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# Investigating Microbial and Host Factors that Modulate Severity of *Clostridioides difficile* Associated Disease

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# INVESTIGATING MICROBIAL AND HOST FACTORS THAT MODULATE SEVERITY OF *CLOSTRIDIOIDES DIFFICILE* ASSOCIATED DISEASE

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# INVESTIGATING MICROBIAL AND HOST FACTORS THAT MODULATE SEVERITY OF *CLOSTRIDIOIDES DIFFICILE* ASSOCIATED DISEASE

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*Clostridioides difficile* is recognized as one of the most important pathogens in hospital and community healthcare settings. The clinical outcome of infection of toxigenic *C. difficile* infection (CDI) ranges from asymptomatic colonization to fulminant pseudomembranous colitis and death. In recent studies, it has been suggested that a high proportion of nosocomial CDI cases are transmitted from asymptomatic carriers which might be acting as infection reservoirs. Understanding what causes the different responses to infection could lead to the development of novel prevention and treatment strategies. Although several explanations have been proposed to explain variations in susceptibility, understanding of the exact mechanisms that underlie the spectrum of variation in CDI disease severity remains limited and further research is needed to determine what factors are responsible for these variations. In this work, we establish different human microbiota-associated (HMA) mouse models. By analyzing innate immune responses to CDI, we demonstrate that these models reproduce differences in disease severity during infection observed in human patients. These differences were largely based on mouse strain (C3H/HeN and C57BL/6J) and independent from *C. difficile* burden or toxin activity. Altogether, our $^{HMA}$mouse models demonstrated the potential to study interactions between microbiome, pathogen and host inflammatory responses in the context of CDI.

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# CHAPTER 1. UNDERSTANDING DISEASE OUTCOMES FOLLOWING INFECTION WITH THE GASTROINTESTINAL PATHOGEN, *CLOSTRIDIOIDES DIFFICILE*

## 1.1 INTRODUCTION

*C. difficile* is a spore-forming, toxin-producing, Gram-positive anaerobic bacillus that causes infectious diarrhea and colitis primarily in health care settings; it is generally associated with the disruption of a "healthy" gut microbiome caused by antibiotic consumption and can cause infections that range from mild diarrhea to pseudomembranous colitis and potential death (Crobach et al., 2018; Collins & Auchtung, 2017). The rise in frequency of *C. difficile* infections that occurred during the first decade of the 21$^{st}$ century led to major concerns about the impact *C. difficile* infections have upon public health and renewed interest in the pathogenesis of *C. difficile* infection (CDI). While strategies to reduce disease transmission have had some success in reducing rates of infection (Al-Omari et al., 2020; Morgan et al., 2019), CDI remains one of the most common causes of hospital acquired infections and is one of only six antibiotic-resistant pathogens designated by the Centers for Disease Control as an urgent threat to public health (2019 CDC report). This review will discuss the history and pathogenicity of *C. difficile*, describe variations in disease outcome and summarize current understanding of potential mechanisms that influence disease severity. In addition, I will examine the role of the host inflammatory responses to CDI in depth to introduce the main focus of this thesis.

## 1.2 *C. DIFFICILE* INFECTION AND DISEASE

### 1.2.1 Burden to public health

*C. difficile* infection has become the leading infectious cause of nosocomial diarrhea in developed countries over the past 20 years , especially in healthcare facilities where *C. difficile*-related excess medical costs were estimated to be as much as one billion dollars in the United States annually presenting a significant financial burden on the healthcare system (Czepiel et al., 2019; CDC, 2019). Asymptomatic carriers, infected patients, contaminated environments and some farm animals are potential infection reservoirs for *C. difficile* (Lim et al., 2020; Bauer & Kuijper, 2015; Knetsch et al., 2014; Samore et al., 1996). Prevalence of *C. difficile* in these reservoirs can be undetectable without strict surveillance.

### 1.2.2 History of disease

Although *C. difficile* was identified in healthy neonates in the 1930s, it was not considered a pathogenic organism until the late 1970s when Bartlett and colleagues isolated *C. difficile* from the stool of patients with pseudomembranous colitis and demonstrated its cytotoxicity *in vitro* and in a hamster model of disease (Hall & O'Toole, 1935; Bartlett et al., 1978). Previously, *C. difficile* was rarely found in the normal intestinal microbiomes of adults, but as the use of antibiotics increased, its role in the pathogenesis of intestinal diseases became more evident (George et al., 1978; Larson et al., 1978). During the last two decades of the twentieth century, *C. difficile*'s clinical relevance was limited to mild and easily controlled cases; however, the incidence and severity of *C. difficile* infection steadily increased at the beginning of the twenty-first century. Increased incidence of infection, disease severity and mortality levels were linked to the emergence of a new *C. difficile* lineage, epidemic ribotype 027 *C. difficile*

(alternatively referred restriction endonuclease analysis group BI or North American pulsed-field electrophoresis 1 or NAP1/BI/027), that was responsible for several outbreaks in North America and Europe (Pepin et al., 2005; Warny et al., 2005, He et al., 2013; McDonald et al., 2005). This ribotype showed high level fluoroquinolone resistance, production of a binary toxin previously rare in *C. difficile* strains and was described as hypervirulent due to increased disease severity typically observed in patients infected with this ribotype. (Warny et al., 2005; Cowardin et al., 2016; He et al., 2013; Spigaglia et al., 2008). Some initial studies pointed to increased sporulation efficiency and production of higher levels of toxins as possible factors contributing to hypervirulence, although later studies indicated that both sporulation efficiency and levels of toxin produced vary among ribotype 027 isolates. Subsequent studies highlighted increased fitness and expanded metabolic capacity as potential factors that led to the emergence of ribotype 027 strains (Robinson et al., 2014; Collins et al., 2018). While some regions have seen declines in the prevalence of infections caused by ribotype 027 strains (Freeman et al., 2020; Eyre et al., 2018), other ribotypes have increased in prevalence and *C. difficile* infection remains one of the most common hospital acquired infections (Lessa et al., 2015).

### 1.2.3 Pathogenicity of toxins.

*C. difficile* enters the body through the ingestion of spores that can be transmitted environmentally or from other patients via fecal-oral route; these spores are able to survive the acidic conditions of the stomach and germinate into vegetative cells once they reach the distal small intestine (Lessa et al., 2012; Sorg & Sonenshein, 2008). The primary site of disease during *C. difficile* infection is the human colon; the anoxic

environment in the lumen permits obligate anaerobic bacteria such as *C. difficile* to survive and, under the right conditions, proliferate and produce enterotoxins that damage the intestinal epithelium in several ways (Smits et al., 2016). Disease is primarily mediated by two virulence factors, toxins A and B, which are encoded by *tcdA* and *tcdB* genes (Fig. 1.1a) (Kuehne et al., 2010). These genes form a chromosomal pathogenicity locus with two regulatory genes *tcdC* and *tcdD* in addition to a *tcdE* porin gene (Govind & Dupuy, 2012). Both toxins are glucosyl transferases that bind to the surface of the intestinal epithelial cells where they are internalized; once internalized, they catalyze the glucosylation of small GTPase proteins of the Rho and Rac family, irreversibly inactivating them (Pruitt & Lacy, 2012). The activity of the toxins leads to depolymerization of the epithelial actin cytoskeleton, disruption of tight junctions and severe epithelial damage due to apoptosis; these mechanisms of compromising intestinal membrane integrity make the host susceptible to transmission of intestinal microorganisms across the epithelial barrier, while also promoting activation of an inflammatory response in the form of cytokines and chemokine production and neutrophil recruitment (Fig. 1.1b) (Abt et al., 2016; Voth & Ballard, 2005; Kelly & Kyne, 2011).

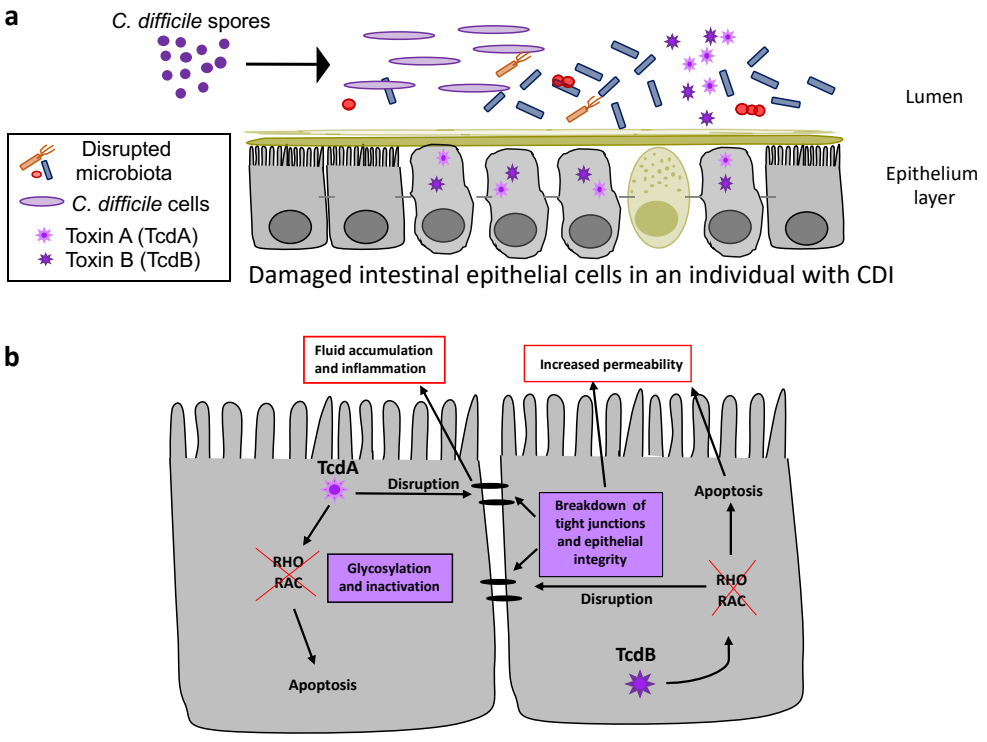


**Figure 1.1 Pathogenicity of *C. difficile* toxins (a)** The indigenous microbiota and its ability to protect against pathogens and other harmful agents can be dramatically reduced by antibiotic treatments. Once a susceptible host becomes exposed to *C. difficile* spores, the favorable environment allows spores to germinate, colonize and subsequently produce enterotoxins that damage the intestinal epithelium. **(b)** *C. difficile* toxins A and B glucosylate and thereby inactivate RHO or RAC GTPases resulting in the breakdown of tight junctions and loss of epithelial integrity leading to inflammation and increased permeability of the host epithelium.

