MICROBIOTA- AND PATHOGEN-SPECIFIC CONTRIBUTIONS TO CLOSTRIDIUM DIFFICILE VIRULENCE IN THE MOUSE MODEL

A Dissertation

Presented to the Faculty of the Weill Cornell Graduate School

of Medical Sciences

in Partial Fulfillment of the Requirements for the Degree

of Doctor of Philosophy

by

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August 2017

MICROBIOTA- AND PATHOGEN-SPECIFIC CONTRIBUTIONS TO CLOSTRIDIUM DIFFICILE VIRULENCE IN THE MOUSE MODEL

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Cornell University 2017

Clostridium difficile is an anaerobic, gram-positive bacterium that is responsible for the majority of hospital-associated gastrointestinal infections. It has been recognized as a pathogen since the 1970s but more recently has become an urgent threat to public health. *C. difficile* produces two powerful toxins that disrupt the integrity of the colonic epithelium and induce a strong inflammatory response. Susceptible individuals experience symptoms that range from mild, self-limiting diarrhea to fulminant pseudomembranous colitis and even death. However, most healthy individuals are protected from *C. difficile* infections so long as they are able to maintain a diverse population of commensal bacteria in their gut. Disruptions to these commensals, often from antibiotic therapy, provide the niche *C. difficile* spores need to germinate, produce toxins, and cause disease. Current first-line therapy for infections is additional antibiotics that lead to a high risk of relapse. In fact, we found that short course antibiotic therapy leaves mice susceptible to additional infections in the days and weeks that the commensal microbiota spends recovering to pre-antibiotic levels. Beyond requiring disruptions to the microbiota before colonization, *C. difficile* is composed of hundreds of different strain subtypes. The variability in disease severity induced by each of these different subtypes has been hampered by diverse sources of human patient data and has confused the literature for years. We found that the mouse model could be used successfully to quantify the differences in disease

burden of phylogenetically diverse *C. difficile* clinical isolates. Our results demonstrate that differences in observed virulence have less to do with the amount of toxin each isolate produces and more to do with its tolerance to secondary bile acids like lithocholic acid. In addition, whole genome sequencing allows us to identify groups of genes that are associated with highly lethal strains. This work emphasizes the need to evaluate the impact of antibiotic therapy and infecting strain when assessing and treating *C. difficile* infections.

BIOGRAPHICAL SKETCH

Brittany B. Lewis completed a Bachelors of Sciences in Neuroscience at the College of William and Mary in 2011 and matriculated into the Weill Cornell / Rockefeller / Sloan Kettering Tri-Institutional MD-PhD program in 2011. She joined Dr. Eric Pamer's laboratory in 2013.

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Lewis B.B., Buffie C.G., Carter R.A., Leiner I., Toussaint N.C., Miller L.C., Gobourne A., Ling L., Pamer E.G. (2015) Loss of microbiota-mediated colonization resistance to Clostridium difficile infection with oral vancomycin compared with metronidazole. **J Infect Dis**.

Abt M.C., **Lewis B.B.**, Caballero S.G., Xiong H., Carter R.A., Susac B., Ling L., Leiner I., Pamer E.G. (2015) Innate immune defenses mediated by two ILC subsets are critical for protection against acute Clostridium difficile infection. **Cell Host Microbe**.

Lewis B.B., Carter R.A., Pamer E.G. (2016) Bile acid sensitivity and in vivo virulence of clinical Clostridium difficile isolates. **Anaerobe**.

Lewis B.B., Carter R.A., Ling L., Leiner I., Taur Y., Kamboj M., Dubberke E.R., Xavier J., Pamer E.G. (2017) Pathogenicity locus, core genome, and accessory gene contributions to Clostridium difficile virulence. **mBio** (*submitted*).

For my family, especially

Mom, Dad, and Megan.

We've traveled the world together,

and you all have kept me

grounded, focused, motivated, and grateful

as I explored my curiosity in the lab.

ACKNOWLEDGMENTS

First I would like to acknowledge the mentorship and support provided by my thesis adviser, Dr. Eric Pamer. Eric is a real-life physician scientist who has demonstrated countless times the unique advantages of living in both the medical and research worlds. He has brought scientific questions to me based on his observations in the hospital and never lets his research questions stray too far from the translational realm. I would also like to thank all members of the Pamer lab for their support, both scientific and personal, during the past four years of my training. Additional thanks goes to my thesis committee members, Dr. Joao Xavier, Dr. David Artis, and Dr. Daniel Mucida, for their comments and advice as my thesis work has evolved. The Tri-Institutional MD-PhD program, especially Ruth Gotian and Dr. Olaf Andersen, have been essential as I transition between medical and graduate work. I am also grateful for the friends and family who have supported me throughout the years. Their interest in my scientific research has reminded me why studying infectious diarrhea is fascinating and important, even during those times when the work has been challenging. Finally, I would like to thank my undergraduate advisor, Dr. Margaret Saha, for being an incredible example of a hardworking, passionate, and successful female scientist.

