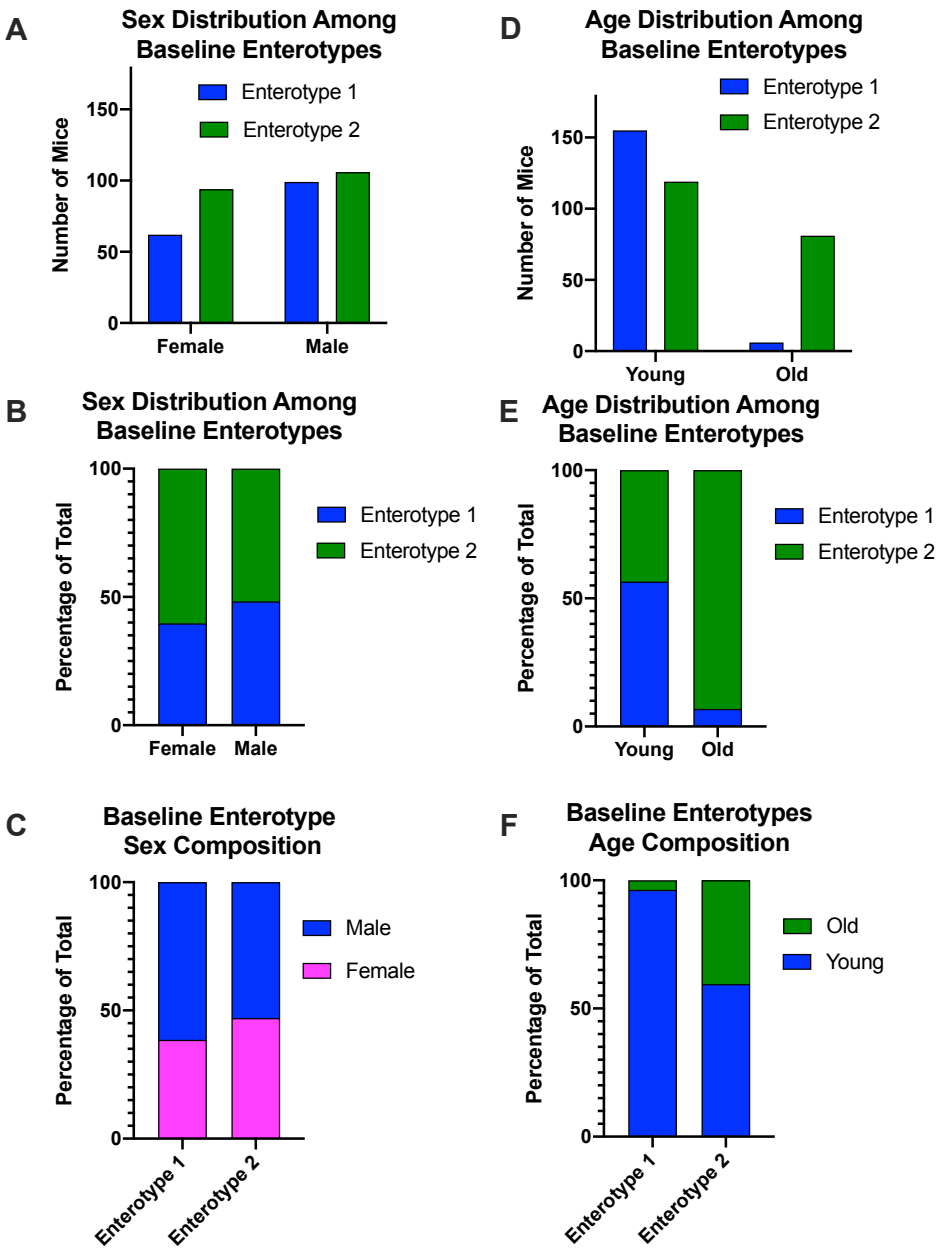


Figure 5.5 Baseline enterotypes are similar by sex but have different age compositions. Histogram showing the number of female and male mice (A) or the number of young and old mice (D) in each of the baseline enterotypes. Percentage of total plot for sex (B) and age (E) showing the breakdown by enterotype. Percentage of each enterotype that is female/male (C) or young/old (F). Sex composition was not different between enterotypes, while age difference was statistically significant by two-sided Fisher's exact test (p 0.0001).

### 5.4.3 Administration of cefoperazone and clindamycin produce new enterotypes associated with specific antibiotic treatment and age

To observe the impact of antibiotic administration on microbial community structure in relation to age and sex, mice were split into cohorts and received either a 10-day course of high dose (0.5 mg/ml) or low does (0.1 mg/ml) cefoperazone. A single intraperotineal injection of high does (10 mg/kg), medium dose (2 mg/kg) or low dose (0.2 mg/kg) clindamycin, or no antibiotics (drinking water for controls of cefoperazone administration and saline injection for controls of clindamycin injection experimental groups). Feces were taken two days after switching to pure water for cefoperazone treatment or one day following clindamycin injection. 16s ribosomal gene sequencing was again performed followed by microbial community analysis and subsequent Dirichlet multinomial mixture (DMM) modeling (Holmes et al., 2012; Koren et al., 2013; Ding & Schloss, 2014; Schloss & Westcott, 2011).

Following administration of antibiotics, a large dispersion and numerous clusters are seen on a PCoA of the microbial features (Figure 5.6 A). DMM modeling determined that there were four distinct enterotypes, visualized by PCoA of OTUs (Figure 5.6 B-C). The four day 0 enterotypes show little differences visually between sex composition (Figure 5.6 D), but age differences are apparent in the PCoA (Figure 5.6 E).

As treatment with antibiotics resulted in these new enterotypes, the next step is to visualize the association between day zero enterotypes and antibiotic treatment (Figure 5.7). Examining the breakdown of each treatment and the resulting enterotype, we see that no antibiotics and the lowest dose clindamycin (0.2mg/kg) result in exclusively day 0 enterotype 2 formation (Figure 5.7 A-B). Moderate dose clindamycin (2mg/kg) and low does cefoperazone (0.1 mg/ml) resulted in majority enterotype 1, with a small portion of enterotype 3. High dose clindamycin (10mg/kg) results in exclusively enterotype 2, while (0.5mg/ml) results in majority enterotype 4, a small quantity of enterotype 3 and a minor number of enterotype 1.

The treatment-enterotype correlation is shown in Figure 5.7 C. Mice with enterotype 4 received exclusively cefoperazone (0.5mg/ml), while mice with enterotype 2 received exclusively clindamycin (10mg/kg). Mice with enterotype 3 received largely cefoperazone (0.5mg/ml). Lastly, enterotype