The potential relative contribution of *tcdA* and *tcdB* in *C. difficile* pathogenesis *in vivo* has been investigated in different animal models. In mouse and human colonic organoids, it has been shown that *C. difficile* strains that lack functional TcdA activity (such as strains in RT017) can induce severe damage to the colonic epithelium and cause a dysfunctional stem cell state that impairs epithelial homeostasis by relying solely on a TcdB-mediated process (Lyras et al., 2009; Mileto et al., 2020). Further, infection of humans with RT017 strains results in clinical disease, indicating that TcdA is not required for pathogenesis. However, deletion mutagenesis studies have shown that deletion of either *tcdA or tcdB* reduce pathogenicity, leading to conflicting interpretations

of whether both TcdA and TcdB were required to cause disease or could independently contribute to disease progression (Lyras et al., 2009, Kuehne et al., 2010). The inconsistent findings point to virulence differences resulting from variations in *C. difficile* strains, experimental animal models, host microbiota composition and antibiotic sensitivity. Overall, it has been demonstrated that TcdB has a major role mediating inflammation and mortality in the murine model and that TcdA has a lighter inflammation impact in mice but is slightly more toxic in hamsters (Carter et al., 2015). Whether TcdA increases the severity of human disease is currently unknown; there have been no known outbreaks caused by strains that encode only TcdA.

In addition, a different binary toxin (also known as *C. difficile* transferase CDT) has also been associated with severe CDI development in some *C. difficile* strains (primarily epidemic ribotypes 027 and 078). This binary toxin ADP-ribosylates actin leading to disruption of the actin cytoskeleton (Gerding, 2014; Abt et al., 2016). Binary toxin is encoded by chromosomal genes *cdtA* and *cdtB,* which are distinct from the chromosomal *tcd* pathogenicity locus (Gerding, 2014; Abt et al., 2016). However, the role of binary toxin in virulence remains unclear (Czepiel et al., 2019).

### 1.2.4 Range of clinical outcomes

Not all patients who become infected with *C. difficile* develop the associated disease. Once a susceptible individual is exposed to *C. difficile*, a wide range of clinical disease outcomes can occur. It has been documented that up to 20% of susceptible individuals who become colonized do not develop diarrhea, and up to two thirds of patients with nosocomial *C. difficile* colonization were asymptomatic for disease (McFarland, et al., 1989; Buggy, et al., 1983). On the other hand, most patients that

develop *C. difficile* infection experience mild diarrhea after an incubation period of 2-3 days; a longer incubation period might also occur, but it varies from person-to-person (McFarland et al., 1989; Samore et al., 1994). Some *C. difficile* infected patients develop abdominal pain, fever, nausea, vomiting, weakness, and loss of appetite generally during or directly after antimicrobial therapy. In the most severe cases, life-threatening symptoms such as dehydration, abdominal distention, hypoalbuminemia with peripheral edema and subsequent circulatory shock can occur leading to severe complications in the form of toxic megacolon, colon perforation, kidney failure, systemic inflammatory response syndrome, septicemia or death (McDonald et al., 2018). Although potential explanations for the variations in susceptibility and disease progression have been proposed, the mechanisms that modulate *C. difficile* infection outcome remain poorly understood and are the focus of ongoing investigations.

## 1.3 C. DIFFICILE CARRIAGE/COLONIZATION

### 1.3.1 Colonization resistance and disruption by antibiotic use.

Antibiotic use is a primary risk factor for developing *C. difficile* infection; this increased risk for infection is likely due to disruption of the indigenous microbiome and subsequent loss of colonization resistance (Abt et al., 2016). One mechanism through which antibiotic treatments alter colonization resistance is to alter the intestinal metabolome (the metabolic products produced by the GI microbiome) which creates a suitable environment for *C. difficile* growth (Theriot et al., 2014). For instance, commensal bacteria can release sialic acid from digestion of host intestinal mucous and convert complex carbohydrates into short-chain fatty acids, two metabolites that are quickly consumed by other members of the microbiome as energy sources (Ng et al.,

2013). However, antibiotic consumption can reduce the number of these competing bacteria causing an excess abundance of those metabolites which *C. difficile* is able to use for growth in the absence of competing organisms (Ferreyra et al., 2014; Theriot et al., 2014; Ng et al., 2013; Wilson & Perini, 1988). Other interactions between the commensal microbiome and *C. difficile* can also limit proliferation, such as bacteriocin production (Corr et al., 2007; Rea et al., 2011). In addition, interactions between the gastrointestinal microbiome and host immune response can influence susceptibility to *C. difficile* infection and can be disrupted by antibiotic treatment; these interactions will be discussed in more detail below (Abt et al., 2016). Therefore, the ability of *C. difficile* to colonize the large intestine can be repressed by both direct and indirect mechanisms of the intestinal microbiome which are disrupted under antibiotic treatments. Most antibiotics have been associated with the development of *C. difficile* infection, including those that are used for treatment. The use of broad spectrum antibiotics, such as penicillins and cephalosporins, clindamycin and fluoroquinolones, has a much higher risk of making the host susceptible to *C. difficile* infection than other antibiotics (Leffler & Lamont, 2015). By restoring the microbiome, through fecal transplantation, the host can re-establish the resistance mechanisms that inhibit *C. difficile* colonization (van Nood et al., 2013).

### 1.3.2 Potential mechanisms of asymptomatic carriage/colonization

Several recent studies have documented that, in contrast with a widely held assumption, a large proportion of hospital associated CDI cases are not due to transmission from other symptomatic CDI cases, but rather from asymptomatic carriers (Curry et al., 2013; Didelot et al., 2012). Although the definition varies among studies, asymptomatic carriers or asymptomatically colonized patients are patients where *C.*

*difficile* and its toxins are detected in stool, but no clinical symptoms appear (Furuya-Kanamori et al., 2015; Crobach et al., 2018). This subset of individuals usually remains undetected unless strict surveillance and infection control methods are implemented. These patients can potentially act as an infection reservoir contributing significantly to the chain of transmission (Didelot et al., 2012; Ziakas et al., 2015; Cohen et al., 2010). Current practice largely underestimates the importance of detecting asymptomatic *C. difficile* colonization in healthcare settings and improvements are needed to minimize the risks of infection caused by asymptomatic patients (Ziakas et al., 2015).

It is not known why toxigenic strains, despite being able to colonize and produce toxins in susceptible individuals, cause severe disease in some patients but not in others. However, potential explanations have been proposed; these explanations include the fact that non-toxigenic *C. difficile* strains or non-pathogenic organisms could be outcompeting toxigenic *C. difficile* strains and allowing them to persist at levels too low to cause disease and/or that differences in immune responses and other host factors could allow asymptomatic colonization in some individuals and not in others (Britton & Young, 2012).

The potential to use a non-toxigenic *C. difficile* strain as a competitor to toxigenic strains has been proposed as a novel prevention and treatment strategy. Pre-colonization with these organisms could exclude toxigenic *C. difficile* strains by outcompeting them a nutritional niche; in addition, non-toxigenic strains could inhibit toxigenic *C. difficile* germination or growth (Sambol et al., 2002). However, Brouwer and colleagues found that transconjugation of the pathogenicity locus from toxigenic to non-toxigenic *C. difficile* strains is possible (Brouwer et al., 2013), indicating that broad-scale

implementation of this approach may not reduce the overall rates of symptomatic *C. difficile* infection.

Other research groups have found that differences in microbiota such as enrichment of *Eubacterium* species and *Clostridium* species other than *C. difficile* distinguish between symptomatic and asymptomatic infection and might have a role in determining disease outcome either by regulating levels of *C. difficile* and/or its toxin or by blocking pathogenic interactions with the host epithelium or immune system (Zhang et al., 2015; Vincent et al., 2016). Buffie and colleagues were able to identify the commensal species *Clostridium scindens*, which promotes conversion from primary bile acids to secondary bile acids, as a *C. difficile* infection-resistant organism, but also concluded that bile acids may not be the only metabolites having a role in the inhibition of *C. difficile* expansion (Buffie et al., 2015).

In addition, substantial evidence has arisen that asymptomatic *C. difficile* colonization has a protective effect against progression to disease through an immune-mediated response (Kelly et al., 2019). Hospitalized patients who later became asymptomatically colonized with *C. difficile* had higher levels of Immunoglobulin G (IgG) to toxin A upon admission than in patients that subsequently developed diarrhea. However, this was only observed in newly *C. difficile*-exposed patients (Kyne & Kelly, 2000). Additionally, Kyne et al. found that patients that became colonized with *C. difficile* and presented low levels of serum IgG antibody against toxin A had a higher risk of developing *C. difficile* diarrhea (Kyne et al., 2000). Mulligan and colleagues observed that the mean optical density for IgA reactive with *C. difficile* somatic-cell antigens was

significantly lower in patients with symptoms than asymptomatic carriers (Mulligan et al., 1993). A study on C57BL/6J mice found that a protective immune response against CDI was generated that included IgG and IgA serum anti-toxin antibodies, as well as mucosal IgA anti-toxin antibodies (Johnston et al., 2014).

Yu et al. reported that CDI elicits a cascade of systemic cytokine production including upregulation of IL-1β, IL-8, IL-16, and IL-17A. These are main cytokines that could serve as markers that mediate *C. difficile* disease (Yu et al., 2017). In addition to these cytokines, upregulation of specific chemokines (Cxcl1, Cxcl2, Ccl2), IL-23, and antimicrobial peptides (Reg3γ) have also characterized severe *C. difficile* infection in numerous human and mouse studies (Sadighi Akha et al., 2013; McDermott et al., 2014, 2015, 2017). Neutrophil infiltration along with tissue damage are also prominent characteristics of patients with pseudomembranous colitis (McDermott et al., 2016). Evaluating these known responses in an asymptomatic colonization model of *C. difficile* could give us a greater insight into how host-specific immune response protect against severe CDI.

### 1.3.3 Microbiome modulation of the mucosal immune response and its impact on *C. difficile* associated disease

As described above, *C. difficile* infection can initiate signaling cascades that result in pro-inflammatory cytokine and chemokine production (Hasegawa et al., 2011). This cytokine signaling also leads to neutrophil infiltration, a hallmark characteristic of *C. difficile* severe infection (Solomon et al., 2013). Further evidence for the role that neutrophils play in disease progression was obtained in studies with IL-23 knock out

mice where neutrophil infiltration was repressed and colonic histopathology was reduced (McDermott et al., 2016). However, transgenic mouse studies in which neutrophil infiltration was reduced have also shown decreased *C. difficile* clearance and an increased mortality rate (Jarchum et al., 2012; Hasegawa et al., 2011). This data suggests that the mechanisms through which neutrophil recruitment is modulated might have an essential role in balancing *C. difficile* clearance with excessive damage to the colonic epithelium.