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LIST OF ABBREVIATIONS

VRE	vancomycin-resistant *Enterococcus*
MRSA	methicillin-resistant *Staphylococcus aureus*
ESBL	extended spectrum beta lactamase
FMT	fecal microbiota transplant
RegIIIγ	regenerating islet-derived protein 3 gamma
TLR	toll-like receptor
SFB	segmented filamentous bacteria
IL	interleukin
LPS	lipopolysaccharide
SCFA	short chain fatty acid
LGG	*Lactobacillus rhamnosus* GG
CDC	Centers for Disease Control
CDI	*Clostridium difficile* infection
IDSA	Infectious diseases society of America
MSKCC	Memorial Sloan Kettering Cancer Center
KPC	carbapenem-resistant *Klebsiella pneumonia*
OTU	operational taxonomic unit
CFU	colony forming unit
MLST	multi-locus sequence type
LCA	lithocholic acid
DCA	deoxycholic acid
OD	optical density
PaLoc	pathogenicity locus

LIST OF SYMBOLS

γ	gamma
α	alpha
β	beta
ϕ	phi

CHAPTER 1

INTRODUCTION

1.1. OVERVIEW

Bacterial pathogens are increasingly antibiotic resistant, and development of clinically effective antibiotics is lagging. Curing infections increasingly requires antimicrobials that are broader spectrum, more toxic, and more expensive, and mortality attributable to antibiotic-resistant pathogens is rising. The commensal microbiota, comprising microbes that colonize the mammalian gastrointestinal tract, can provide high levels of resistance to infection, and the contributions of specific bacterial species to resistance are being discovered and characterized. Microbiota-mediated mechanisms of colonization resistance and pathogen clearance include bactericidal activity, nutrient depletion, immune activation, and manipulation of the gut's chemical environment. Current research is focusing on development of microbiota-based therapies to reduce intestinal colonization with antibiotic-resistant pathogens, with the goal of reducing pathogen transmission and systemic dissemination.

1.1.1. Introduction

Acquisition of antibiotic resistance by bacterial pathogens is one of the most important challenges facing modern medicine. Antibiotic therapy has driven the emergence of resistant bacteria that threaten the health of increasing numbers of people each year(38). Antibiotic resistance has become so widespread that the World Health Organization(249) reports that it is now "one of the biggest threats to global health, food security, and development." Antibiotic resistance among bacteria such as

Salmonella enterica Typhimurium, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, and many other pathogens is leaving patients and their physicians with a shrinking repertoire of treatment options. In addition to antibiotic-resistant bona fide pathogens, a number of less pathogenic bacterial species have acquired very high levels of antibiotic resistance and have emerged as common and difficult to treat causes of infection. Strains of *Enterococcus faecium*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Enterobacter cloacae* can be highly antibiotic resistant and cause systemic infection in hospitalized patients. Extensive resources are required to limit patient-to-patient transmission of these strains within health care environments. Physicians are often forced to use newer, broad-spectrum antibiotics or older, more toxic drugs when treating patients with systemic infections caused by these organisms.

Mammals are colonized by complex commensal bacterial populations, referred to as the microbiota, with wide-ranging physiologic effects. In many settings the microbiota contributes to health and disease resistance, whereas in other circumstances, it exacerbates inflammatory disease pathogenesis. While the term "microbiome" was coined only in 2001(131), the discovery that mucosal surfaces are covered with diverse "animalcules" is attributed to Antony van Leeuwenhoek and his use of the microscope in the seventeenth century(133). The microbiota contains a diverse array of bacteria, viruses, fungi, and protozoans, but the bacterial component has been most extensively investigated. These studies are beginning to reveal how disruptions to bacterial populations affect health and disease. Although diet and other environmental factors can influence the composition of the human microbiota, administration of antibiotics has the most dramatic and long-term impact, in some cases leading to permanent loss of many bacterial taxa(54, 55). In this review, we summarize recent work on three groups of bacterial pathogens that are readily suppressed by a complex, antibiotic-naive microbiota but that can thrive and cause

disease in patients with a disrupted microbiota: *Clostridium difficile*, vancomycin-resistant *Enterococcus* (VRE), and multidrug-resistant *Enterobacteriaceae*. These resistant pathogens have arisen in part because of widespread antibiotic use, and they thrive in the setting of antibiotic-induced microbiome dysbiosis. In addition, we review strategies to remediate antibiotic-induced damage to the microbiota and to enhance resistance to these infections(27).

1.2. ANTIBIOTIC TREATMENT COMPROMISES THE MICROBIOTA AND INCREASES SUSCEPTIBILITY TO NOSOCOMIAL INFECTIONS

1.2.1. *Clostridium difficile*: Emergence and relationship to antibiotic therapy

Clostridium difficile is a gram-positive, anaerobic bacterium that causes antibiotic-associated diarrhea and colitis and results in the majority of gastroenteritis-associated deaths in the United States(92). It was identified as the cause of antibiotic-associated pseudomembranous colitis in 1978 following the demonstration that *C. difficile* culture supernatants caused cell rounding and death in in vitro cell cultures(83). The early part of the 21st century was marked by a large increase in both the number and the severity of *C. difficile* colitis cases in the United States. In 2013, the Centers for Disease Control and Prevention(38) classified the threat of *C. difficile* as urgent and called for intensified investigation of this pathogen. *C. difficile* infects at least 453,000 Americans annually, leading to approximately 29,000 deaths and in excess of $4.8 billion in health care costs(134). Previous antibiotic exposure, hospitalization, advanced age, compromised immune system, and some forms of cancer treatment, such as hematopoietic stem cell transplantation, are associated with increased risk of developing *C. difficile* colitis(116).