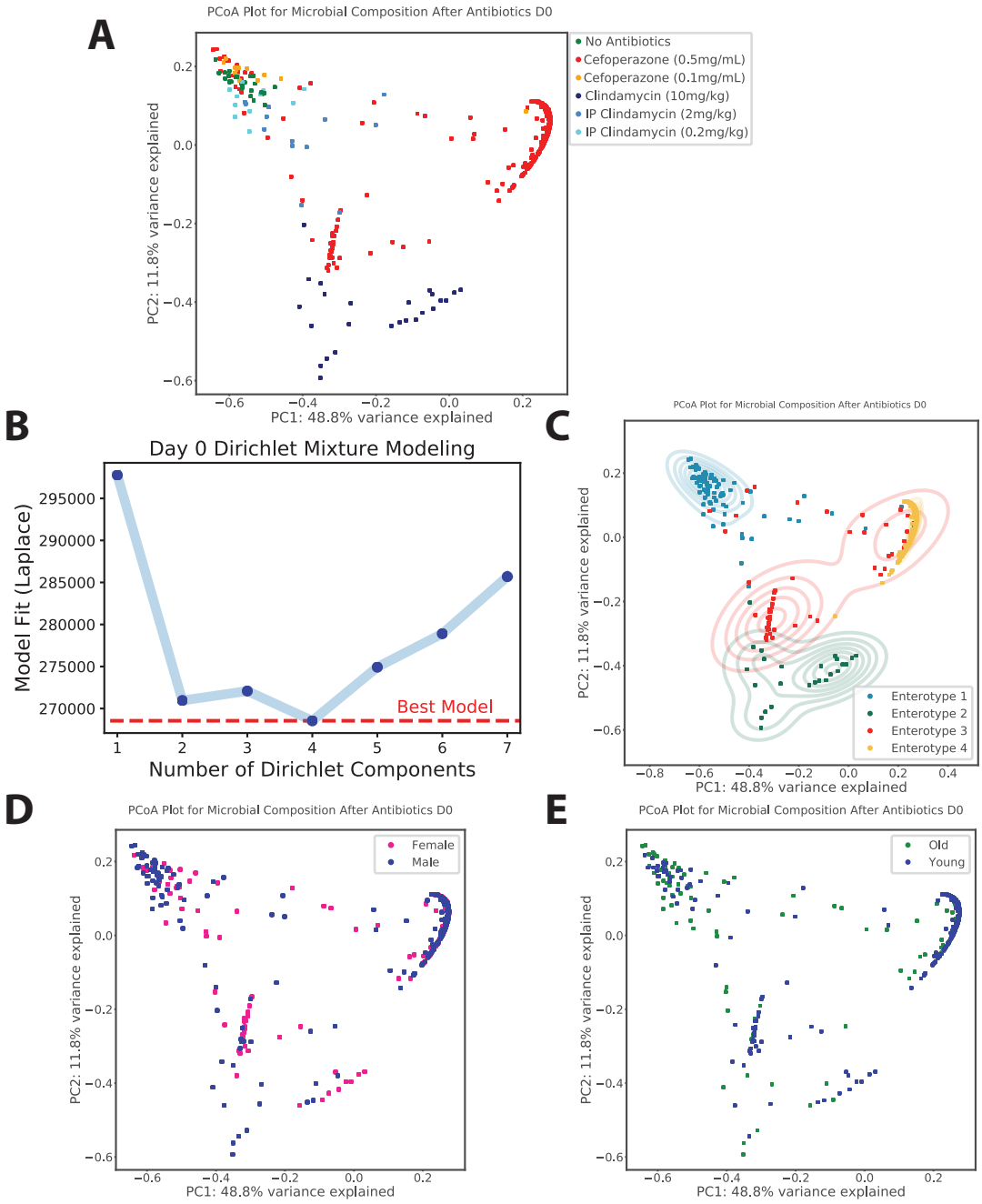


Figure 5.6 Day 0 (after antibiotic administration) microbial community analysis using Dirichlet mixture modeling and Principal Coordinate Analysis. B) Dirichlet mixture modeling Laplace fitting results using feces microbial OTUs from day 0, after the administration of the antibiotics. The best model is represented by the red, dashed line indicating the model with four enterotypes is the best fit. B-D) Principal coordinate plot using baseline microbial OTUs for each mouse. Principal coordinate one explains 48.8% of the variance while principal coordinate two explains 11.8% of the variance. The plot is colored by antibiotic treatment (A), DMM enterotype (C), sex (D) and age (E).

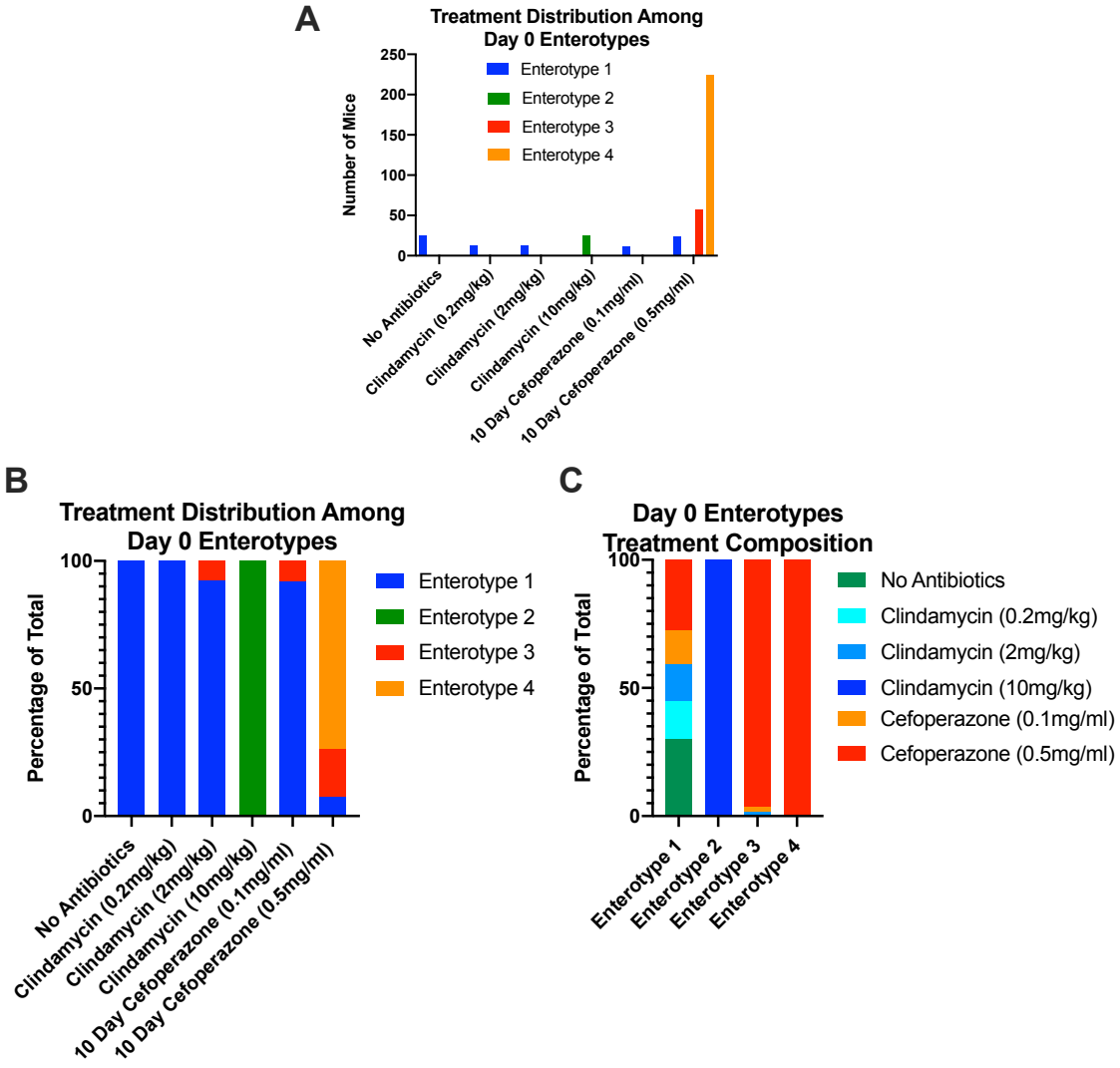


Figure 5.7 Impact of antibiotic treatment on the development of day 0 enterotypes. A) Histogram showing the number of mice with each enterotype by treatment received. B) Percentage of total plot showing the proportion of resulting enterotype by treatment received. C) Percentage of total plot for each enterotype showing the percentage of each treatment the mice included in that enterotype received.

1 was associated with no antibiotics, the lowest doses of antibiotics and some mice that received cefoperazone (10mg/kg). This indicates that enterotype 1 is composed of most likely the baseline microbial community witnessed before antibiotic treatment, with some mice not responding to the highest does of cefoperazone. Sex composition was not different between day 0 enterotypes, while age composition was statistically different between the day 0 enterotypes by Fisher's exact test ($p < 0.0001$; see Figure 5.8).

Sex composition was not different between day 0 enterotypes, while age composition was statistically different between the day 0 enterotypes by Fisher's exact test ($p < 0.0001$; see Figure 5.8). However, as each treatment has different numbers of young and aged mice, it is appropriate to look at the breakdown of enterotype for each category of sex and age individually (Figure 5.9). Old mice have a different response to cefoperazone (0.5mg/ml) with increased number of enterotype 1 and enterotype 3 (Figure 5.9 A, $p < 0.0001$). A similar difference is seen between female and male mice in response to cefoperazone (0.5mg/ml) with an increase in the number of enterotype 1 (Figure 5.9 B, $p = 0.0005$). There are also subtle differences in the response to low dose clindamycin and cefoperazone between young and old and between female and male mice.

### 5.4.4 Enterotype microbial communities differ most drastically across the families of *Lactobacillaceae*, *Enterobacteriaceae*, *Verrucomicrobiaceae*, *Bacteroidaceae*, and *Porphyromonadaceae*

All the operational taxonomic units (OTUs) were combined into the associated bacterial families. Then, relative abundances were calculated per mouse by dividing the number of sequences associated with each family by the total sequences observed in the fecal sample. The relative abundance of the bacterial families with the highest importance for baseline enterotype classification are shown in Figure 5.10 in order of importance. Baseline enterotype 1, which was associated primarily with young mice, had on average higher relative abundance of *Porphyromonadaceae*, *Verrucomicrobiaceae*, *Erysipelotrichaceae*, and *Bifidobacteriaceae*, while baseline enterotype 2, which was associated with old mice, had higher relative abundance of *Lactobacillaceae*, *Bacteroidaceae*, and *Lachnospiraceae*.

Following antibiotic treatments, there were four enterotypes present in the mouse cohort at day