Data from additional mouse studies have demonstrated that timing and magnitude of immune responses might have a significant role in regulating *C. difficile* disease outcomes. Mice deficient in innate lymphoid cell (ILC) signaling rapidly succumb to disease following infection. This deficiency can be rescued through restoration of Type 1 ILCs, suggesting that these cells can play a protective role during *C. difficile* infection (Abt et al., 2015). Signals produced by the commensal microbiome, most notably *Bacteroides fragilis* polysaccharide A, are known to promote ILC1 expansion through stimulation of dendritic cell secretion of IL-17 (Duan et al., 2010). Recruitment of eosinophils via IL-25 and IL-33 signaling also provide protection during CDI and are modified by the presence of the microbiome (Buonomo et al., 2016; Frisbee et al., 2019)). As described above, ILC3 responses promote (e.g., IL-23) and antagonize (e.g., IL-22) intestinal damage during *C. difficile* infection (Nagao-Kitamoto et al., 2020; Buonocore et al., 2010). Microbiome composition is known to regulate secretion of IL-22 and IL-23 as well as regulate the balance between activities of Th17 and regulatory T cells (Abt et al., 2015; Atarashi et al., 2011; Ivanov et al., 2009; Mazmanian et al., 2008; Atarashi et al., 2013). This regulation could also play a role in restoring immune homeostasis during resolution of *C. difficile* infection.

From this data, it is clear that there are many mechanisms through which the microbiome could potentially modulate the activity of the immune response to *C. difficile* and influence the outcome of infection. Yet, further research is necessary for defining specific immune response pathways or other mechanisms of protection against disease. Studying these interactions will provide a better understanding of *C. difficile* pathogenesis potentially leading to better predictive modeling of disease progression and identification of new therapeutic targets to regulate disease outcome.

### 1.3.4 Mouse models of *C. difficile* associated disease

Numerous animal models, including hamsters, rabbits, piglets, mice, and rats, have been used to evaluate CDI. Infection in these models can be initiated by a variety of antibiotic regimens followed by experimental challenge or simply environmental exposure to *C. difficile* (Best et al., 2012). The hamster model has been one of the most well described and extensively employed models to study CDI. The hamster model displays several pathophysiological features observed in humans. However, if left untreated, infection is rapidly fatal within the first 48 hours from infection. The observed rapid fatality is one of the key differences with human disease along with the fact that hamsters also generally do not develop diarrhea. Thus, the hamster model serves as a model to test interventions that prevent death. For this reason, many researchers have transitioned to mouse studies.

As in human disease, mice develop intestinal pathology primarily involving the colon. It has also been reported that mice can exhibit a spectrum of disease that includes asymptomatic colonization (Chen et al., 2008), in contrast to the previously mentioned fulminant and highly lethal pathology shown on hamster models (Babcock et al., 2006;

Lusk et al., 1978). Given that the metabolic functionality and composition of the intestinal microbiota of conventional mice or other animal models vastly differ from the human gut microbiota in many aspects, gnotobiotic animals colonized with human microbiota might be a better model to help overcome those differences and have a more close resemblance of microbiome-host interactions in the context of CDI (Hirayama, 1999; Tlaskalová-Hogenová et al., 2011). The human gut microbiota can retain its bacteriological and enzymatic characteristics when it is associated with gnotobiotic rodents, therefore the human microbiota-associated mice model is a convenient way to study causal relationships between relevant microbes to human disease and the host pathology (Licht et al., 2007; Hirayama et al., 1995; Du et al., 2015; Collins et al., 2015). In the context of CDI, the $^{HMA}$mouse model has the potential to reveal important interactions between specific human fecal microbes, *C. difficile* and the host immune system that are important for the disease pathology of CDI observed in human beings and facilitate studies on the potential mechanisms of protection against the severity of disease in humans.

## 1.4 CONCLUSIONS

*C. difficile* infection is an important threat to public health for which a better understanding of disease pathophysiology is highly needed. While many studies have made progress towards understanding microbiome-mediated mechanisms for preventing *C. difficile* colonization and toxin production, the role of host inflammatory responses to CDI and how they vary between symptomatic and asymptomatic disease are not fully understood. While, there is evidence of a major role of the innate immune system in

regulating disease severity, further research is needed to understand the exact mechanisms underlying variation in disease outcome. The work discussed in the following chapter will describe the work that I have done to develop and characterize appropriate mouse models for studying interactions of microbiome and host factors that contribute to differences in disease severity during CDI.

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# CHAPTER 2. DIFFERENCES IN IMMUNE RESPONSES CONTRIBUTE TO ALTERED LEVELS OF DISEASE SEVERITY BETWEEN C3H/HEN AND C57BL/6J MICE INFECTED WITH *C. DIFFICILE*

## 2.1 ABSTRACT

*Clostridiodes difficile* has become the leading infectious cause of antibiotic-associated diarrhea in developed countries over the past 20 years. It has achieved a steadily rising global incidence of infection and with this, an increase in mortality, especially in hospitals and community healthcare settings. When a susceptible individual is exposed to *C. difficile*, multiple outcomes are possible, including asymptomatic colonization, mild diarrhea, pseudomembranous colitis, and death; however the mechanisms underlying these variations in disease severity are currently not fully understood. In this study, we established different human microbiota-associated (HMA) mouse models that demonstrated clear differences in disease severity after *C. difficile* exposure independent from *C. difficile* burden or toxin activity. We observed that mouse strain background (C3H/HeN and C57BL/6J) had the largest impact on disease outcome and that donor microbiome also influenced disease severity. Characterization of the initial inflammatory responses after infection revealed a clear difference in levels of inflammation during CDI based upon host genetic background. Altogether, the results of this study demonstrated the potential of the $^{HMA}$mouse model to study interactions between microbiome and host inflammatory responses in the context of CDI.

## 2.2 INTRODUCTION

*Clostridioides difficile* is the leading cause of nosocomial gastrointestinal infections and has been categorized as a serious public health problem due to its high rates of disease, morbidity and mortality over the last couple of decades (Martin et al., 2016). *C. difficile* infection occurs after the ingestion of spores by a susceptible host, most commonly associated with antibiotic treatments that disrupt the host indigenous microbiome. However, the clinical manifestation of disease can range from mild to moderate diarrhea to severe life-threatening conditions that include pseudomembranous colitis, toxic megacolon, and death. However, the mechanisms underlying these variations in disease outcome are not known (Leffler & Lamont, 2015).

While there have been substantial advances identifying commensal bacterial species and mechanisms that can prevent *C. difficile* colonization (Britton & Young, 2012), the role of the host immune defenses and their interactions with the host microbiome in limiting disease severity are less well understood (Saleh & Petri, 2019). Evidence continues to be reported that supports the idea that the type of immune response mounted by the host against *C. difficile* infection has a major role in modulating severity and determining outcome of disease (El Feghaly et al., 2013; Saleh & Petri, 2019). Ultimately, a better understanding of the protective and pathogenic properties of immune responses during CDI is crucial to identify targets for potential immunotherapies and strategies for prevention (Saleh & Petri, 2019).

The innate immune signaling pathways linked to innate lymphoid cells (ILCs) have previously been associated with the early host defense to CDI (Abt et al., 2016). ILCs, which reside primarily at mucosal surfaces, largely maintain homeostasis with the indigenous microbiome by preventing harmful inflammatory responses to nonpathogenic microbes (Seo et al., 2020). ILCs also have a prominent role regulating the early responses to infections by different pathogenic microbes at mucosal barriers and contribute to clearance of pathogens and restoration of intestinal integrity after infection by rapid production of cytokine signaling (Britanova & Diefenbach, 2017).

Functional subsets of ILCs have been identified where ILC1, ILC2 and ILC3 share similarities in transcription factors and cytokine signatures with Th1, Th2 and Th17 cells types respectively (Klose et al., 2014; Serafini et al., 2015; Walker et al., 2013). Type 1 ILCs secrete IFN-γ which can increase phagocytic mechanisms. Loss of IFN-γ signaling can lead to increased vulnerability to disease progression during *C. difficile* infection as shown by Abt and colleagues (Abt et al., 2015). Mouse studies have previously shown that Type 2 ILCs have a protective role against *C. difficile*. For example, interleukin 25 (IL-25), a cytokine regulated by Type 2 ILCs whose levels are also dependent on the microbiota, becomes suppressed during CDI; IL-25 restoration can induce eosinophil, basophil, and mast cell accumulation at sites of inflammation reducing mortality and tissue pathology (Buonomo et al., 2016). Neutrophil infiltration, along with secretion of IL-1β, IL-17 and GM-CSF is characteristic of ILC type 3 immune responses. Neutrophil activation and tissue infiltration have long been considered a hallmark of *C. difficile* infection although neutrophils can have both protective and

pathogenic roles mainly due to the lack of target cell specificity (Kelly et al., 1994; Jose & Madan, 2016; Saleh & Petri, 2019). From these studies, it is clear that there are complex interacting roles for ILC1, ILC2 and ILC3 responses during infection and more work is needed to characterize how these pathways interact with each other and the microbiome to influence disease outcome during infection.

Here, we establish different human microbiota-associated (HMA) mouse models that demonstrate clear differences in disease severity after *C. difficile* exposure independent from *C. difficile* burden or toxin activity. To develop these models, we colonized mice from two genetic backgrounds (C57BL/6J and C3H/HeN) with fecal microbiota from three healthy human donors. We observed that mouse strain background had the largest impact on disease outcome, as C57BL/6J mice showed the most severe manifestation of disease following infection. We also observed that donor microbiome influenced disease severity, with one donor microbiome leading to reduced disease severity in C57BL/6J mice compared to the other microbiomes tested.

We focused on a subset of $^{HMA}$mice colonized with a single human fecal sample that represented both an asymptomatic (C3H/HeN) and severe disease (C57BL/6J) presentation to characterize the initial inflammatory responses and the level of epithelial damage after infection. We hypothesized that severe disease would correlate with increased levels of inflammatory markers associated with ILC Type 3 responses, while a more protective effect would be due either to reduced levels of ILC Type 3 responses or upregulation of ILC Type 1 and 2 associated markers. We observed that several inflammation markers were highly upregulated in the C57BL/6J mice and expressed at lower levels in C3H/HeN mice. Only one inflammatory marker, IL-1β was induced at

higher levels in C3H/HeN mice. Thus, reduced disease severity in C3H/HeN mice appear to be primarily due to reduced activation of ILC Type 3 responses rather than upregulation of ILC Type 1 and 2 responses. Altogether, we demonstrate the potential of the $^{HMA}$mouse models to study interactions between microbiome and host inflammatory responses in the context of CDI.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Establishment of human-microbiota associated mice ($^{HMA}$mice)

Germ-free C57BL/6J and C3H/HeN were administered, via oral gavage, one of three human derived fecal microbiota samples (designated MA, MB and MC) that were collected from healthy donors (Donor A, B and C) that had not taken antibiotics for at least three months. Donors varied by age: donor A was a person of advanced age (>65 year old), donor B was a child (5-17), and donor C was an adult (18-65); information about sex of the donors was not collected. Samples were collected and administered under protocols approved by IRB panels at Baylor College of Medicine (H-38014) and the University of Nebraska-Lincoln (#18585). Samples were screened for *C. difficile* presence by selective plating on the *C. difficile* enrichment medium, taurocholate-cefoxitin-cycloserine-fructose agar (TCCFA), to confirm their *C. difficile*-negative state. Following an initial establishment over the course of two weeks, mice were housed in individually ventilated cages with *ad libitum* access to autoclaved water and food and bred in a specific-pathogen-free facility where their microbiota was passed to their progeny. Sequencing of the V4 region of the 16S rRNA gene of representative progeny mice from each $^{HMA}$mouse line (Appendix A) was used to analyze differences in

microbiome content such as relative taxa abundance, alpha and beta diversity measures as previously described (Auchtung et al., 2020).