A healthy, diverse community of commensal microbes is essential for protection against *C. difficile*--associated disease(3, 194). Brief treatment with antibiotics can be sufficient to disrupt the host's microbiota-mediated resistance to *C. difficile* infection; many classes of antibiotics can induce profound, long-lasting shifts in the microbiota that enable *C. difficile* germination and outgrowth(4, 29, 30, 135, 209, 216). Treatment with metronidazole or oral vancomycin is only partially effective at curing *C. difficile* infection. This is in part because it does not restore protective commensal microbial populations and recurrence is therefore common(26). Fidaxomicin, a newer antibiotic introduced to treat *C. difficile* infections, has a narrower antibacterial spectrum. It preserves some colonic anaerobic taxa and is associated with a lower risk of relapse(44, 124, 182), but because of its high cost, it is rarely used as first-line treatment of *C. difficile* infection. *C. difficile* can persist in aerobic environments as heat-, acid-, and alcohol-tolerant spores that are notoriously difficult to eradicate, partially explaining high rates of infection in health care settings and high rates of recurrence in treated patients(51, 124, 182).

1.2.2. Vancomycin-resistant *Enterococcus*: Emergence and relationship with antibiotic therapy

Enterococci are gram-positive diplococci that constitute a population of commensal bacteria that reside in the gastrointestinal tract of most animals, albeit as minority members of the microbiota. In contrast to *C. difficile*, which remains susceptible to a wide range of antibiotics, members of the *Enterococcus* genus have become resistant to many clinically useful antibiotics, including vancomycin. VRE was first described in 1986(130, 226), and current estimates suggest that 30% of health care--associated *Enterococcus* infections are now vancomycin resistant(38). Most VRE infections are caused by *E. faecium*, with smaller contributions from *E. faecalis*

and *E. avium*(257). Intestinal colonization with VRE is asymptomatic. However, gut colonization can lead to dangerous, difficult to treat systemic infections if the bacteria traverse the intestinal epithelium and enter the bloodstream. Antibiotic treatment can lead to blooms of VRE in the gastrointestinal tract, and studies in patients undergoing allogeneic hematopoietic stem cell transplantation demonstrated that intestinal VRE colonization precedes VRE bacteremia(224). Later studies reported that VRE domination (when more than 30% of bacteria in the sample belong to one VRE operational taxonomic unit) results in a ninefold increased risk of VRE bacteremia(214). In addition, posttransplantation patients who harbor high levels of *Enterococcus* spp. in their large intestines are more likely to suffer from gastrointestinal graft-versus-host disease(99) and have worse survival outcomes(239).

Intestinal domination by VRE bacteria can usually be traced to previous antibiotic administration. This has been demonstrated most clearly in mouse models: while antibiotic-naive mice are able to clear an oral challenge with VRE, mice pretreated with antibiotics experience a profound VRE bloom and remain persistently colonized(60, 153, 172, 224). In humans, complete eradication of VRE from the gut using antibiotics alone has proven challenging: One group found that patients thought to have successfully cleared VRE colonization had for years been harboring low levels of these organisms, which then rebounded to high levels following antibiotic treatment(11). In addition, colonization by VRE is associated with other hospital-acquired infections, including methicillin-resistant *Staphylococcus aureus* (MRSA), *C. difficile*, and multidrug-resistant, gram-negative bacteria(63).

1.2.3. Multidrug-resistant *Enterobacteriaceae*: Emergence and relationship with antibiotic therapy

Antibiotic use is also linked to an increase in the number of infections with gram-negative bacteria in the *Enterobacteriaceae* family. The *Enterobacteriaceae* include familiar pathogens in the genera *Escherichia*, *Citrobacter*, *Klebsiella*, *Serratia*, *Salmonella*, *Enterobacter*, *Yersinia*, and *Proteus*. All members of the *Enterobacteriaceae* family can reside in the gastrointestinal tract: Some remain local and cause intestinal inflammation and diarrhea, while others cross the epithelium to disseminate systemically. A common characteristic of bacteria in the *Enterobacteriaceae* family is the high prevalence of multidrug resistance(102). The proportion of strains producing extended-spectrum β-lactamases (ESBLs) (which confer resistance to many β-lactam antibiotics) and carbapenemases is increasing around the world(249). Not surprisingly, bloodstream infections with these organisms are often preceded by gut colonization and are associated with high mortality(20, 38).

1.2.4. Recovery of the microbiome is key to eradicating infections that arise in the setting of dysbiosis

Restoration of the microbiota to its preantibiotic state is critical to achieve long-lasting protection against pathogens that exploit dysbiosis to cause disease. One way to restore the microbiome is through fecal microbiota transplantation (FMT), a practice that was first described in China in the fourth century as a treatment for intestinal maladies(80) but that has recently been demonstrated to be effective for recurrent *C. difficile* infection. In contrast to antibiotic treatment, which removes bacterial communities, FMT therapy introduces microbes en masse from a healthy donor into a diseased gut. In the modern medical era, FMT was initially described as a treatment for pseudomembranous enterocolitis by Eiseman and colleagues in

1958(69), but it was not subjected to a randomized controlled trial until 2013(229). This later study and others have shown remarkable relapse-free cure rates (greater than 90%) of *C. difficile* that surpass the efficacy seen with conventional antibiotics(89). Advances in sequencing technology have ushered in a wave of studies that investigated the bacterial populations that are present before and after antibiotic therapy and have correlated the loss of members of the phyla *Firmicutes* and *Bacteroidetes* with susceptibility to *C. difficile* infections in both mice and humans(40, 220).