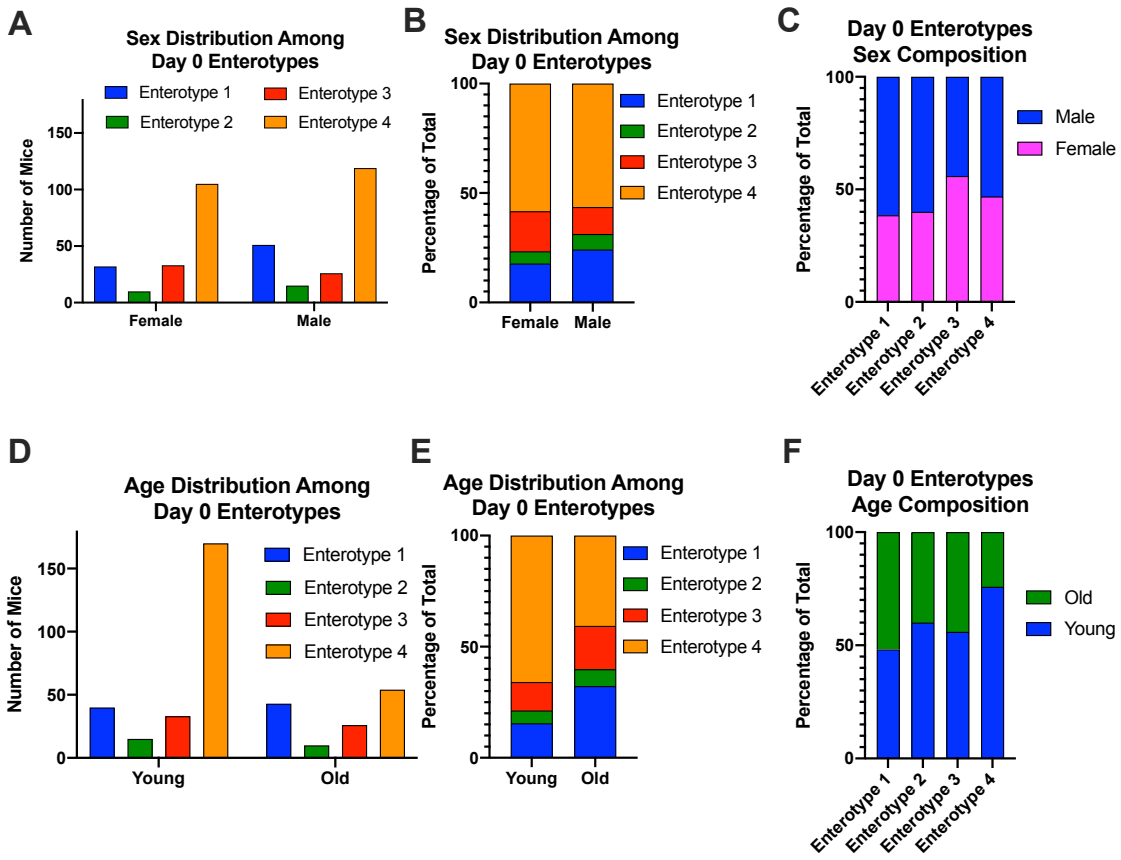


Figure 5.8 Day 0 enterotypes are similar by sex but have different age compositions. Histogram showing the number of female and male mice (A) or the number of young and old mice (D) in each of the day 0 enterotypes. Percentage of total plot for sex (B) and age (E) showing the breakdown by day 0 enterotype. Percentage of each day 0 enterotype that is female/male (C) or young/old (F). Sex composition was not different between day 0 enterotypes, while age composition was statistically different between the day 0 enterotypes Fisher's exact test (p 0.0001).

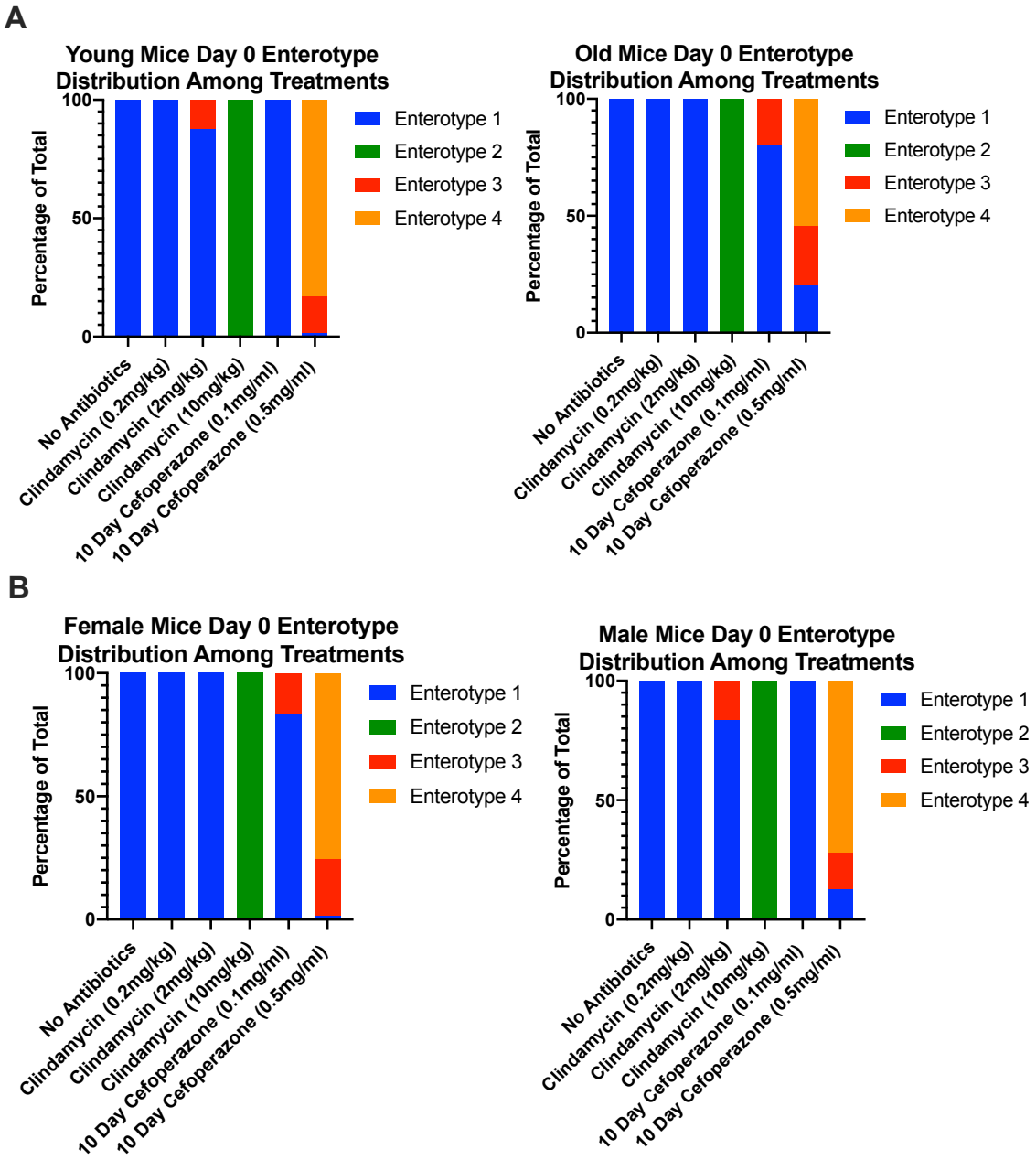


Figure 5.9 Association of each treatment and day 0 enterotypes for female, male, young, and old mice. A) Percentage of total plot including only young (left) or old (right) mice showing the resulting day 0 enterotype for each treatment. B) Percentage of total plot including only female (left) or male (right) mice showing the resulting day 0 enterotype for each treatment. We see a statistical difference in the resulting enterotypes in response to high does cefoperazone (0.5mg/kg) between young and old mice (p 0.0001, Chi-square) and between female and male mice. (p = 0.0005, Chi-square).

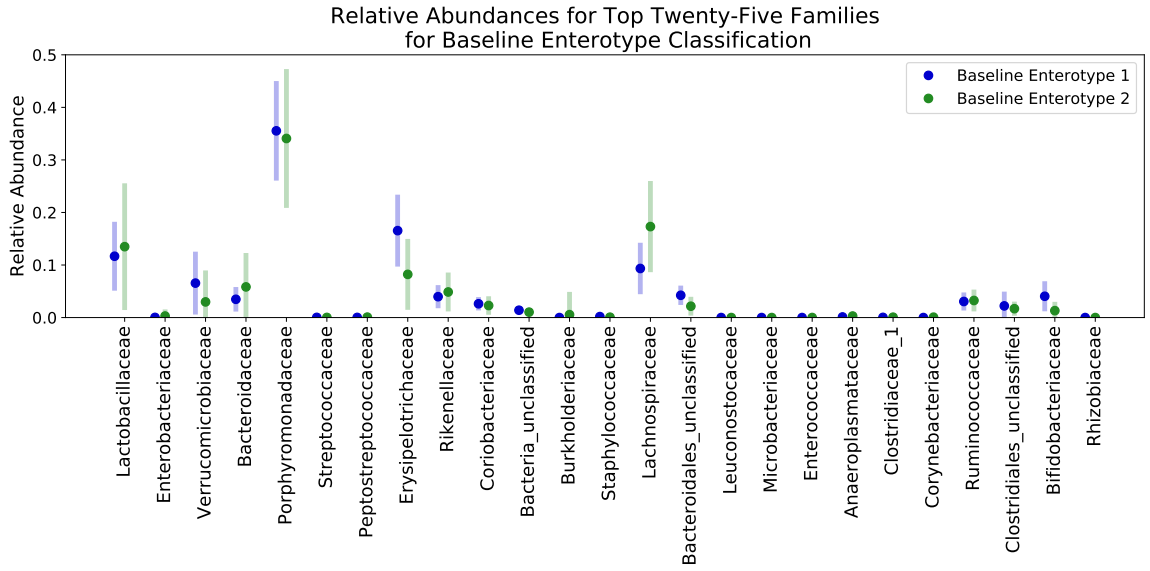


Figure 5.10 Relative abundance of the top twenty-five most important bacterial families for baseline enterotype classification. The mean is represented as a point and the bars represent standard deviation.