### 2.3.2 *C. difficile* infection experiments in $^{HMA}$mice.

In order to compare *C. difficile* colonization in $^{HMA}$mouse models, we performed infection experiments by administering an antibiotic cocktail consisting of kanamycin (0.4 mg $ml^{-1}$), gentamicin (0.035 mg $ml^{-1}$), colistin (850 U $ml^{-1}$), metronidazole (0.215 mg $ml^{-1}$), and vancomycin (0.045 mg $ml^{-1}$) for 4 days in drinking water. A day after mice were transitioned to fresh water, a single intraperitoneal injection of clindamycin (10 mg/kg) was administered as described by Collins and colleagues (Collins et al., 2015). Exposure to $10^4$ spores of *C. difficile* ribotype 027 strain CD2015 spores via gavage followed after 24 hours from antibiotic treatment completion as previously described by Robinson (Robinson et al., 2014). Mice were monitored for weight loss, visible signs of disease such as hunched posture, loss of ambulation, presence of watery stool, and death for 3 or 7 days following initial *C. difficile* infection. A subset of mice was euthanized on day 3 to determine levels of histopathogical damage and/or to measure levels of immune responses during the peak of disease severity, as previous studies had demonstrated resolution of symptoms by day 7 of infection (Collins et al., 2015). Fecal samples were collected at different time points following infection to determine the levels of *C. difficile* colony forming units (CFU) and relative toxin units (RTU) per gram of feces as previously described (Auchtung et al., 2020).

### 2.3.3 Measuring levels of *C. difficile* CFU and toxin activity present in fecal samples.

In order to determine the CFU of *C. difficile* present in mouse fecal samples, the fecal pellets were weighed and then homogenized in 0.5 ml anaerobic PBS, serially diluted at 1:10 dilutions and spot plated on TCCFA plates containing erythromycin and rifampicin that were previously pre-reduced in an anaerobic chamber. Plates were incubated at 37°C for 24-36 hrs. Colonies were counted and used to calculate the number of CFU/g of fecal mass.

To measure the amount of relative toxin units (RTU)/g of feces in the mouse fecal samples, Vero cell cytotoxicity assays were carried out as described (Robinson et al., 2014). Vero cells were grown to near confluence in 150 $cm^2$ flasks in DMEM + 10% FBS in an incubator at 37°C with 5% $CO_2$. Cells were trypsinized, seeded at $10^5$ cells/ml into 96-well microtiter plates, and incubated for at least 4 hours at 37°C with 5% $CO_2$. Fecal samples were weighed and resuspended in 0.5 ml sterile PBS, centrifuged, and their supernatants filtered through sterile 0.2 μm PVDF membranes. Filtrates were serially diluted and overlaid on the Vero cell seeded plates. Plates were incubated at 37°C with 5% $CO_2$ for 36-48 hours, and then examined for cell rounding. RTU were determined based upon the last dilution at which 80% of cells were rounded. RTU/g of feces were calculated using the recorded dilution factor.

**2.3.4 RNA isolation and expression analysis**

Cecal RNA was isolated from 1 cm cecal tissue sections collected at necropsy and stored in RNAlater (Thermo Fisher). Cecal tissue was homogenized and RNA was extracted with RNeasy Mini kit (Qiagen) according to manufacturer's protocol with optional on-column DNase I digestion. RNA concentration and purity were determined by measuring absorbance at $A_{260}$ and $A_{280}$. cDNA generation and qRT-PCR for relative

gene expression were performed using One-Step SuperScript III Platinum SYBR Green kit (Invitrogen) with 2 µl RNA and the primer sets in Table 1 (1 µM final concentration) on a QuantStudio 5 Real-Time PCR instrument (Thermo Fisher). The cycling conditions were: 3 minutes at 50° C for cDNA synthesis, a denaturation step for 5 minutes at 95°C followed by 40 cycles of 95°C for 15 seconds and at 60°C for 30 seconds, and a 1 minute hold step at 40°C after. Relative expression levels between mice challenged with *C. difficile* and uninfected controls (n=1 uninfected control/mouse background) were determined using the $2^{-\Delta\Delta Ct}$ method, and GAPDH was used as the internal control. Results are the average of triplicate 10µl reactions.

| Gene | Sense | Sequence |
|---|---|---|
| IL-17 | Forward | 5'-AAG GCA GCA GCG ATC ATC C-3' |
| | Reverse | 5'-GGA ACG GTT GAG GTA GTC TGA G-3' |
| IFN-γ | Forward | 5'-GGCCATCAGCAACAACATAAGCGT-3' |
| | Reverse | 5'-TGGGTTGTTGACCTCAAACTTGGC-3' |
| IL-1β | Forward | 5'-AAG GGC TGC TTC CAA ACC TTT GAC-3' |
| | Reverse | 5'-ATA CTG CCT GCC TGA AGC TCT TGT-3' |
| IL-23 | Forward | 5'-CCT GCT TGA CTC TGA CAT CTT C-3' |
| | Reverse | 5'-TGG GCA TCT GTT GGG TCT C-3' |
| IL-25 | Forward | 5'-CGGAGGAGTGGCTGAAGTGGAG-3' |
| | Reverse | 5'-ATGGGTACCTTCCTCGCCATG-3' |
| IL-33 | Forward | 5'-TCCAACTCCAAGATTTCCCCG-3' |
| | Reverse | 5'-CATGCAGTAGACATGGCAGAA-3' |
| IL-12(p40) | Forward | 5'-ACA TCA AGA GCA GTA GCA GTT C-3' |
| | Reverse | 5'-AGT TGG GCA GGT GAC ATC C-3' |
| IL-6 | Forward | 5'-TCC AGT TGC CTT CTT GGG AC-3' |
| | Reverse | 5'-GTG TAA TTA AGC CTC CGA CTT G-3' |
| KC | Forward | 5'-TCG CCA ATG AGC TGC GCT GTC-3' |
| | Reverse | 5'-GCT TCA GGG TCA AGG CAA GCC-3' |
| MIP-1a | Forward | 5'-CAGCCAGGTGTCATTTTCCT-3' |
| | Reverse | 5'-CTGCCTCCAAGACTCTCAGG-3' |
| MIP-2 | Forward | 5'-GAG CTT GAG TGT GAC GCC CCC AGG-3' |
| | Reverse | 5'-GTT AGC CTT GCC TTT GTT CAG TAT C-3' |
| GAPDH | Forward | 5'-GGAGGAACCTGCCAAGTATG-3' |
| | Reverse | 5'-TGGGAGTTGCTGTTGAAGTC-3' |

**Table 1. Primer pair sequences for inflammatory markers tested.**

### 2.3.5 Histopathological lesion scores

Cecum and colon tissue from a subset of representative mice were evaluated for tissue damage because of infection. For this, a 1 cm section was collected from the cecum

and colon at necropsy and fixed in 10% buffered formalin. Tissues were embedded in paraffin and sectioned prior to hematoxylin and eosin (H&E) staining, visualization and scoring at Iowa State University (Department of Veterinary Pathology). H&E-stained tissues were scored by a licensed pathologist blinded to treatment conditions for epithelial tissue damage, neutrophil infiltration, and mucosal edema as described (Shelby et al., 2019).

### 2.3.6 Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity was used to assess the levels of neutrophil/granulocyte infiltration in cecum tissue as outlined by Haarberg and colleagues with a few modifications (Haarberg et al., 2015). Cecum segments were collected at day 3 of *C. difficile* infection. Samples were stored in 15% DMSO and 0.1 mM phenylmethylsulphonyl fluoride (PMSF), immediately frozen on dry ice, and stored at -80°C. Intestinal samples were thawed, blotted, trimmed to roughly 35 mg and homogenized in PBS with 0.1 mM PMSF and 0.02% hexadecyltrimethylammonium bromide (CTAB). Tissue homogenates were sonicated at an amplitude of 5, pulse on for 4 seconds, pulse off for 1 second, for 20 seconds total. The tissue sonicates were then centrifuged at 250 X g for 15 min and the clarified supernatant used for the MPO assay. The reaction mixture consisted of 150 µl of supernatant, 50 µl of 0.78 mg/mL 3,3',5,5'-tetramethylbenzidine and 50 µl of 5 mM hydrogen peroxide in a total volume of 250 µl. Reactions were incubated at room temperature for 3 minutes and terminated by the addition of 50 µl of 2N $H_2SO_4$. Absorbance was measured at 405 nm and activity in each sample was compared to a standard curve generated from 2-fold serial dilutions of mouse

peripheral blood mononuclear cells and a CTAB negative control. The MPO activity was expressed as the relative units of enzyme per gram of wet weight of tissue.

### 2.3.7 Statistical analysis

Statistical analyses were performed using the GraphPad Prism version 8.4.2 for macOS, GraphPad Software, San Diego, California USA. Differences in percentage of body mass loss, *C. difficile* loads and toxin activity in feces were determined by Kruskal-Wallis test with uncorrected Dunn's test. Additionally, a Mantel-Cox log rank test was used to determine significant differences in level of mortality between C57BL/6J mice colonized with the three different donor's microbiomes. Two sample unpaired t tests with Welch's correction determined significance for changes in MPO activity and clinical sickness scores and one-way ANOVA with uncorrected Fisher's LSD test for differences in histopathology scores. Significance of differences in cytokine levels between C57BL/6J and C3H/HeN mice were determined with a non-parametric Friedman test. Correlation between percentage of initial body mass loss and relative gene expression was determined with simple linear regression analysis. All significance was determined at p-value$< 0.05$.