In contrast to the investigation of FMT for *C. difficile* infections, less work has focused on the potential of FMT to eliminate VRE from the gut. Ubeda et al.(222) and Caballero et al.(32) demonstrated that administration of feces from an antibiotic-naive mouse to mice dominated by VRE can lead to clearance of the pathogen from the gastrointestinal tract. Fractionation of the colonic microbiota of antibiotic-naive mice demonstrated that VRE clearance is dependent upon obligate anaerobic bacterial populations and that *Barnesiella* abundance is inversely correlated with VRE levels in both mice and humans (**Figure 1.1**)(222). Studies using an anaerobic in vitro system found that the human fecal microbiota is sufficient to eliminate VRE and depends on anaerobic bacteria(62, 100). While the ability of FMT to clear VRE from the gastrointestinal tract of patients has not been tested in a randomized controlled trial, an anecdotal report suggests that reestablishing a diverse microbiota can reduce VRE colonization(66).

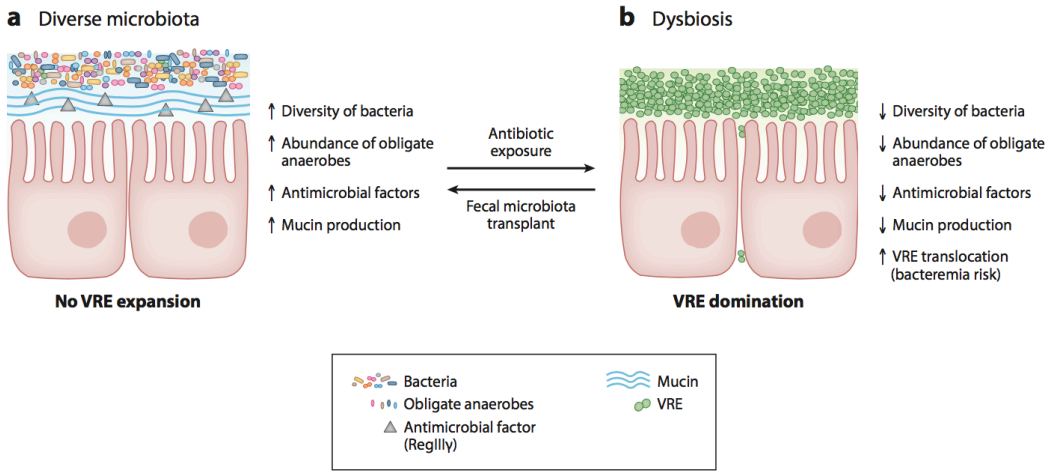


Figure 1.1. Characteristics of healthy microbiota and dysbiotic microbiota following exposure to vancomycin-resistant *Enterococcus*. (**A**). The healthy microbiota is characterized by a highly diverse population of commensal bacteria including obligate anaerobes. Expression of antimicrobial peptides is high, and the mucous layer is thick. (**B**). Following antibiotic exposure, VRE can bloom to high levels. Bacteria that translocate across the epithelium may cause bacteremia.

FMT therapy also shows promise in clearing colonization with multidrug-resistant *Enterobacteriaceae*. Studies in mice demonstrate that antibiotic-naive animals quickly clear infections with pathogenic *E. coli* but that short-course antibiotic treatment permits sustained colonization with these organisms(228, 241). Metronidazole, which has activity against anaerobic bacteria, is associated with increased severity of *C. rodentium* infections(248). FMT clears intestinal colonization with carbapenem-resistant *K. pneumoniae*(32), and a diverse, complex microbiota can clear infections with nontyphoidal *Salmonella* diarrhea in mice(70). Further studies demonstrate that a consortium of 15 commensal bacterial strains cultured from resistant mice can provide high levels of resistance against *Salmonella enterica* Typhimurium(28).

Evidence of FMT efficacy in humans is limited to case reports for infections other than *C. difficile* colitis. Bilinski and colleagues used a fecal transplant to treat an

immunocompromised patient colonized with both β-lactamase-positive *K. pneumonia* and ESBL-positive *E. coli*(19). They found that rectal swabs collected one month after transplantation yielded neither *K. pneumonia* nor *E. coli*. Another study reported that a single FMT (originally prescribed for a relapsing *C. difficile* infection) had the added benefit of clearing infections with carbapenem-resistant *Enterobacteriaceae*, MRSA, multidrug-resistant *Acinetobacter baumanii*, and VRE(46).

1.3. MECHANISMS OF MICROBIOTA THERAPY EFFECTIVENESS

1.3.1. Commensal bacteria can combat pathogens both indirectly and directly

The mechanisms by which commensal bacterial strains enhance resistance against pathogens are complex and often overlap. In the following sections we describe inhibitory mechanisms that range from direct, i.e., bacterium-to-bacterium inhibition, to indirect, i.e., involving commensal bacterial induction of host immune defenses or modification of host factors that inhibit pathogens.