0. The top five most important bacterial families for day zero enterotype classification are shown in Figure 5.11, and the next twenty-five most important are shown in Figure 5.12. Day 0 enterotype 1, which was associated with no antibiotic treatment and low dose clindamycin (0.2mg/kg and 2mg/kg) and cefoperazone (0.1mg/ml and to a lesser extent 0.5mg/ml), resembles the baseline enterotypes the most, with similar levels of *Lactobacillaceae*, *Enterobacteriaceae*, *Verrucomicrobiaceae*, *Bacteroidaceae*, and *Porphyromonadaceae*. Day 0 enterotype 2, which was formed following high dose clindamycin (10mg/kg), is associated with the highest levels of *Enterobacteriaceae*, much higher than the other enterotypes. Day 0 enterotype 3, which was associated with high dose cefoperazone and to a less extent low dose cefoperazone (0.1mg/ml) and medium dose clindamycin (2mg/kg), had higher levels of *Lactobacillaceae*, *Porphyromonadaceae*, Streptococcaceae, Peptostreptococcaceae, and Staphylococcaceae. Lastly, day 0 enterotype 4, which was produced by high dose cefoperazone (0.5mg/ml), was composed of almost entirely *Lactobacillaceae* and had lower relative abundance of all the other bacterial families examined.

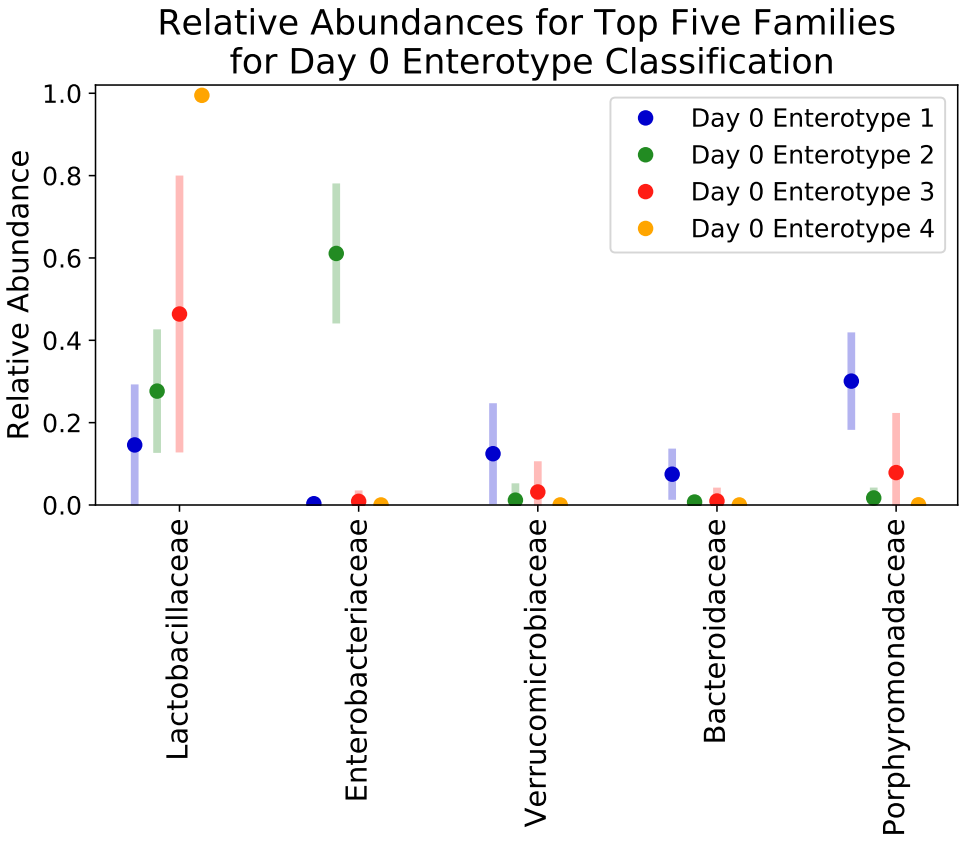


Figure 5.11 Relative abundance of the top five most important bacterial families for day 0 enterotype classification. The mean is represented as a point and the bars represent standard deviation.

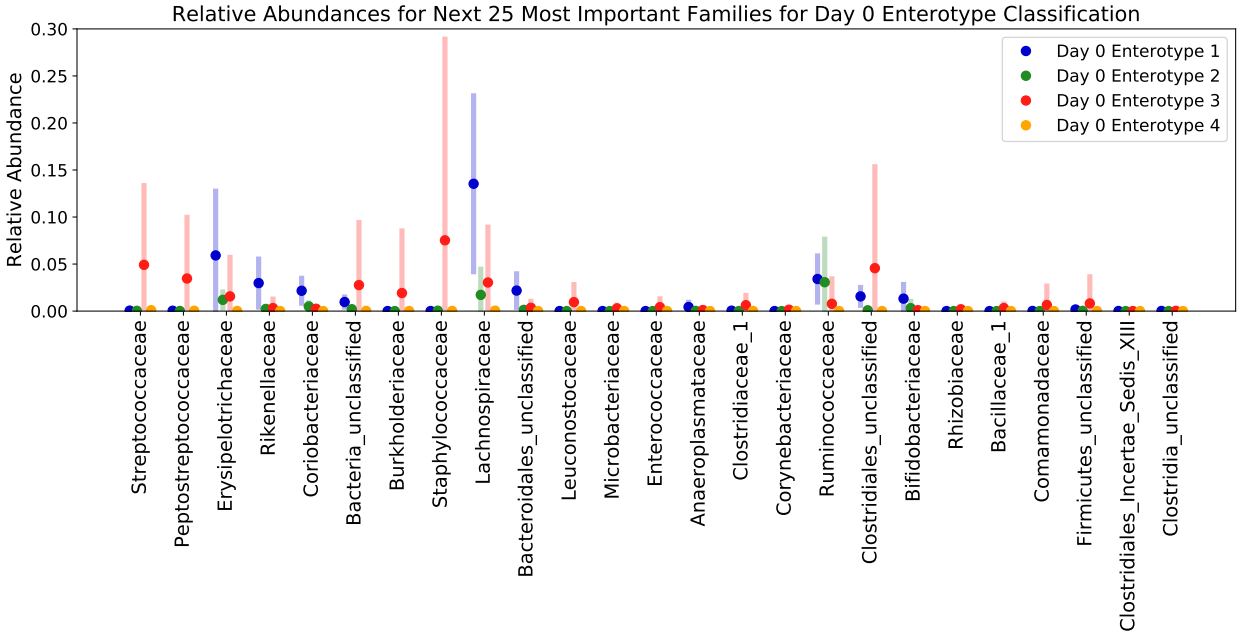


Figure 5.12 Relative abundance of the next twenty-five most important bacterial families for day 0 enterotype classification. The mean is represented as a point and the bars represent standard deviation.

### 5.4.5 Antibiotic-induced enterotypes are associated with *C. difficile* colonization and subsequent disease severity

At baseline, there were two enterotypes associated with the age of the mice. After differential antibiotic treatment, four day 0 enterotypes developed that were related to the antibiotic treatment received. The next goal is to determine if the day 0 enterotypes are associated with initial colonization by *C. difficile* and subsequent disease severity (Figure 5.13).

The first stage of *C. difficile* infection is initial colonization. To determine if any enterotypes were resistant to colonization, *C. difficile* colony forming units per gram feces was determined at one day post infection. Of all infected mice in the cohort, mice with day 0 enterotype 1 were resistant to colonization as they have substantially lower *C. difficile* burden compared to mice in the other three enterotypes at one day post infection levels (Figure 5.14 A, $p < 0.0001$). This reduced *C. difficile* burden in mice with day 0 enterotype 1 was seen for 630g and VPI 10463 infections, but not for infection with R20291 (Figure 5.14 B-C, $p < 0.0001$ for 630g and VPI 10463). In summary, day 0 enterotype 1 appears to be resistant to *C. difficile* colonization except in rare cases, while

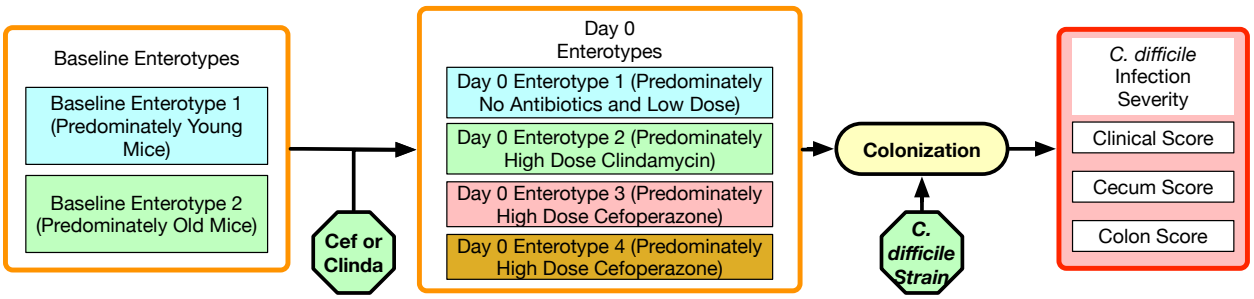


Figure 5.13 Diagram of experimental design showing the baseline enterotypes with age association, transition to day 0 enterotypes with treatment associations, and finally infection with *C. difficile* and utilized metrics of disease severity. Antibiotic treatments are either 10-day cefoperazone (0.1mg/ml or 0.5mg/ml), single intraperitoneal injection of clindamycin (0.2mg/kg, 2mg/kg, or 10mg/kg). Mice were infected with either 630g (mild virulence), R20291 (moderate virulence), or VPI 10463 (high virulence) strain of *C. difficile*. Cef = cefopearzone, Clinda = Clindamycin.

enterotypes 2, 3, and 4 result in high *C. difficile* colonization at one day post *C. difficile* spore challenge.