## 2.4 RESULTS

### 2.4.1 *C. difficile* associated disease severity is influenced by mouse strain and microbiome.

In initial studies, we colonized male and female gnotobiotic C57BL/6J and C3H/HeN mice with fecal samples from three healthy donors (MA, MB and MC; more details on the microbiome composition of these $^{HMA}$mice can be found in Appendix A). These $^{HMA}$mouse lines were bred under specific pathogen-free conditions and progeny

mice were tested for susceptibility to *C. difficile* infection following treatment with a combination of antibiotics known to induce susceptibility to *C. difficile* in other mouse models. Disease severity for the seven days following infection was monitored through daily measurement of body mass, and observation for symptoms of severe disease including diarrhea, hunched posture, difficulty breathing and loss of ambulation. After initial *C. difficile* exposure, we observed a clear distinction in disease severity following infection between C57BL/6J mice and C3H/HeN (Fig. 2.1a-c). Consistent with previous studies (Collins et al., 2015), C57BL/6J mice exhibited more severe disease as evidenced by 5-17% body mass loss, presence of diarrhea and mortality in a subset of mice 48-72 hours following infection. In contrast, we observed little to no declines in body mass (0-6%), nor any signs of overt disease or mortality in C3H/HeN (Fig. 2.1d).

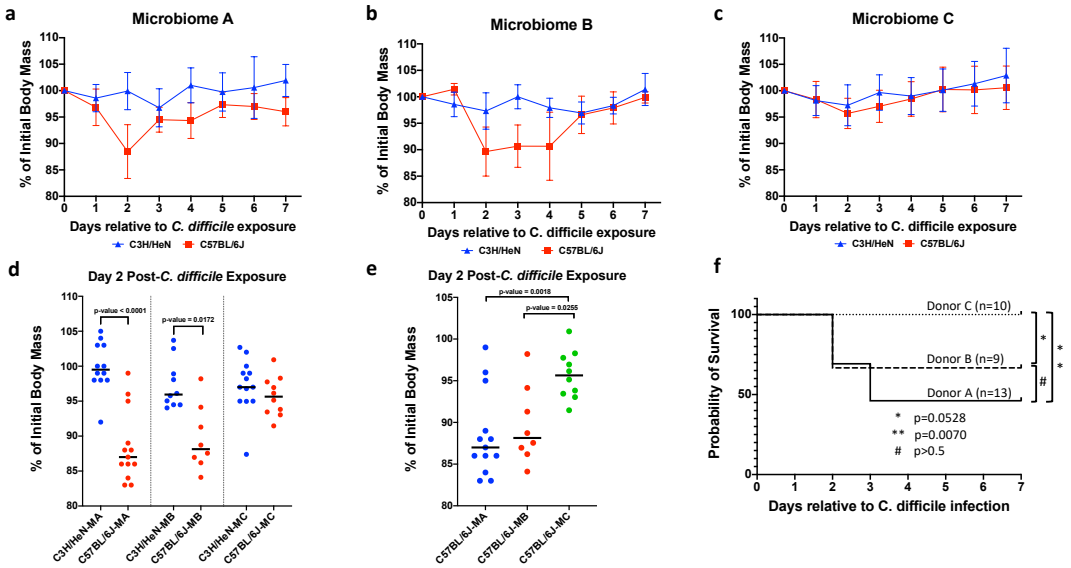


**Figure 2.1. Differences in loss of body mass and mortality in different models of $^{HMA}$mice. (a, b & c)** Percentage of initial body mass was monitored daily after initial *C. difficile* exposure across the different mice strain-microbiome configurations**.** Points represent the medians and error bars represent the interquartile ranges. In a, n=13 C57BL/6J, n=12 C3H/HeN; in b, n=9 C57BL/6J, n=10 C3H/HeN; in c, n=10 C57BL/6J, n=13 C3H/HeN **(d & e)** Differences in initial body mass on day 2, the most severe point of infection. Data is re-plotted from panels a-c; statistical significance was determined with Kruskal-Wallis test. **(f)** Probability of survival of C57BL/6J $^{HMA}$mice following C. difficile challenge. Statistical significance was determined with Mantel-Cox log rank test.

In addition, the composition of donor microbiome used to colonize C57BL/6J also impacted disease severity. C57BL/6J mice colonized with microbiomes from donor A and B (MA and MB mice) showed a statistically significant decrease in body weight and mortality compared to mice colonized with microbiome from donor C (MC mice) which exhibited smaller decreases in body mass (6±3% (MC) vs 13±4% (MA) or 12±2% (MB)) at 48 hours, the point of infection at which mice had lost the largest percentage of body mass (Fig. 2.1e-f). Altogether, these data indicate that there are specific host and microbiome interactions that influence disease severity following *C. difficile* infection.

**2.4.2 Differences in disease severity are not due to altered levels of *C. difficile* colonization or toxin production.**

One potential explanation for differences in disease severity between mouse lines could be due to differing levels of *C. difficile* or its toxin, as previous studies have demonstrated that mice with lower levels of *C. difficile* exhibit less severe disease (Reeves et al., 2011; Collins et al., 2015). Therefore, we sought to measure the levels of *C. difficile* colonization and toxin activity in our *C. difficile* infected mice to determine if there were differences in *C. difficile* colonization levels or toxin production that could explain disease outcome. To accomplish this, fecal samples were collected at different time points throughout the experiments and levels of *C. difficile* and toxin were measured. Examining levels of *C. difficile* in feces, we observed that all mouse strains had similar levels of *C. difficile* in feces on day 2 after infection. On day 4 of infection *C. difficile* levels in C3H/HeN were similar to (MC) or higher (MA) than in C57BL/6J mice (Fig. 2.2a). Similarly, we observed that levels of *C. difficile* toxin activity measured by cytotoxicity assays were not significantly different across the microbiome-mouse

configurations on day 2 or day 4 of infection (Fig. 2.2b). These data not only demonstrate the ability of *C. difficile* to colonize and produce toxin in all $^{HMA}$mice tested but also indicate that additional factors are likely influencing disease outcome.

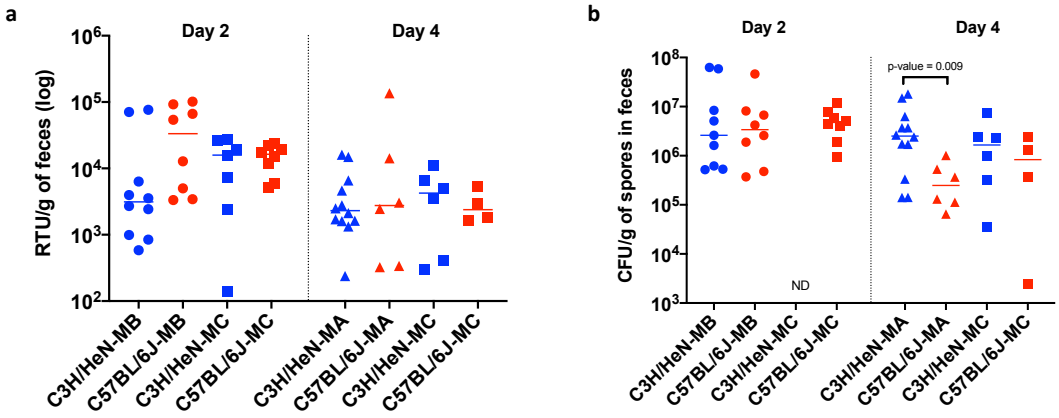


**Figure 2.2. Levels of CFU and toxin activity in feces from C57BL/6J and C3H/HeN $^{HMA}$mice.** **(a)** Levels of *C. difficile* relative toxin units per gram of feces on day 2 and 4 after initial infection determined with cytotoxicity assays. **(b)** Levels of *C. difficile* loads present on day 2 and 4 after initial infection determined with cytotoxicity assays. Statistical significance between mice groups was determined with Kruskal-Wallis test. Day 2 and day 4 sample collections belong to different experiments. ND indicates data not collected. Data was not collected from seven C57BL/6J-MC shown in Figure 2.1 as mice died prior to fecal sample collection.

### 2.4.3 Mice with severe disease exhibit increased intestinal damage and neutrophil tissue infiltration.

After determining that differences in *C. difficile* colonization or toxin production was unlikely to determine differences in overt signs of disease, we decided to focus on better characterizing host responses to infection in different mouse-microbiome configurations. To streamline these studies, we focused on a single mouse-microbiome pair, microbiome MB-colonized C57BL/6J and C3H/HeN mice. In addition to monitoring body weight, clinical sickness scores were determined for these studies taking into consideration changes in behavior and stool characteristics. Consistent with body weight data from initial studies, C57BL/6J mice showed significantly higher disease

scores in contrast with C3H/HeN where we observed little to no signs of disease (Fig. 2.3a).