1.3.2. Competition for essential cations can help defend against enteric infections

The intestinal lumen of mammalian hosts is an environment that poses significant challenges to microorganisms that colonize it, and many exogenous bacteria are unable to survive or persist in the gut. For example, microbial growth can be inhibited by the sequestration of essential cations. Neutrophils recruited to the gut in response to infection release calprotectin and lipocalin 2 (among other antimicrobial factors), which chelate zinc, manganese, and iron(56). Cation sequestration is effective in inhibiting the growth of many pathogenic microbes. However, certain members of the *Enterobacteriaceae* family have evolved ways to avoid the chelation and proliferate within the inflamed gut(18). *Salmonella* serovar Typhimurium produces the

manganese transporters MntH and SitABCD, which allows it to acquire manganese and provides a survival advantage over certain *E. coli* strains(56). Members of the same *Salmonella* serovar similarly circumvent zinc chelation using a zinc transporter, ZnuABC(139), and they produce a number of siderophores that scavenge iron(52). Interestingly, while *Salmonella* bests *E. coli* in the context of manganese starvation(56), the *E. coli* strain Nissle 1917 utilizes its own powerful siderophores to outcompete *Salmonella* during states of inflammation-induced iron depletion(52, 56).

1.3.3. Cooperative interactions between commensal microbes and the host response

An important mechanism by which the microbiota can combat intestinal infections is through the influence of bacteria on host responses. The commensal microbiota interact closely with the host immune system, in the process promoting additional levels of defense against invading pathogens. The presence of commensal microbes induces expression of regenerating islet-derived protein III-gamma (RegIIIγ), an antimicrobial C-type lectin that defends against gram-positive bacteria(24, 25, 37). Depletion of commensal species following antibiotic treatment drastically lowers RegIIIγ expression in the gut, thereby increasing the risk of extraintestinal VRE dissemination (**Figure 1.1**)(24, 214). RegIIIγ expression, along with expression of the α-defensin cryptdin 2, mediates the protective effect produced by a commensal strain of *E. faecium* against infection with *S. enterica* Typhimurium: *E. faecium* produces a peptidoglycan hydrolase called SagA that can protect against infections with *S. enterica* or *C. difficile* when expressed ectopically on commensal strains unrelated to *E. faecium*(165, 175). In addition, systemic flagellin or oral LPS [agonists for Toll-like receptor 5 (TLR5) and TLR4, respectively] are able to restore RegIIIγ expression after antibiotic treatment and improve host defense against VRE

challenge(24, 117). This effect is not limited to bacterial agonists of TLR signaling. The TLR7 agonist resiquimod mimics the effect of viral infection and can decrease VRE colonization in antibiotic-treated mice, again in a RegIIIγ-dependent manner(1).

The relationship between commensal bacteria and host immune responses is not limited to the induction of antimicrobial peptides. For example, Zeng and colleagues(254) identified an antigen on the surface of some gut commensal species that triggers the production of IgG antibodies. These antibodies recognize conserved antigens found on gram-negative pathogens and are important for the opsonization and clearance of both *E. coli* and *Salmonella* infections. In addition, colonization of epithelial-adherent segmented filamentous bacteria (SFB) or commensal *Clostridia* strains is sufficient to induce accumulation of Th17 cells, potent signals for neutrophil recruitment and pathogen clearance(10). Epithelium-derived molecules such as IL-22RA1 protect mice from *C. rodentium* infections by increasing production of fucosylated oligosaccharides, an important nutrient for anaerobic commensal symbionts(169). In times of stress, like that induced by exposure to TLR ligands, the host sheds fucosylated proteins into the gut lumen, which are then metabolized by the commensal bacteria that provide protection against *Citrobacter* infections(170). However, the beneficial increase in immune tone can be exploited to the host's detriment. One member of the *Enterobacteriaceae* family, *Proteus mirabilis*, can stimulate production and release of IL-1β from intestinal monocytes and thus amplify inflammation after intestinal injuries(195).

One feature of commensal organisms is their ability to persist in the presence of host antimicrobial factors. Many members of the *Bacteroidetes* phylum escape inflammation-mediated clearance by removing a single phosphate group from their LPS molecules, conferring resistance to antimicrobial peptides like polymyxin B(47). Certain pathogens, however, are able to escape the immune response stimulated by

commensal microbes. Among these bacteria is *E. coli*: Pathogenic strains are able to expand to high levels in the inflamed gut using a nitrate respiration pathway(206, 245). Future strategies that employ microbial cues to boost the host response may therefore be challenged by the ability of pathogens to evade and potentially even exploit inflammatory responses.

1.3.4. Carbohydrate metabolism by the microbiota affects pathogen outgrowth

One major nutrient source for commensal microbes is dietary carbohydrates. Complex carbohydrates can be metabolized to short-chain fatty acids (SCFAs; primarily acetate, propionate, and butyrate) or released as monosaccharides such as sialic acid(144). The presence or absence of specific members of the microbiota influences the production of SCFAs and organic acids, which in turn influences the pathogen's ability to expand to high densities. For example, certain strains in the *Bifidobacterium* genus metabolize carbohydrates to acetate. Increased acetate levels protect mice from a lethal challenge with Shiga toxin--producing *E. coli* by stimulating the host inflammatory response and preventing an increase in epithelial barrier permeability(78). In a similar mechanism, the butyrate produced by SFB and benign *Clostridia* species is known to be important in decreasing the colonization and dissemination ability of *S. enterica* Typhimurium(107, 181).