Once the mice were colonized, peak disease develops at four days post infection (DP4) for 630g infection and at two days post infection (DP2) for R20291 and VPI 10463 infection. Systemic disease is measured by clinical score, which is composed of scores for posture, coat, activity, eyes/nose, diarrhea, and weight loss. Low scores indicate no signs of disease, while scores towards the maximum of 20 indicate severe disease. As expected, mice infected with 630g show the lowest signs of disease, mice infected with R20291 show moderate signs of disease, and mice infected with VPI 10463 show severe signs of disease severity (Figure 5.15 A).

In terms of day 0 enterotypes, no differences were statistically noticeable for mice infected with 630g or R20291. However, substantial differences were apparent during VPI 10463 infection. With VPI 10463 infection, mice with day 0 enterotype 1 developed lower clinical scores than mice with day 0 enterotype types 3 and 4 (Figure 5.15 A, $p < 0.0001$). This is consistent as day 0 enterotype 1 mice had lower levels of colonization at day 1 post infection. However, mice with day 0 enterotype 2, which were colonized, also exhibited lower clinical scores compared to mice with enterotype 3 ($p = 0.0013$) and mice with enterotype 4 ($p = 0.0013$).

Local intestinal damage was measured by taking representative histology slides from the cecum and colon and scoring them for edema, epithelial destruction and inflammatory cell infiltration.

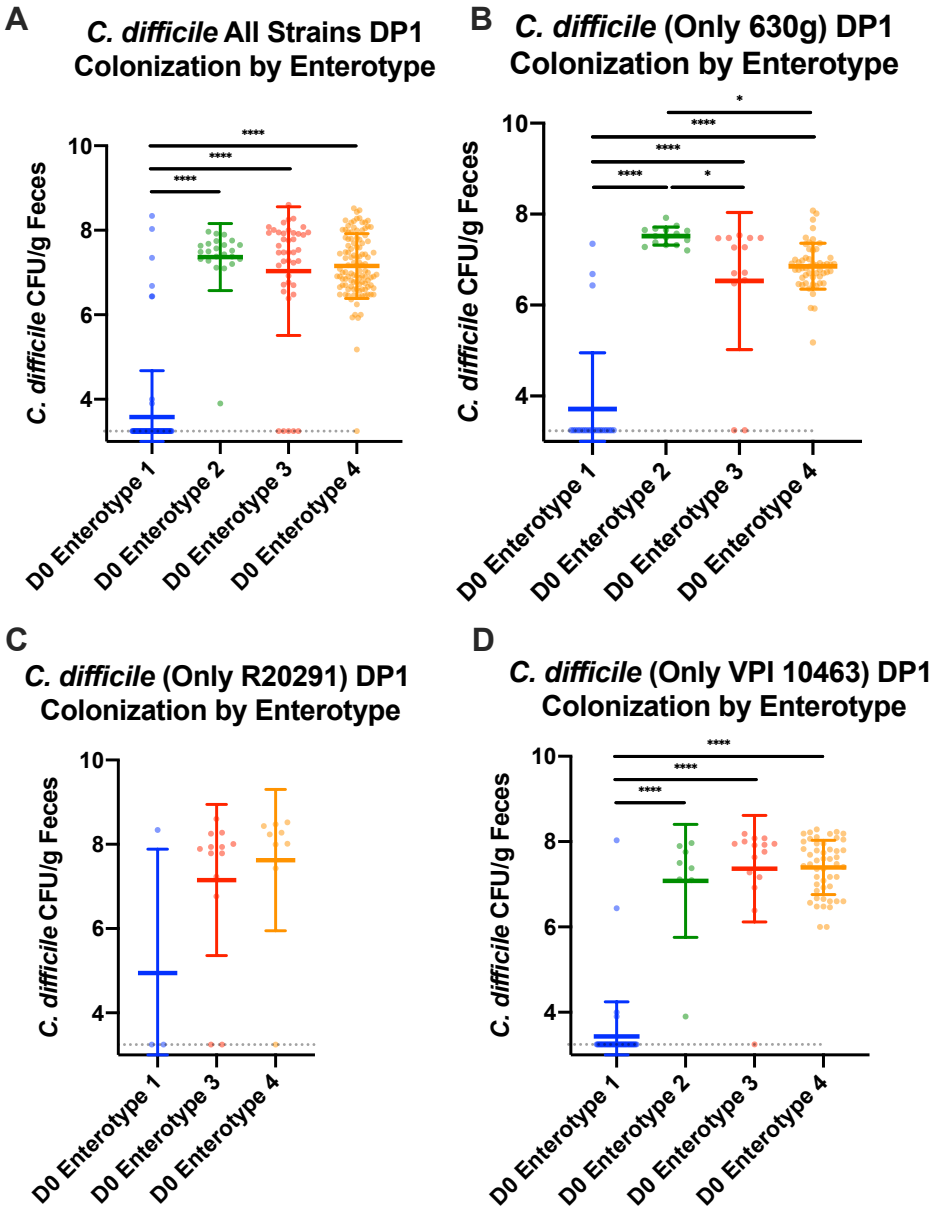


Figure 5.14 *C. difficile* burden by enterotype and infecting strain at one day post infection. Fecal content was taken at one day post infection (DP1) and *C. difficile* CFU/g feces was determined. A) *C. difficile* burden per enterotype with all mice. Plot showing *C. difficile* burden per enterotype for only mice infected with strain 630g (C), R20291 (B), or VPI 10463 (D). Center line represents the mean and whiskers show the standard deviation. Gray dotted line shows limit of detection for CFU/g feces. Analysis consisted of a one-way ANOVA followed by a Tukey multiple comparison adjustment. *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

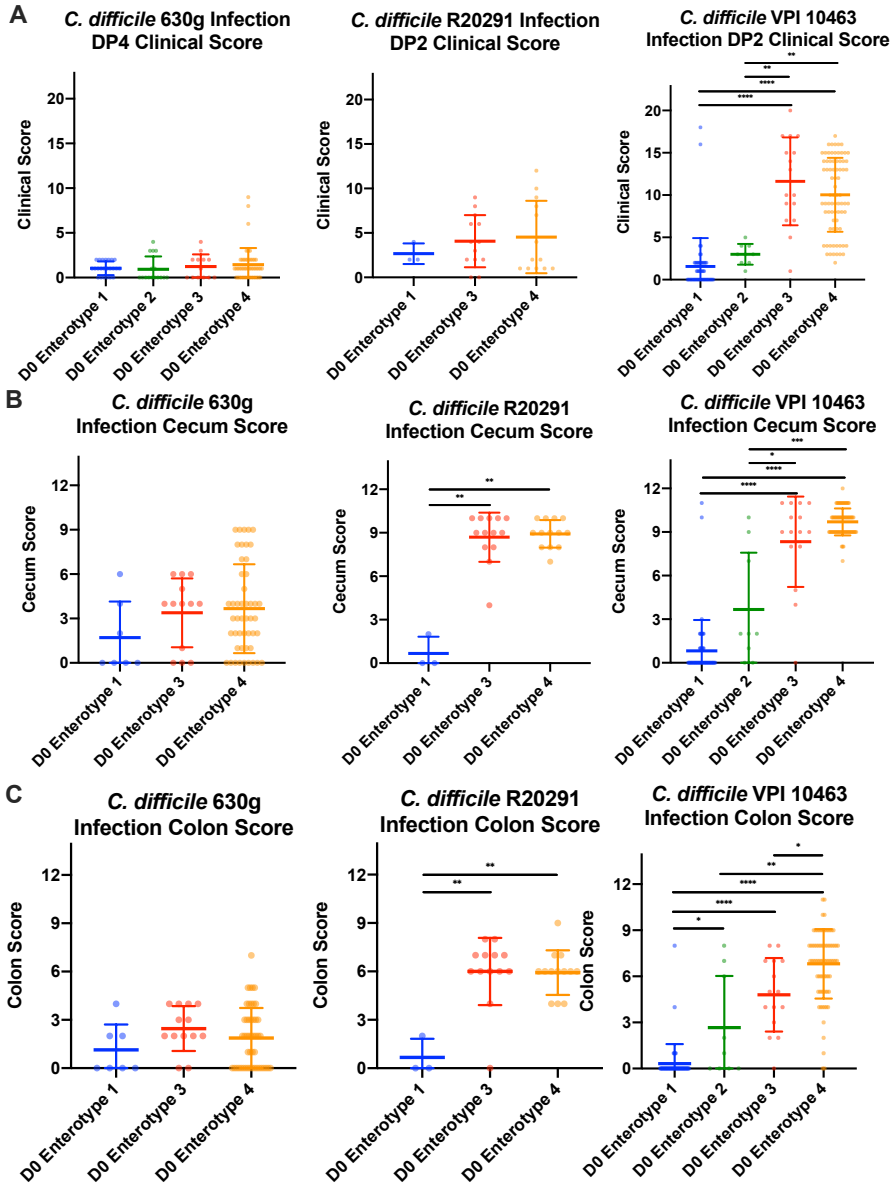


Figure 5.15 *C. difficile* infection systemic and intestinal disease severity for each *C. difficile* infecting strain by day 0 enterotype. A) Clinical score by day 0 enterotypes at time indicated for mice infected with 630g (left), R20291 (middle), or VPI 10463 (right) strain of *C. difficile*. Clinical score is a composite of posture, coat, eyes/nose, activity, diarrheal signs, and weight change with a range from 0 (no disease) to 20 (severe disease). Cecum scores (B) and colon scores (C) by day 0 enterotypes at euthanization for mice infected with 630g (left), R20291 (middle), and VPI 10463. Cecum and colon slides were scored for edema, epithelial damage, and inflammatory cell infiltration with scores ranging from 0 to 12. Gray dotted line shows limit of detection for CFU/g feces. Analysis consisted of a one-way ANOVA followed by a two-stage step-up method (Benjamini, Krieger, and Yekutieli) false discovery rate adjustment for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Mice infected with 630g showed the lowest signs of cecal (Figure 5.15 B) and colon (Figure 5.15 C) damage, with no differences seen between day 0 enterotypes. Mice infected with R20291 had limited cecal and colon damage with day 0 enterotype 1, but higher cecal and colon damage with day 0 enterotypes 3 and 4 (cecal: $p = 0.0034$ and $p = 0.0034$, colon: $p = 0.0042$ and $p = 0.0088$). Only one mouse with enterotype 1 infected with R20291 was colonized and did have lower cecum and colon damage compared to the other enterotypes.