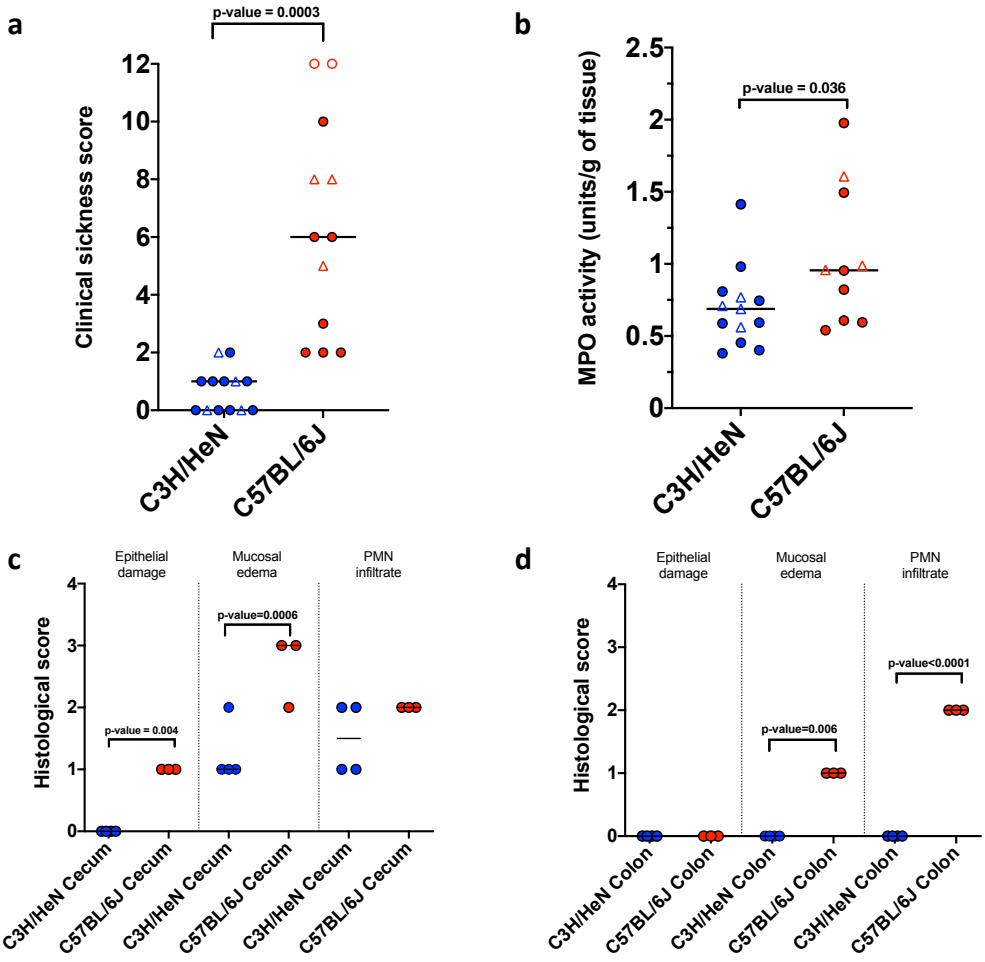


**Figure 2.3. Donor B-colonized (MB) C57BL/6J mice present elevated measures of disease severity in contrast to C3H/HeN mice. (a)** Clinical sickness scoring was determined by evaluating percent of body weight loss, stool characteristics and changes in behavior. Scores ranged from least (0) to most (4) severe for each category adding to a total score of 12. Open circles indicate mice that died at a time before tissue harvesting for which no tissue was collected for MPO analysis. C3H/HeN n=13, C57BL/6J n=12 **(b)** Levels of neutrophil/granulocyte infiltration in cecum tissue was determined with myeloperoxidase activity assay. C3H/HeN n=13, C57BL6J n=10. Open triangles indicate the subset of mice used for histopathology analysis. **(c)** Fixed cecal and **(d)** colon tissue from a subset of C57BL/6J and C3H/HeN mice were blindly scored for epithelial cell loss, mucosal edema, and PMN infiltrate. Scores ranged from least (0) to most (4) severe for each measure. Statistical significance was determined with two sample unpaired t tests with Welch's correction for changes in MPO activity and clinical sickness scores, and one-way ANOVA with uncorrected Fisher's LSD test for differences in histopathology scores.

To evaluate the level of damage and cellular responses in mouse tissue as a consequence of infection we used MPO activity assay (Fig. 3b) and histopathological scoring (Fig. 2.3c-d). Histopathological analysis of cecal and colon tissue was performed on a representative subset of microbiome MB-colonized C57BL/6J and C3H/HeN mice (Fig. 2.3c-d). C3H/HeN mice showed low levels of polymorphonuclear leukocyte (PMN) infiltration, mucosal edema and no epithelial cell loss in cecal and colon tissue collected from mice at necropsy 72 hours after initial infection, while the majority of these markers of severe disease were elevated in the C57BL/6J mice. Cecal changes were more substantial than those in the colon, suggesting that at the time of necropsy (72 hours after infection), disease is localized in the cecum. Therefore, further analysis focused on characterizing inflammation in the cecum.

Neutrophil infiltration was independently assayed in these mice by measuring the activity of myeloperoxidase (MPO), an enzyme that is produced at high levels by neutrophils. Consistent with the evidence of PMN infiltration in tissue, we observed higher levels of MPO activity in the cecum of C57BL/6J mice in comparison to C3H/HeN mice (Fig. 2.3b). These data correlate well with the differences in disease severity previously observed and indicate that C57BL/6J exhibit signs of a stronger ILC3 response.

### 2.4.4 Differences in disease severity correlate with differences in expression of a subset of pro-inflammatory cytokines

The higher levels of epithelial damage and neutrophil activity in the cecum of sick mice led us to hypothesize that differences in host immune response could be responsible for modulating disease severity in the different mouse backgrounds. More specifically,

reduced disease severity in C3H/HeN mice could be due either to lower levels of induction of immune pathways that increase disease severity (ILC3 response) or due to higher levels of induction of immune pathways that decrease disease severity (ILC2 response, and to a lesser extent ILC1 response.) Therefore, we identified 11 cytokine or chemokine analytes (IFN-γ, IL-1β, IL-17A, Il-23, IL-25, IL-33, IL-12(p40), IL-6, KC, MIP-1α and MIP-2) from previous reports that have been identified as leading to increased or decreased disease severity during *C. difficile* infection to test our hypothesis.

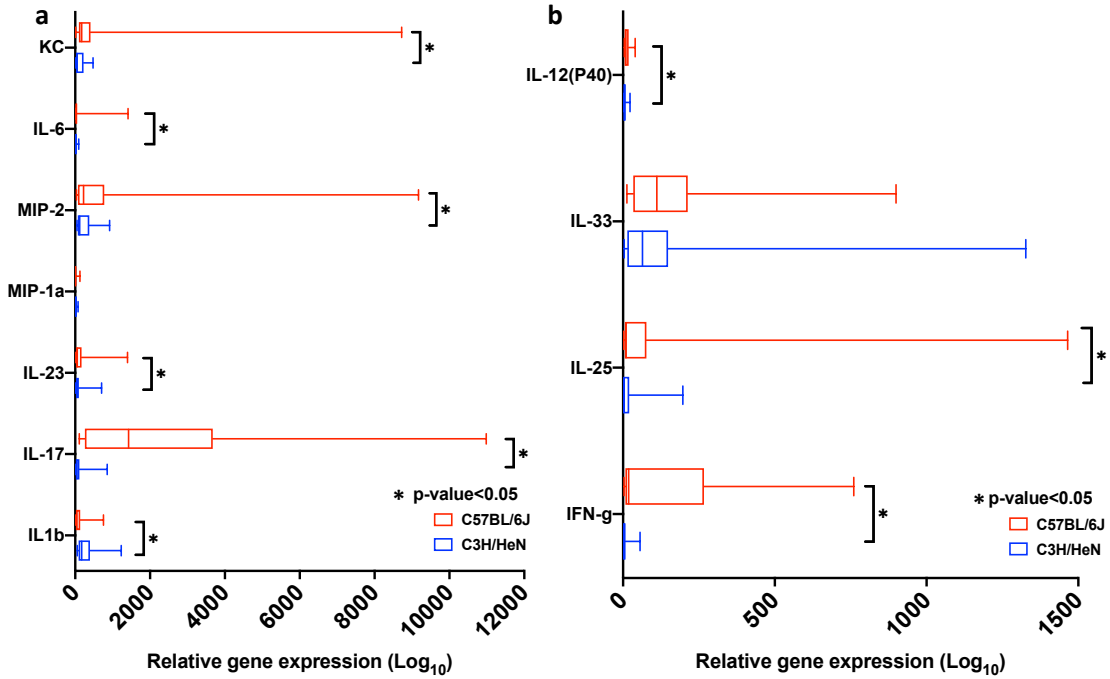


**Figure 2.4. Expression of inflammatory markers upregulated in C57BL/6J mice.** Levels inflammatory cytokines and chemokines associated with ILC type 3 **(a)** or type 1/2 **(b)** responses were measured from cecal tissue through RT-qPCR. Statistical significance ($p<0.05$) of differences in cytokine levels between C57BL/6J and C3H/HeN mice were determined with a non-parametric Friedman test.

We compared levels of expression of these genes in *C. difficile*-infected mice to uninfected mice of the same strain background through RT-qPCR. We found that levels of all analytes with the exception of IL-1β, IL-33 and MIP-1α were induced to

significantly higher levels in the ceca of microbiome MB-colonized C57BL/6J mice compared to C3H/HeN mice (Fig. 2.4a). Interestingly, IL-1β was the only analyte that had increased levels in C3H/HeN mice cecum. When we examined relative expression of analytes more strongly associated with ILC type 1 or 2 responses that have been associated with protective mechanisms against severe disease, we observed that IL-25, IFNγ, and IL-12(p40) were also significantly higher in C57BL/6J mice (Fig. 2.4b). Altogether, the results are consistent with increased immune activation in C57BL/6J mice relative to C3H/HeN mice.

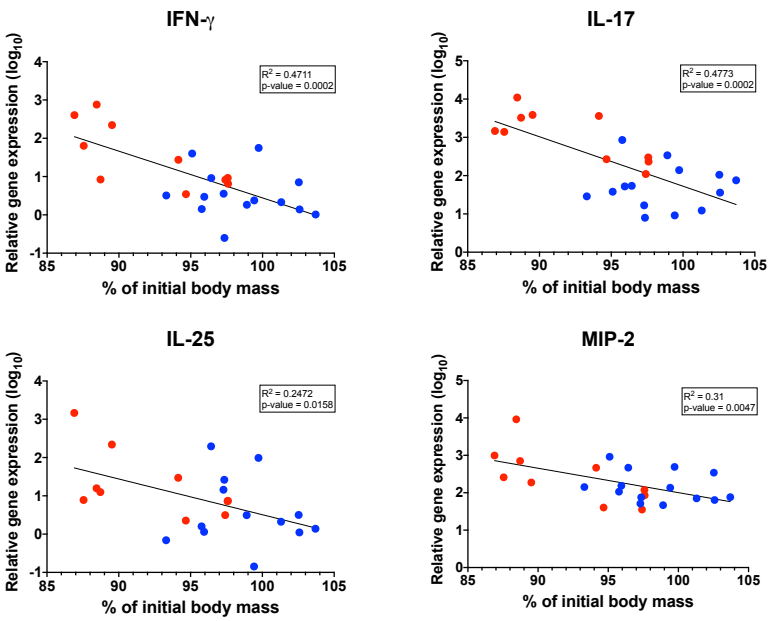


**Figure 2.5. Inflammation markers correlate with loss of body mass.** Simple linear regression tests showed association between relative gene expression of inflammatory markers and loss of body mass in C57BL/6J mice ceca.

While we observed overall increased expression of pro-inflammatory cytokines and chemokines in C57BL/6J mice compared to C3H/HeN mice, we also observed levels of induction varied within these two populations of *C. difficile*-infected mice. We were interested in determining to what extent variations in levels of cytokine induction

paralleled differences in disease severity. To determine if these markers correlated with one of our previously observed measure of disease severity (body mass), we performed simple linear regression tests on every analyte against the percentage of body mass loss. We discovered that four analytes, IFN-γ, IL-17A, IL-25 and MIP-2, had significant correlation ($P < 0.05$) between increased gene expression and increased body mass loss (Fig. 2.5). Altogether, this data indicates that a subset of immune markers correlate with body mass loss, one of our measures of disease severity, while the other markers show little correlation.

**2.5 DISCUSSION**

In the current study, we established lines of C57BL/6J mice and C3H/HeN $^{HMA}$mice colonized with different fecal samples and demonstrated clear differences in disease severity due to differences in mouse background and microbiome composition. Two of three lines of C57BL/6J $^{HMA}$mice exhibited severe disease and mortality during *C. difficile* infection that was consistent with previous studies of *C. difficile* infection in $^{HMA}$mice (Collins et al., 2015); the third line of C57BL/6J $^{HMA}$mice exhibited less severe disease, likely due to differences in microbiome composition that reduce disease severity. Studies of the effect of microbiome composition on disease severity in C57BL/6J mice will be the focus of future studies.