The commensal species *Bacteroides thetaiotaomicron* possesses a sialic acid catabolism operon capable of freeing sialic acid from mucosal carbohydrates. In the antibiotic-naive state, commensal bacteria consume sialic acid so that it remains at low levels in the gut. However, antibiotic treatment increases sialic acid concentrations in the large intestine, which both *C. difficile* and *S. enterica* Typhimurium can metabolize, leading to their growth and expansion. Lacking sialidase genes themselves, *C. difficile* and *S.* Typhimurium rely on the presence of *B. theta* as they

expand and cause disease(155). In addition to benefitting from *B. thetaiotaomicron*'s sialic acid catabolism, *C. difficile* reduces succinate (again produced during the commensal's fermentation of dietary carbohydrates) to form butyrate. Succinate, in this case, is used by the pathogen to regenerate NAD^+, and the absence of most commensals allows succinate levels to rise in the postantibiotic state(72).

1.3.5. Bile acid levels affect *C. difficile*'s ability to germinate and expand in the gut

The microbiota also influences the growth of pathogens like *C. difficile* through its effects on bile acid metabolism (**Figure 1.2**). Bile acids are amphipathic molecules produced in primary form in the liver and converted to secondary bile acids by specific members of the commensal microbiota(148). The relative concentrations of primary and secondary bile acids serve as key signals for *C. difficile* spore germination and growth: Primary bile acids such as taurocholate and cholate induce spore germination(174, 242, 243), whereas secondary bile acids such as deoxycholate and lithocholate inhibit outgrowth of toxin-producing vegetative cells(118, 215). Critical to *C. difficile*'s ability to germinate from metabolically dormant spores are the relative concentrations of various members of the bile acid pool. For example, cecal extracts from antibiotic-naive mice, which have a high relative concentration of inhibitory secondary bile acids, do not support outgrowth of *C. difficile* spores(84). Antibiotic administration decreases the population of bile acid--converting commensal species and thus leads to a drastic drop in secondary bile acid levels and a relative increase in germination-promoting primary bile acids. In this setting, *C. difficile* can grow to high densities. Successful FMT is accompanied by a return of secondary bile acid levels to preantibiotic levels(238). In addition, administration of a four-bacteria

mix containing a species (*Clostridium scindens*) with the bile acid conversion operon *bai* is sufficient to provide protection against *C. difficile* challenge(29).

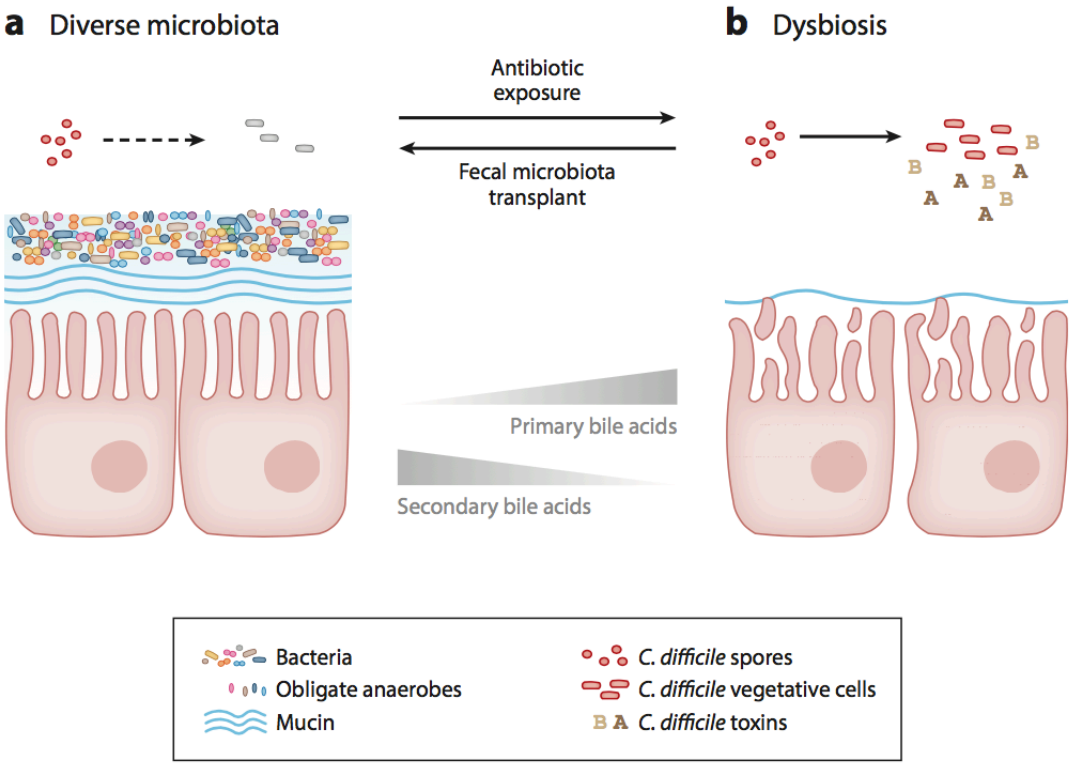


Figure 1.2. Relative concentrations of primary and secondary bile acids affect *C. difficile* colonization ability. (**A**). The healthy microbiota contains commensal bacteria with bile acid conversion enzymes. Secondary bile acid concentration is high, and *C. difficile* spores (red cocci) are unable to germinate. (**B**). The dysbiotic microbiota is characterized by a higher concentration of primary bile acids, which promote germination and production of toxins A and B from *C. difficile* cells (red bacilli).