For mice infected with *C. difficile* VPI 10463, mice with day 0 enterotype 1 exhibited low cecum and colon damage as expected for the lower colonization levels. The cecum scores were lower for mice with day 0 enterotype 1 compared to day 0 enterotype 3 ($p < 0.0001$) and day 0 enterotype 4 ($p < 0.0001$). Interestingly, while mice with day 0 enterotype 2 were colonized, the cecum score was substantially lower compared to mice with day 0 enterotype 3 ($p = 0.0294$) and day 0 enterotype 4 ($p = 0.0009$). Colon damage was similarly lower in mice infected with VPI 10463 with day 0 enterotype 1 compared to those with enterotype 2 ($p = 0.0423$), enterotype 3 ($p < 0.0001$), and enterotype 4 ($p < 0.0001$). Interestingly, there is an increase colon damage score as you move from enterotype 0 to enterotype 4 with VPI 10463 infection. In the setting of VPI 10463 infection, mice with day 0 enterotype 2 have lower colon scores than enterotype 4 ($p = 0.002$), and mice with day 0 enterotype 3 have lower colon scores than mice with enterotype 4 ($p = 0.032$).

In summary, mice with day 0 enterotype 1 have low colonization and low clinical, cecum, and colon scores. Mice with day 0 enterotype 2 are colonized with *C. difficile* but do not show high disease severity. Mice with day 0 enterotype 3 and 4 show severe signs of disease when infected with R20291 and VPI 10463, with enterotype 4 associated with even higher colon scores than enterotype 3.

## 5.5 Discussion

*C. difficile* infections have a high incidence and result in a wide range of outcomes from mild diarrhea to severe colitis and death. The intestinal microbiota is vital to both colonization resistance against *C. difficile* and for the complete recovery after *C. difficile* infection. However, a gap in our knowledge is the association of the microbiota with age and subsequent disease severity.

In this work we show that old mice have a different baseline enterotype from young mice, associated with higher relative abundance of *Lactobacillaceae*, *Bacteroidaceae*, and *Lachnospiracea* and lower relative abundance of *Porphyromonadaceae*, *Verrucomicrobiaceae*, *Erysipelotrichaceae*, and *Bifidobacteriacea*.

### 5.5.1 Enterotype Correlations

Following treatment with either no antibiotics, clindamycin, or cefoperazone, new enterotypes are formed that are associated with subsequent *C. difficile* colonization and disease severity. The day 0 enterotypes are separated by their characteristics (their overall microbial diversity and the specific families of bacteria dominating their microbial structure) as well as by the outcomes occurring most commonly in each enterotype. In Figures 5.11 and 5.12, we showed the relative abundances for bacterial families by enterotypes. In Figure 5.16, we perform the same analysis but at genus-level instead of at Family-level to better determine the effects of specific genuses.

#### 5.5.1.1 Enterotype 4

Low microbial diversity correlates with worse responses to CDI, both in terms of infection rates and disease outcomes (Chang et al., 2008; Theriot & Young, 2015; Kriss et al., 2018). This holds true in adult and elderly patients (Rea et al., 2012). In Figure 5.16, *Lactobacillus* is the dominant microbe in the Day 0 Enterotype 4 community to almost total exclusion of all other genuses. *Lactobacillus* correlates to susceptibility to CDI (Seekatz & Young, 2014), and low diversity also correlates to CDI Hopkins & Macfarlane (2002) found that in older patients, CDI correlated with presences of *Lactobacillus*, as well. That our Enterotype 4 suffered the worst disease of all the enterotypes is not surprising based on its low microbial diversity pre-infection and the presence of solely members of Genus *Lactobacillus*. Enterotype 4 was composed of mostly young mice.

#### 5.5.1.2 Enterotype 3

The cohort included in Enterotype 3 was successfully infected with *C. difficile*, and had disease with the second-worst severity among all enterotypes. Compared to Enterotype 4, which had very low microbial diversity pre-infection, Enterotype 3 had a more diverse microbiota before infection

with *C. difficile*. The fact that the Enterotype 3 mice were healthier than the Enterotype 4 mice is consistent with results in humans that healthier older patients tend to have more diverse microbiota (Rea et al., 2012). Enterotype 3 was composed roughly half of young mice, and half of old mice. The results of Hopkins & Macfarlane (2002) showed that while healthy elderly patients tend to have diverse *Bacteroidetes* (seen in Enterotype 1 of this work, but not in Enterotype 3), older patients with CDI damage tended to have more *Lactobacillus*, *Enterobacteriaceae*, and *Enterococcus*. In Figure 5.16, we show that Enterotype 3's two most plentiful bacterial genuses pre-infection are *Lactobacillus* and unclassified *Enterobacteriaceae*. Of all four enterotypes, Enterotype 3 has the highest level of Genus *Streptococcus*, which correlates with higher levels of CDI (Schubert et al., 2015).

#### 5.5.1.3 Enterotype 2

Enterotype 2 contained mice that were successfully colonized by *C. difficile*, but whose disease severity was relatively low. The mice in this enterotype were treated with high doses of Clindamycin (10 mg/kg). The majority of the microbiota pre-infection in this enterotype was genuses *Lactobacillus* and *Staphylococcus*, with varying genuses within Family *Enterobacteriaceae* being the single most populous species. The presence of a large number of *Enterobacteriaceae* is unique to Enterotype 2, as the other enterotypes have very minimal values. Hopkins & Macfarlane (2002) found that *Enterobacteriaceae* generally correlates with CDI in elderly human patients, and Reeves et al. (2011) also found a correlation between *Enterobacteriaceae* and susceptibility to CDI in mice.

#### 5.5.1.4 Enterotype 1

From Figure 5.16, we see that Day 0 Enterotype 1 has as its most plentiful genuses *Lactobacillus*, unclassified *Porphyromonadaceae*, and *Akkermansia*. In the first two cases (*Lactobacillus*, unclassified *Porphyromonadaceae*), our findings are consistent with previous literature which has found some species of *Lactobacillus* to be protective against and even antagonistic to *C. difficile* (ex: *Lactobacillus* reuteri; Rao & Young, 2017; Mills et al., 2018), and *Porphyromonadaceae* to generally be protective (Seekatz & Young, 2014; Rodriguez et al., 2016). Enterotype 1 is most similar to the initial baseline enterotypes. Enterotype 1 also has the largest population of *Bifidobacteriaceae*, which

was also present in the baseline enterotypes and more plentiful in baseline Enterotype 1 (which was associated with younger mice). *Bifidobacteriaceae* interact positively with butyrate-producing anaerobic bacteria, the presence of which correlates negatively with CDI (Antharam et al., 2013).

*Akkermansia* (first identified as a single-species genus in 2004; Derrien et al., 2004)is an apparently beneficial microbe (Naito et al., 2018)which correlates against diabetes and obesity (Yassour et al., 2016) and has effects that may be context-dependent (Schubert et al., 2015). In mouse experiments, it was protective against immune-mediated liver damage (Wu et al., 2017). [1] Donta et al. (1982) found that TcdB can damage rat liver cells. This work finds that the presence of *Akkermansia* in the Day 0 microbiota of the mice correlates with subsequent non-colonization after exposure to *C. difficile*. Although previous work has not robustly addressed the effect of *Akkermansia* on CDI susceptibility and severity, here we find that the presence of *Akkermansia* pre-infection correlates with non-susceptibility to CDI in our mouse model.

### 5.5.2 Future Directions

The results of this work show the main modes (enterotypes) of *C. difficile* infections across antibiotic, CDI strain, and mouse state. From our pre-infection study of mice microbiota, we can classify the mice into four main modes of reaction to CDI. The clear next step of this work is to construct a predictive model that takes the information gained from the enterotype analysis about the correlations between pre-infection state and post-infection disease severity and use it to predict an individual's outcome before the disease has progressed significantly. This type of predictive model would help assign treatment urgency and priority of a larger cohort of patients.

Interestingly, a number of mice treated with cefoperazone maintained their microbial composition similar to baseline enterotypes, resulting in the day 0 enterotype 1. This indicates that even with high dose cefoperazone, some mice will not be as drastically impacted. This is a potential future research direction, looking at the reason why specific mice fail to respond to cefoperazone.

Future work could also determine the extent to which the protective correlative effect of *Akkermansia* against CDI depends on the larger microbial context – that is, does *Akkermansia* act against

[1] We note that *Akkermansia*'s family is sometimes called *Akkermansiaceae*, but has in the past been categorized as *Verrucomicrobiaceae* and is listed as such in the Silva reference files used in this work and so is categorized as *Verrucomicrobiaceae* in the plots in this chapter.