Unexpectedly, C3H/HeN infected mice showed little to no signs of overt disease despite similar levels of colonization with toxin-producing *C. difficile*; the similarities in disease presentation in these mice and asymptomatically colonized patients prompted further research on the mechanisms that could be protecting these mice from disease. Our initial studies focused on additional clinical markers of disease severity – epithelial

damage in infected mice and immune cell infiltration. We found that C57BL/6J infected mice exhibited significantly higher levels of epithelial damage and immune cell infiltration (neutrophil and other PMN) than C3H/HeN mice. Neutrophil activation and recruitment are prominent characteristics of *C. difficile* infection which are often elicited by Type 3 ILC signaling (Saleh & Petri, 2019).

We hypothesized that severe disease in C57BL/6J mice would correlate with increased levels of inflammatory markers associated with ILC Type 3 responses, while reduced disease severity in C3H/HeN mice could be due either to reduced levels of ILC Type 3 responses or upregulation of ILC Type 1 and 2 associated markers previously linked to reducing disease severity during *C. difficile* infection. Our results are consistent with the initial hypothesis, that reduced disease severity in C3H/HeN mice is more likely due to reduced levels of induction of pathways associated with increased disease severity rather than elevated expression of pathways associated with potentially protective effects during infection.

Other potential differences between C3H/HeN and C57BL/6J mice could be contributing to the lower levels of induction observed in C3H/HeN mice. One potential mechanism could be that the kinetics of immune responses during *C. difficile* infection may vary between C3H/HeN and C57BL/6J mice, as differences in infection kinetics between C3H/HeN and C57BL/6J infected with *Citrobacter rodentium* have been previously reported (Carson et al., 2020). In C3H/HeN mice, earlier colonization of *C. rodentium* leads to earlier upregulation of IFNγ and IL-22 that leads to more severe disease than is observed in C57BL/6J mice infected with *C. rodentium*. If similar colonization dynamics occurred during *C. difficile* infection, induction of these responses

could help to mitigate initial disease severity. We will test this hypothesis in future studies, where we plan to measure induction of cytokines at earlier points during infection. The current studies were designed using disease progression in C57BL/6J as a model for sampling, which is why tissue harvesting occurred three days after *C. difficile* exposure at the point when mice are just beginning to recover (Collins et al., 2015). Other potential mechanisms to investigate include differences in receptor intoxication of epithelial cells and differences in bile acid pools that may impact *C. difficile* pathogenicity.

## 2.6 CONCLUSIONS

These newly established $^{HMA}$mouse models expand the tools available for studying how differences in microbiome-host interactions influence *C. difficile* disease progression. In addition to demonstrating that mouse genetic backgrounds have large impact on disease susceptibility, we also demonstrated that natural variation in microbiome composition can also influence disease outcome. While the results presented here clearly demonstrate differences in levels of inflammation during infection based upon both host genetic background and microbiome composition, future work is needed to determine the specific mechanisms that govern these differences. In the final chapter of this thesis, I will discuss potential future research directions to better understand how differences in responses to *C. difficile* infection may differentiate asymptomatic from symptomatic *C. difficile* infection.

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# CHAPTER 3. FURTHER CHARACTERIZATION OF IMPACTS OF HOST GENETICS AND MICROBIOME COMPOSITION ON *C. DIFFICILE* DISEASE SEVERITY

## 3.1 OVERVIEW

*C. difficile* infection can cause a wide spectrum of clinical manifestations ranging from asymptomatic carriage or colonization to fulminant disease with toxic megacolon or death. Although the basis for these variations is not fully understood, continuously growing evidence indicate that interactions between host, microbiome and pathogen factors such as the activation of the innate mucosal immune signaling might have a significant role modulating disease severity (Collins & Auchtung, 2017; Abt et al., 2016). The development of appropriate mouse models that replicate differences in disease severity during *C. difficile* infection could provide essential pre-clinical tools to facilitate understanding of host-microbiome-pathogen interactions that reveal important insights into how these interactions impact *C. difficile* virulence and disease outcome in infected patients.

Towards this goal, we have established $^{HMA}$mouse models for which two phenotypes were observed, a state with little to no evidence of overt disease similar to asymptomatic colonization in patients and a state presenting symptomatic disease that varied in severity. These were largely based on the mouse genetic background (C3H/HeN and C57BL/6J respectively) and to a lesser degree on the specific microbiome they were colonized with. One major finding in our study was that, regardless of whether or not mice had symptomatic illness, they were colonized with comparable levels of *C. difficile*

and shed similar levels of *C. difficile* toxin in their feces, indicating that *C. difficile* had no obvious difficulty colonizing the intestinal tracts of these mice and subsequently producing its toxins. From this data, it was clear that there are both host genetic and microbiome-mediated mechanisms to reduce severity of disease when exposed to *C. difficile* toxins. Host genetic differences were apparent in comparisons between C3H/HeN and C57BL/6J mice colonized with microbiomes MA and MB, whereas microbiome-mediated differences were apparent in C57BL/6J colonized with different donor microbiomes (donor C compared to donors A and B).

### 3.2 IMPACT OF MICROBIOME COMPOSITION ON DISEASE SEVERITY

Several mechanisms by which the intestinal microbiota inhibit *C. difficile* have been proposed. These include the transformation of bile acids, which have impactful consequences on *C. difficile* spore germination and cell growth (Sorg and Sonenshein, 2008, 2009); niche exclusion, where the use of non-toxigenic *C. difficile* strains could be preventing toxigenic strains colonization (Merrigan et al., 2003; Sambol et al., 2002); and the production of antimicrobial agents such as bacteriocins (Rea et al., 2010). However, these mechanisms are thought to primarily limit the initial levels of *C. difficile* colonization, and so are unlikely to be causing the variations in disease outcome we observed.

Although the broad microbiome analysis we conducted did not show obvious trends that could explain the disease differences, the study was limited in sample size and did not covered the progression of disease. Future studies to characterize these differences will take advantage of a more extensive 16S rRNA gene sequencing to consider changes in microbiome composition over more time points from the beginning

of disease, with a specific emphasis on how differences in the abundance of bacteria that produce short chain fatty acids (SCFA) change over the course of disease. Previous studies have found that SCFA have been associated with modulation of host immune system and metabolism (Correa-Oliveira et al., 2016; Koh et al., 2016). Several studies have observed that a drastic reduction in SCFA after antibiotic treatments increase susceptibility to CDI (Antharam et al., 2013; Theriot et al., 2014), and a restoration of butyrate concentrations in the intestine have proven to attenuate disease severity in mice (Fachi et al., 2019). This data indicates that SCFA-producing bacteria have the potential to be used in protective strategies against CDI. We will also use microbiome transfer experiments to provide clearer mechanistic insights into how microbiome composition may impact disease severity.

### 3.3 IMPACT OF HOST IMMUNE RESPONSE ON DISEASE SEVERITY

In addition to differences in overt signs of disease, one prominent difference that we observed in our study was differences in the levels of neutrophil activity in the ceca of the mice. As was consistent with differences in disease severity between C3H/HeN and C57BL/6J mice, there was little neutrophil infiltration in C3H/HeN mice and elevated neutrophil infiltration in C57BL/6J mice. To further understand potential mechanisms for differences in disease presentation, we measured differences in expression of several pro-inflammatory cytokines between C3H/HeN and C57BL/6J mice. Cytokines were selected based upon previously reported roles in disease progression in C57BL/6J mice (Saleh and Petri, 2019; Abt et al., 2016). Consistent with differences in disease presentation, several pro-inflammatory cytokines were significantly elevated in C57BL/6J mice compared to C3H/HeN mice on day 3 of infection. Levels of cytokines were measured on day 3 of

infection, which immediately follows the point of largest body mass loss during infection in C57BL/6J mice. Only one cytokine, IL-1β, was significantly elevated in C3H/HeN mice. While these studies demonstrated reduced induction of pro-inflammatory mechanisms, these studies failed to provide broader insights into the underlying mechanisms that contribute to these differences. As described in the discussion for Chapter 2, one hypothesis is that the kinetics of disease progression may differ between C3H/HeN and C57BL/6J mice. Doing more extensive analysis from the beginning of infection, including broader gene expression and proteomics analysis, could provide insights into whether this hypothesis is correct. Once we identify specific pathways, comparative genomics analysis could be performed to look at the differences between both mice strains and the correlations to disease severity at a deeper level.

It is also possible that other mechanisms might have a more significant role in providing protection against CDI. *C. difficile* produces a paracrystalline surface protein array, known as the S-layer, composed of surface layer proteins (SLPs) (Ní Eidhin et al., 2008). It has been demonstrated that SLPs have a role in binding of *C. difficile* in the gastrointestinal tract, but they could potentially have additional roles as virulence factors as it has been shown for other bacteria such as *Campylobacter fetus* and *Aeromonas salmonicida* (O'Brien et al., 2005; Grogono-Thomas et al., 2000; Sára & Sleytr, 2000). SLPs have also been associated to innate immune system mechanisms for recognition of *C. difficile* where they could be playing an important role for activating the inflammatory responses (Ryan et al., 2011). Therefore, we will also examine whether differences in SLPs receptors could play a role in modulating disease severity in our $^{HMA}$mouse model.

Altogether, we believe the research described in this thesis has the potential to contribute in the understanding of microbe-host interactions that regulate susceptibility and disease outcome in the context of CDI and lead to the development of novel targeted treatments adjunct to antibiotics and preventative strategies.

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**APPENDIX A**

To produce $^{HMA}$mice, we colonized germ-free C57BL/6J and C3H/HeN mice with microbiomes from three different healthy donors (Donor A, B and C). These mice were bred under specific-pathogen-free conditions and their microbiota was subsequently passed to their progeny. We collected fecal samples from different $^{HMA}$mice progeny mice at 6-8 weeks of age in order to examine their microbial composition with 16S rRNA gene sequencing. DNA was extracted by bead beating fecal pellets with 0.1mm beads, followed by BioSprint 96 One-For-All Vet kit processing. DNA was then amplified in duplicate with Phusion polymerase using Illumina barcoded primers 515F and 806R (Collins et al., 2015), then sequenced on a MiSeq using 2 x 250 kits. Generated fastq files were processed by mothur 1.41.3, removing chimeras identified by uchime, mapping sequences against Silva release 132, and clustering OTUs at 99% identity (Schloss et al., 2009). To remove sample bleedover, the median + quartile value for a given OTU in 74 controls (*Bacillus subtilis* 168 or ZymoBIOMICS microbial community standard) were removed from all samples prior to rarefaction. The level of rarefaction was chosen to maximize the number of samples and the reads/sample.