1.3.6. Bacteriocins target and kill competing bacteria

Several members of the commensal microbiota produce small molecules that directly target neighboring bacteria. One example of direct inhibition comes from bacteria related to the Clostridiales cluster XIVa, which secrete an unidentified metabolite capable of inhibiting expression of *Salmonella* invasion genes in vitro(8).

Many bacteria synthesize bacteriocins, small molecules that kill other bacteria with high specificity.

Of the known bacteriocins, lanthipeptides are of special clinical interest because of their specificity and stability. This subset of bacteriocins possesses multiple thioether cross-linked amino acids (termed lanthionines) that confer stability against proteases, heat, and oxidation(6, 59, 158, 190, 227). Lanthipeptides target gram-positive bacteria and are produced by a variety of commensal and pathogenic organisms. More than half of *E. faecalis* strains contain a plasmid that encodes the Bac41 family of bacteriocins(122). This plasmid contains both bacteriocin effector genes and encodes immunity factors that protect the parent bacteria from self-destruction. Bacteriocins lyse target bacteria by interfering with cell wall integrity, yet specificity and selectivity are sufficient to preserve microbial diversity in the gut(122).

1.4. THERAPEUTICS DERIVED FROM THE COMMENSAL MICROBIOTA

1.4.1. Limited effectiveness of antibiotics for the treatment of infections resulting from a compromised microbiota

Antibiotics remain the principal treatment for gastrointestinal infections with *C. difficile* and systemic infections with VRE and antibiotic-resistant *Enterobacteriaceae*. As antibiotic resistance increases, however, alternative approaches that exploit the ability of commensal bacteria to confer resistance to colonization may provide important alternative avenues to combat infections and reduce their transmission. FMT therapy holds some promise for treatment of *C. difficile* and multidrug resistant bacterial infections, but there are several important drawbacks: Donor screening requires testing for bacterial, viral, and protozoan pathogens, and it is costly and time consuming. Furthermore, current technologies are

unable to completely determine fecal composition, raising the possibility that uncharacterized components may adversely affect the FMT recipient(114, 162, 184, 191, 250). Given these concerns, there is a strong rationale to shift from collecting and administering complex and incompletely defined fecal samples to assembling and delivering precisely defined mixtures of commensal microbes for specific clinical interventions. Ultimately, the goal of microbiota-based therapies is to eradicate the offending microbe from the gut with minimal disruption to the commensal flora and minimal toxicity to the host. The following sections describe the current status of microbiota-based therapies, focusing on bacterial consortia, conventional probiotics, bacteriocins (specifically those in the lanthipeptide class), and phage therapy.

1.4.2. Microbiota therapy for intestinal infections arising in the setting of dysbiosis

As previously outlined, the pathogen most extensively studied with regards to microbiota therapy is *C. difficile*. Most studies of FMT for *C. difficile* infections have used fresh fecal samples, requiring coordination between fecal donation and recipient preparation for FMT, which can be logistically challenging. An important step forward was the demonstration that feces can be frozen for prolonged periods and thawed with minimal loss of effectiveness(252). This enables donors who pass rigorous screening protocols to provide multiple samples that are frozen and stored until they are required for administration. Other studies have demonstrated that fecal samples can be frozen as capsules and administered orally, with 90% overall cure rates that are on par with freshly prepared fecal transplants, with no serious treatment-related adverse events(251). Other groups have confirmed and expanded these studies, determining that fecal samples can be frozen in glycerol for more than six months and even freeze-dried without losing clinical efficacy(45, 94, 98, 132, 219).

The ability of assembled commensal bacterial strains to confer resistance to *C. difficile* in humans was first demonstrated in 1989. Tvede & Rask-Madsen(220) identified a consortium of ten facultative aerobic and anaerobic bacteria that eliminated *C. difficile* toxin from the stool of five patients and led to bowel colonization with commensal *Bacteroides* species. Addition of two bacterial strains to the mix resulted in a 63% cure rate from recurrent *C. difficile* infections(221). A mix of 33 bacterial isolates obtained from a healthy donor was found to cure two patients of hypervirulent *C. difficile* infections and keep them symptom free for at least six months(168). Higher success rates were reported when more organisms were included in the transplant mix, whether as standardized microbiota suspensions(29, 159) or as the ethanol-stable spore fraction of healthy donor samples(115, 159). In mice, microbes such as Lachnospiraceae, *B. thetaiotaomicron*, or a mix of six phylogenetically diverse bacteria have been shown to ameliorate *C. difficile*--associated disease(72, 126, 155, 178, 211). In particular, members of the taxa Ruminoccocaceae, Faecalibacterium, and Clostridia cluster XIVa are associated with resistance to infection(29, 110, 192, 196), while members of the phylum *Proteobacteria* (specifically *Gammaproteobacteria*) are often found in the antibiotic-treated, *C. difficile*--susceptible state(193, 200, 236).

Commensal bacterial species that have been rendered antibiotic resistant by genetic engineering may provide opportunities for enhancing colonization resistance in the setting of ongoing antibiotic treatment. For example, when Stiefel and colleagues colonized mice with a β-lactamase-producing strain of *B. thetaiotaomicron* and administered a dose of ceftriaxone, they found that the presence of the antibiotic-inactivating enzyme in the gut allowed the microbiota to recover and prevent overgrowth with VRE or *C. difficile*(211).