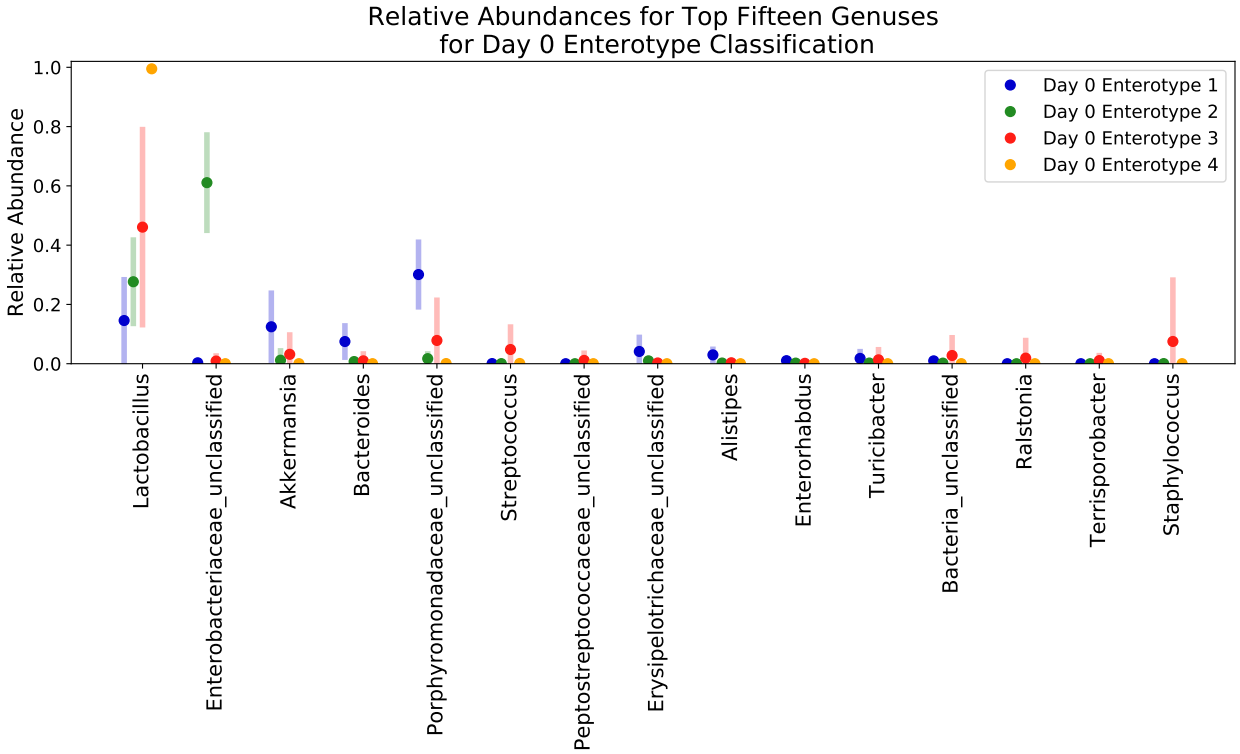


Figure 5.16 Relative abundance of the top fifteen most important bacterial genuses for day 0 enterotype classification. The mean is represented as a point and the bars represent standard deviation. This figure was constructed the same way as Figure 5.11 and Figure 5.12, but one level lower on the phylogenetic tree.

CDI on its own, or in which specific microbial composition does it act protectively?

Although Day 0 Enterotypes 3 and 4 have similar levels of disease severity, they may have different times and extent of colonization resistance recovery. Another fruitful avenue of future study is to examine the long-term outcomes of mice in these groups (perhaps with lower levels of infection so the experiment does not have to be truncated due to mouse health), and determine if and when the mice get back to their baseline microbiota or what other factors in recovery differ between the enterotypes. Additionally, it could be useful to look at giving therapies or probiotics to mice in these groups at day 0 before any progression of CDI and see if you can arrest damage or move mice into Enterotype 1.

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# CHAPTER VI

# Conclusion

The work presented throughout this thesis is the culmination of my graduate training studying the pathogenesis and severity of *Clostridioides difficile* infections (CDI). My graduate journey began with studying the current state of *C. difficile* infections within the United States and progressed towards elucidating the associated drivers of disease severity. In this thesis, I examined the burden that *C. difficile* infections place on the healthcare system, which has led to rapid innovation targeting each aspect of the pathogenesis, including preventing by restoring the natural microbiota before colonization, treating acute disease through novel antibiotics which have lower impact on the native microbiota, and reducing recurrence (Chapter II). I then used patient data to identify patients at high risk and who require more aggressive therapy, which I found could be done at time of diagnosis using serum biomarkers that predict adverse *C. difficile* infection outcomes (Chapter III). I then studied the impacts that aging has on the response to *C. difficile* infection by altering the levels of systemic inflammatory mediators and reducing the population of neutrophils and eosinophils in the injured intestines (Chapter IV). Then, I studied specific host factors (age and sex) and antibiotic treatments that contribute to the development of specific enterotypes in mice that correlate with *C. difficile* colonization and disease outcomes. I subsequently found that both systemic biomarkers and microbial community structure can be used to estimate adverse outcomes in mouse models of *C. difficile* infections (Chapter II). This concluding chapter will summarize each of these findings and discuss the implications for future work based on these results.

## 6.1 The emerging landscape of *C. difficile* infection prevention, acute therapy, and recurrence reduction

With the rising incidence during the early 2000s following CDU epidemics and the emergence of NAP1/BI/027 strains of *C. difficile*, the annual number of *C. difficile* infections in the United States has risen to approximately 500,000. While current therapies including the use of fidaxomicin and vancomycin are effective in a majority of cases, there are still substantial rates of mortality and recurrence.

A group of therapies are emerging that aim to prevent the initial colonization of *C. difficile* following disruption of the natural gut microbiota. High risk individuals such as those receiving systemic antibiotics can be treated with medications, such as probiotics (ex: BioK or VSL#3) aimed at restoring the natural microbiota. Similar strategies can be utilized for the restoration of the microbiota following successful *C. difficile* infection therapy for the reduction of recurrence. Bacterial replacements for recurrence are currently being studied (SER-109, CBM588, MET-2, RBX2600, CP101). Further research is necessary to identify the specific bacteria that are necessary for colonization resistance, a goal being pursued by multiple researchers studying both clinical samples and animal models. Identifying specific keystone species will inform future probiotics and bacterial replacement therapies. An alternative strategy is to block the colonization of *C. difficile* through the development of vaccines (ex: VLA84, ACAM-CDIFF, PF-06425090, CDVAX) that could be distributed to those at highest risk (residents of nursing homes for example).

During acute disease, specific therapies can be utilized to reduce the risk of adverse outcomes. A few examples of emerging options are therapies directed at binding to active toxin (Calcium Aluminosilicate, GT267-004, GT160-246), immune modulatory therapies, and antibiotics that can effectively reduce *C. difficile* burden while limiting the deleterious impact on native bacteria (Cadazolid, CRS3123, LFF571, MCB3681, Nitazoxanide, Ramoplanin, Ridinilazole, Surotomycin, Tigecycline). These therapies need to be further tested and appropriately targeted as they can be costly and have unclear side-effect profiles.

We believe that the limited spectrum antibiotics currently being developed will be one of the first classes of therapies utilized in clinics with a substantial impact in reducing recurrence by allowing

the natural microbiota to recover during and shortly after therapy. As mentioned above, another important category of emerging therapy are bacterial replacement therapies. Further clinical trials will help identify the most promising *C. difficile* therapies for prevention, acute treatment, and reduction of recurrence. Continued monitoring of these trials is paramount for both clinicians and researchers, as even failures will give additional insight into the pathogenesis of *C. difficile* and help narrow which bacterial species are necessary for colonization resistance and recovery after successful treatment. The next step is judiciously applying these therapies by producing accurate models for the identification of those individuals with *C. difficile* infection at highest risk for adverse outcomes.

## 6.2 Utilizing predictive models and biomarkers to determine patients at high risk of initial infection and subsequent adverse outcomes.

The high focus on preventing and treating *C. difficile* infections has led to extensive efforts to identify patients with *C. difficile* infections and their effective management. With the rapid rise of new diagnostic techniques, clinicians are faced with the difficulty of defining which patients require no treatment (asymptomatic *C. difficile* colonization or *C. difficile* colonization with co-morbid gastrointestinal disease such as inflammatory bowel disease), standard treatment (standard course of antibiotics and supportive care), and aggressive treatment (higher dose antibiotics, enemas, ICU admission, or early surgical assessment).

Extensive research efforts have produced guidelines for defining mild, moderate, and severe *C. difficile* for determining appropriate therapy. Models are being formulated to utilize a variety of data in electronic medical records ranging from past medical history, vital signs, and demographic information to predict the risk of initial colonization to adverse outcomes (LaBarbera et al., 2015; Oh et al., 2018; Li et al., 2019). An additional avenue is utilizing serum biomarkers assess the risk of adverse outcomes once the diagnosis of *C. difficile* has been made. As we have shown, a panel of inflammatory markers including HGF, procalcitonin, IL-8, IL-2R$\alpha$, IP-10, and CXCL-5 can be utilized at time of diagnosis to predict the risk of adverse outcomes in patients with *C. difficile* infections.