Beta diversity analysis from the bacterial communities formed in our $^{HMA}$mouse models showed that the communities formed in $^{HMA}$mice segregated primarily by human fecal donor sample (Fig. A1a). Communities from each human fecal donor sample vastly diverged from their respective human sample inoculum along axis 1 with 19.1-23.1% of variation explained. Additional separation was observed along axis 2 (10.8-17.8% of variation), which separated mice based primarily upon mouse genetic background (Fig. A1b-d), although communities formed in microbiome C colonized mice were more

similar between C3H/HeN and C57BL/6J mouse strains than were communities formed in $^{HMA}$mice colonized with the other two microbiomes (MA and MB). Analysis of alpha diversity measures indicated that communities formed in C3H/HeN mice had lower levels of total OTUs when colonized with all three human microbiomes and reduced Shannon Diversity in mice colonized with microbiomes B and C (Fig. A1e); the microbiome from donor A, which started with the highest overall diversity and number of OTUs, had the most dramatic reduction in diversity compared to the starting inoculum.

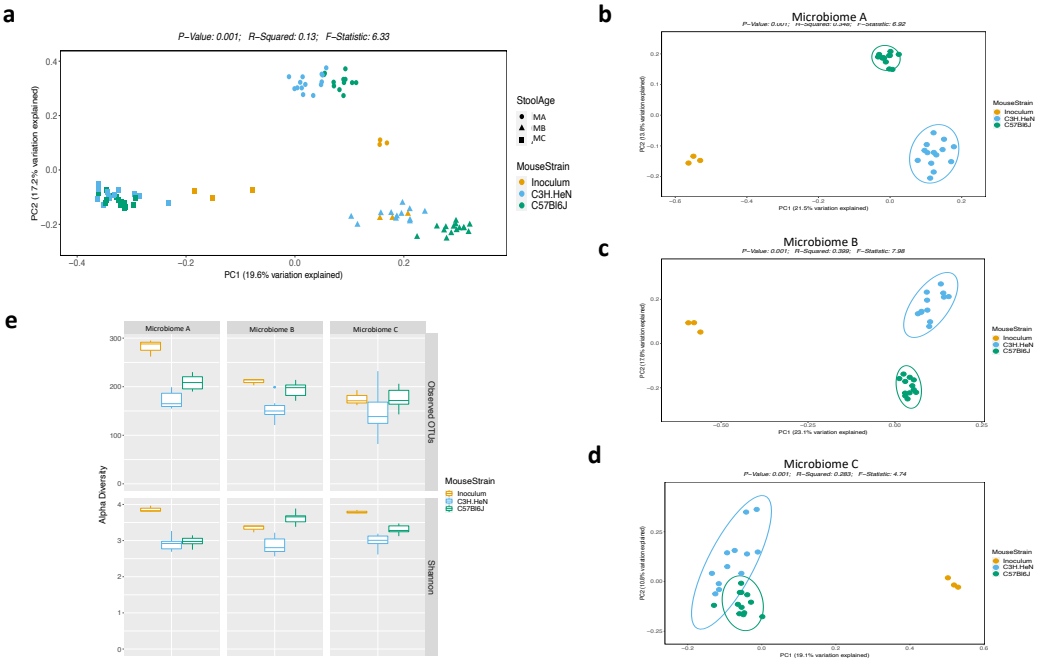


**Figure A1. Measurements of alpha and beta diversity from $^{HMA}$mice. (a-d)** Clustering of bacterial communities formed in different $^{HMA}$mice colonized with three distinct healthy donor's microbiomes based upon principal coordinates analysis (PCoA) of Sorensen dissimilarities **(e)** Measurements of number of OTUs observed and Shannon diversity from the different microbiome-mouse combinations. For mouse samples, n=12 replicates/HMAmouse line, except for C3H/HeN-MA, n=14; for donor inocula, n=3 technical replicates. In a-c, statistical significance of groupings was determined through PERMANOVA.

The relative abundance of the top 20 bacterial genera shifted in both mouse strains from the initial inoculum (Fig. A2). One trend that was obvious for all $^{HMA}$mice was an overall increase in the abundance of *Bacteroides* in all donor colonized mice

compared to the starting inoculum. We also observed a reduction in levels of *Agathobacter,* a genus of *Lachnospiraceae*, in donor B and C colonized mice compared to the inoculum. Of note, Donor A colonized mice had lower overall levels of *Bacteroides* and increased in *Muribaculaceae* compared to donor B and C-colonized mice.

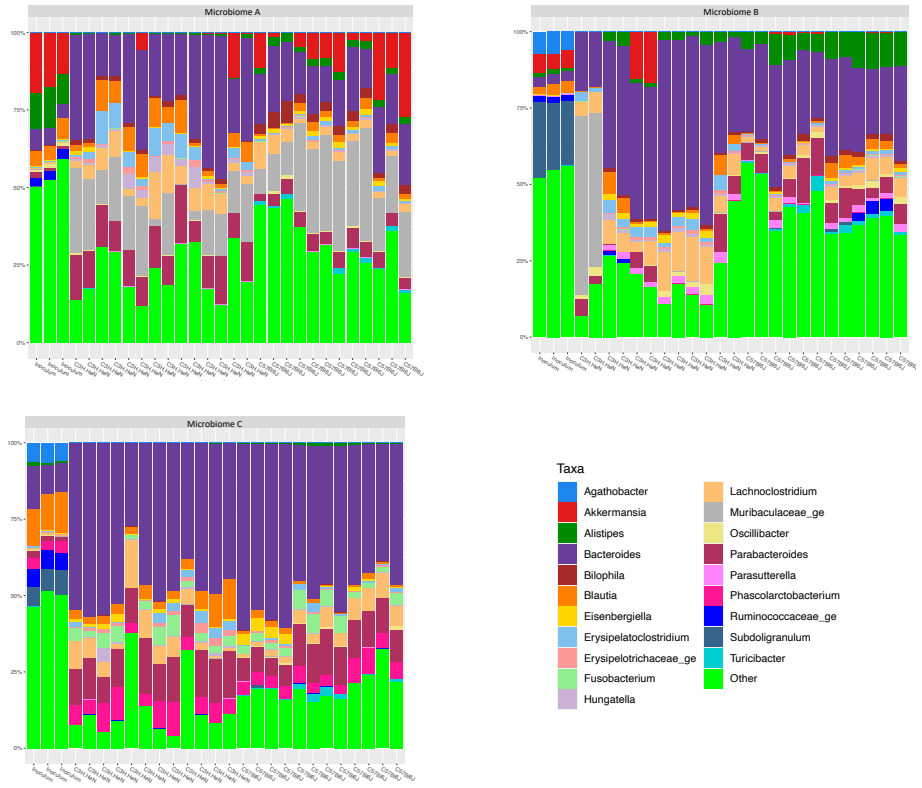


**Figure A2. Top 20 taxa that differ significantly between $^{HMA}$mice and fecal inocula.** Stacked bar plots showing the relative abundance of bacterial genera of replicate fecal inocula and $^{HMA}$colonized mice distinct communities. Data are relative abundances of sequences assigned at the genus level from inoculum, n=3; C3H/HeN-MA, n=14; C3H/HeN-MB and MC, n=12; C57BL/6J-MA, MB and MC, n=12.

To better understand the differences in beta-diversity that we observed between C3H/HeN and C57BL/6J mice colonized with the same fecal samples that we observed in Fig. A1, we used Mann-Whitney testing to identify genera among the top 20 most abundant genera that differed significantly between C3H/HeN and C57BL/6J mice when

colonized with the same fecal sample. While there were several differences that were unique to each donor microbiome (Figure A3), we also observed trends consistent across all three fecal donor samples that may indicate selection imposed by host genetic background. Specifically, we observed significantly higher levels of *Alistipes* and *Turicibacter* in the C57BL/6J mice and higher levels of *Blautia* in C3H/HeN colonized with microbiomes A and C. As mentioned above, the proportions of *Bacteroides* species was elevated compared to the starting inoculum in all $^{HMA}$mice, with *Bacteroides* species constituting 12-60% of reads detected. Overall, we did not observe obvious patterns that could be linked to differences in CDI disease severity and further research is needed to draw definitive conclusions.

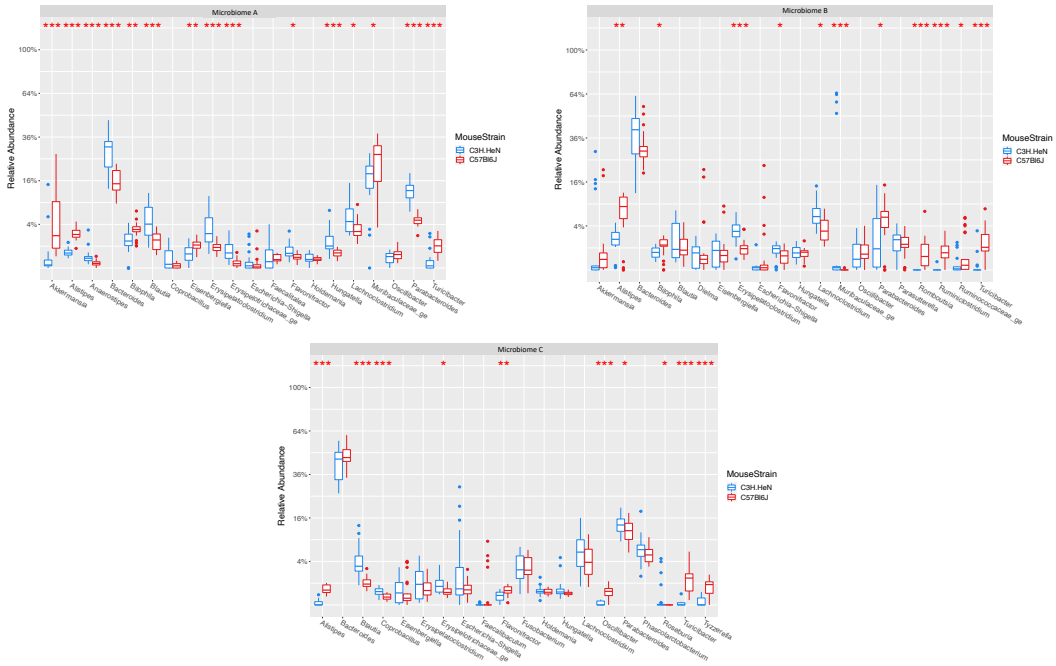


**Figure A3. Top 20 genera that differ significantly between C3H/HeN and C57BL/6J $^{HMA}$mice.** Significant taxa that differentiated between C3H/HeN and C57BL/6J HMA mice organized by donor's microbiome. The OTUs are classified to the genus level and significance ($p<0.05$) was determined with Mann-Whitney test.

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