Our model was able to outperform both IDSA severity criteria and Elixhauser comorbidity index for the prediction of adverse outcomes (ICU admission, colectomy, or death within 30-days of diagnosis). Using our model, a patient found to be positive would have an increased risk of developing adverse outcomes and could be treated with more aggressive therapy. Additionally, our model could be used to assist clinical trial enrollment for therapies to reduce overall mortality by enriching the patient cohort with higher risk individuals. While our model for predicting adverse outcomes did validate in a mouse CDI experimental cohort, further research needs to be performed to validate the model in external and larger *C. difficile* infection cohorts. This is an important step as any model has the potential to be overfit for the specific cohort it was designed on, necessitating an external validation step in future research. Additionally, the specific biomarkers highlighted are important immune modulators and should be further studied in association with *C. difficile* infection pathogenesis in animal models. Specifically, research into the development of a rapid biomarker panel for clinical decision making may allow physicians to determine the appropriate early interventions for reducing morbidity and mortality in a judicious and repeatable manner.

An aspect of *C. difficile* infection that needs additional study is the impact of co-morbidities on *C. difficile* infection outcomes and treatment success. We know that specific disorders alter the immune response and the gastrointestinal environment, including Crohn's and Ulcerative Colitis. As our model is based on inflammatory mediators, diseases that alter the immune response, such as AIDS and immunosuppressant therapies for transplants, may impact the prediction of adverse outcomes. Additionally, alterations to diet and the local immune response may have drastic and long-standing implications on the intestinal microbial community structure. We have have shown that specific enterotypes within the intestines are associated with colonization and subsequent disease progression in *C. difficile* infection. Further research is needed to understand the implications of age, dietary habits and gastrointestinal diseases, such as inflammatory bowel disease, on *C. difficile* infections.

## 6.3 Aging alters levels of systemic inflammatory mediators and reduces the intestinal localization of neutrophils and eosinophils during severe *C. difficile* infections

Age is also a key player in *C. difficile* infection outcomes as older individuals experience higher rates of mortality, which may be related to immunosenescence, comorbidities and risk of exposure. Age has been shown to be associated with increased risk and severity of *C. difficile* infection (George et al., 1979; Reeves et al., 2011; Warren et al., 2012; Shin et al., 2018; George et al., 1979; Warren et al., 2012; Peniche et al., 2018). These factors need to be studied in relation to predictive models and their effects accounted for in future model iterations. To further the knowledge surrounding the impact of aging on *C. difficile* infection, we utilized our mouse model of *C. difficile* infections.

As *C. difficile* infection pathogenesis begins in the gut, difficulties in appropriately responding to the local disease could have large implications of disease progression. Past studies have shown that *C. difficile* infections are associated with increased neutrophil accumulation within the intestine, which can lead to tissue breakdown and pseudomembranous colitis as the intestinal barrier is disrupted. Intestinal innate immune response and eosinophil response has also been shown to be important for acute disease and recurrence. In our study, we examined the systemic and local immune response to mild and severe *C. difficile* infection in young and age mice. We found, similar to past studies, that severe *C. difficile* infection leads to an increase in systemic immune mediators such as KC (CXCL-1) and subsequent intestinal neutrophil and immune cell populations. However, aged mice were found to have reduced cecal infiltration of neutrophils and eosinophils during severe *C. difficile* compared to young mice. This occurred with altered systemic immune mediator levels, with decreased levels of CXCL-1 (important for neutrophil recruitment and activation) and increased IL-17A and circulating white blood cells and eosinophils. Eosinophils are quickly being appreciated as playing a potentially vital role in *C. difficile* infection, with reports of increased intestinal eosinophils being associated with reduced mortality and peripheral eosinophil counts shown to be predictive of *C. difficile* infection severity and mortality in patients (Buonomo et al., 2016; Cowardin et al., 2016; Kulaylat et al., 2018). One study suggests that *C. difficile* binary toxin suppresses colonic eosinophil response in a toll-like receptor 2 (TLR2) dependent manner, impairing

the potential protective effect of eosinophils during *C. difficile* infection (Cowardin et al., 2016).

Further research is needed to look at the exact mechanism of eosinophil protection and the implications of aging on eosinophil response in *C. difficile* infections. Animal models can be utilized to examine the molecular pathways associated with eosinophil recruitment and subsequent action in the setting of acute *C. difficile* infection and recurrence. Pharmaceuticals impacting eosinophil function can be utilized to assess the impact on *C. difficile* infection severity. Additionally, future research will need to assess the impact of aging not only on acute infection, but on initial colonization and subsequent recovery after successful recovery from *C. difficile* infection.

## 6.4 Intestinal bacterial enterotypes correlate with *C. difficile* colonization and disease outcomes in our murine model

As the disease starts locally within the intestines due to the impact of *C. difficile* toxins, stool biomarkers of inflammation such as fecal calprotectin could also be used to assess the inflammatory state of the intestines. We know that the microbial community structure is vital for colonization and disease development. As such, another avenue of research for predicting adverse outcomes is looking specifically at the microbial community structure, as the components and abundances of bacterial populations will affect the risk of initial infection and disease severity. As 16S rRNA DNA sequencing techniques and the methods of bacterial community type analysis are becoming faster and more readily accessible, fecal community structure may hold a tremendous potential for the prediction of the risk initial infection and adverse outcomes.

In our study presented in chapter 5, we found that at baseline older mice (18-28 months) have on average a different microbial community structure (enterotype) then young mice (2-3 months old) from the same breeding colony and housing facility. The enterotype associated with older mice had higher relative abundance of *Lactobacillaceae*, *Bacteroidaceae*, and *Lachnospiraceae*, while the enterotype associated with younger mice had on average higher relative abundance of *Porphyromonadaceae*, *Verrucomicrobiaceae*, *Erysipelotrichaceae*, and *Bifidobacteriaceae*. At baseline, there were no differences between the distribution of females and males between these enterotypes.

Following antibiotic treatments, we found that a high dose injection of clindamycin (10mg/mk)

produced a distinct enterotype from mice treated with a 10-day course of high dose cefoperazone (0.5mg/ml). The enterotype associated with high dose clindamycin had the highest levels of Enterobacteriaceae and moderate levels of *Lactobacillaceae*. This enterotype was associated with low disease severity when colonized by highly virulent strains of *C. difficile* (R20291 and VPI 10463). This indicates a potentially protective role of the microbial species present in this enterotype (designated day 0 enterotype 2).

In contrast, the enterotype associated with high dose cefoperazone was composed of almost entirely *Lactobacillaceae* and had lower relative abundance of all the other bacterial families examined, indicated a lower diversity of the community. This enterotype was associated with the highest resulting systemic, cecum and colon damage when mice with this enterotype were infected with high virulence *C. difficile* (strains R20291 and VPI 10463). This indicates that low diversity and high abundance of *Lactobacillaceae* could lead to high severity of *C. difficile* infection. Treatment with low dose cefopearzone (0.1mg/kg) resulted in an enterotype with higher levels of *Lactobacillaceae*, *Porphyromonadaceae*, *Streptococcaceae*, *Peptostreptococcaceae*, and *Staphylococcaceae*. Mice with this enterotype also developed high disease severity when infected with highly virulent strains of *C. difficile*.

However, not all mice receiving high dose cefoperazone developed these susceptible enterotypes associated with severe disease. In fact, a number of mice treated with both high and low dose cefoperazone maintained an enterotype similar to the baseline enterotypes that was resistant to *C. difficile* colonization. Similarly, mice treated with low dose clindamycin (0.2mg/kg or 2 mg/kg) maintained this protective enterotype, indicating the importance of antibiotic dose for rendering an individual susceptible to *C. difficile* infection. Interestingly, *Akkermansia* was the third most important genus of bacteria for the classification of this enterotype and was found to be the highest in this colonization resistant enterotype.

These results indicate that age is an important factor in the microbial community structure at baseline and that the specific antibiotic and dose impact the development of susceptible enterotypes. These enterotypes are not only related to the risk of becoming infected with *C. difficile* but are associated with the degree of disease severity that may develop. Further research is needed to determine what specific attributes of these enterotypes, such as specific bacterial species, are either

protective or permissive to severe disease development in *C. difficile* infections. Disease outcomes in mice infected with *C. difficile* are affected by various host and pathogen features. In particular, it would be useful to determine the predictive capacity of specific host features (age and sex), pathogen features (strain virulence), and microbial community features (baseline and following antibiotics). Additional studies can be performed to monitor the recovery of these enterotypes following antibiotic administration to see if there is a difference in time and extent of recovery of original colonization resistance.

## 6.5 Final Thoughts

Tremendous effort is being made to reduce *C. difficile* infections, including reducing the incidence of initial infection, severe disease outcomes, and recurrence after successful treatment. The findings of this thesis showcase the complexity of *C. difficile* infections and the difficulty in appropriately aiming therapies to reduce mortality and morbidity. We find promising results for the application of a serum biomarker model to determine high-risk individuals at time of *C. difficile* infection diagnosis. If an cost-effective and rapid panel can be developed using these markers, its application would allow for allocating expensive and higher-risk emerging therapies effectively to individuals who will develop adverse CDI outcomes. Utilizing our mouse cohort, we show that age, sex, infecting strain, and microbial community structure impact the risk of initial *C. difficile* colonization and subsequent CDI disease progression. These findings highlight that one key aspect to understanding CDI is full exploration of the heterogeneity found within the host immune response, intestinal microbial community, and the specific infecting strain of *C. difficile*.

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