1.4.3. Specific members of the microbiota confer protection against specific intestinal pathogens

Mammalian hosts benefit from the complex, diverse microbiota composed of a broad array of anaerobic bacterial species because they provide resistance to a wide range of pathogens(22, 97). Pathogens differ, however, in terms of which commensal bacterial strains provide resistance. Enterotoxigenic *E. coli*, for example, is inhibited by *Bifidobacterium* species and their introduction of acetate into the intestinal lumen(78). *Bifidobacterium longum* and *B. thetaiotaomicron*, upon monocolonization of the murine intestine, induce transcription of distinct genes in intestinal epithelial cells, perhaps reflecting their different composition and/or localization in the gut(201). Commensal *E. coli* strains are effective in combating the epidemic *E. coli* O157:H7 strain; in this setting, the commensal strains eliminate the pathogenic *E. coli* O157:H7 from the gut by nutrient depletion(129, 146). Resistance to *C. difficile*, on the other hand, can be mediated by *Clostridium scindens*, a bacterial species that converts primary to secondary bile acids, which inhibit growth of the vegetative form of *C. difficile*(29). In contrast, members of the family *Porphyromonadaceae* may be more important when decolonizing patients following *Enterobacteriaceae* infections(222). Oral gavage of commensal *Lactobacillus* species is sufficient to protect against *C. rodentium* infections and improve epithelial barrier integrity(231).

1.4.4. Over-the-counter probiotics and prebiotics and resistance to infections caused by *C. difficile*, vancomycin-resistant *Enterococcus*, or antibiotic-resistant *Enterobacteriaceae*

The idea that specific bacterial species or strains might confer health benefits dates back to over 110 years ago, to Elie Metchnikoff's postulate that ingestion of yogurt containing lactobacilli led to the longevity of certain human populations in

Bulgaria. Foods and supplements said to contain health-promoting bacterial species, often referred to as probiotics, have become a multibillion-dollar industry that, for a number of reasons, has mostly evaded regulation by the US Food and Drug Administration(163). The ability of over-the-counter probiotics to confer resistance to *C. difficile*, VRE, and drug-resistant *Enterobacteriaceae* has been studied. Among these, *Bacillus coagulans*, *Lactobacillus rhamnosus* GG (LGG), and the yeast *Saccharomyces boulardii* have been studied the most extensively and demonstrate some benefits, but they do not clear the gut of *C. difficile* or other antibiotic-resistant pathogens(61, 64, 75, 76, 147, 207, 212, 213). *S. boulardii* reduces the inflammatory response to *C. difficile* infection in vivo, and supernatants of *S. boulardii* cultures reduce *C. difficile* toxin--mediated damage to epithelial cells in vitro(120). Orally ingested *S. boulardii* also appears to reduce the adverse impact of amoxicillin-clavulanate administration on the fecal microbiota(111). Foods containing probiotic microbes, such as kefir (a fermented milk product that contains several strains of live bacteria), also provide some, albeit limited, benefits. It is likely that the levels of colonization resistance conferred by the obligate anaerobic bacterial species that constitute the intestinal microbiota will be difficult to achieve with the more palatable, oxygen-tolerant probiotic bacterial species currently available to the public.

An indirect method to help boost commensal species and restore colonization resistance to infections is with prebiotics, orally ingested products such as complex carbohydrates and fibers that can affect microbiota composition by favoring the expansion of potentially desirable commensal species. Shifting from meat-based to vegetable-based diets alters microbiota composition, a process that is reversible when the diet reverts(50). Plant-derived extracts such as eugenol and lavender oil are reported to promote bacterial diversity and resistance to *C. rodentium* infection(12, 247), and rice bran-enhanced diets can temporarily reduce fecal shedding of *S.*

enterica in mice(121). A polysaccharopeptide from the mushroom *Trametes versicolor*, upon administration to humans, alters the microbiota composition; however, the health implications of the alterations are unclear(161). In contrast, diets enhanced with a prebiotic, either inulin or short-chain fructo-oligosaccharides, had no protective effects when investigated in an in vivo model of *C. rodentium* infection(108).

1.4.5 Bacteriocins as therapeutics

While administration of live probiotic agents for prevention and clearance of antibiotic-resistant intestinal infections can be effective, there are risks associated with the administration of live bacteria to highly immunocompromised patients or patients with compromised mucosal barrier function. Targeting the colonization resistance mechanisms with nonreplicating microbial products or molecules may inhibit invading pathogenic species without exposing the host to live bacteria. Bacteriocins, and lantibiotics in particular, can specifically target some of the pathogenic species that have become highly antibiotic resistant (**Table 1.1**). Some bacteriocins have relatively broad specificity for gram-positive bacteria; the best characterized of these is nisin, which is synthesized by *Lactococcus lactis*. Nisin has antimicrobial activity against a large range of clinical *C. difficile* isolates(127, 128) as well as antibiotic-resistant *Enterococcus* species. Oral administration of nisin-secreting *L. lactis* bacteria, for example, reduced VRE colonization in the gut. This effect was bacteriocin specific, as inoculating mice with a pediocin-producing strain of bacteria did not reduce intestinal VRE colonization(152). Nisin also has bactericidal activity against a variety of clostridial strains but preserves some of the commensal microbiota and is not absorbed in the gastrointestinal tract(15, 39, 74). Another bacteriocin produced by *Lactococcus lactis*, named lacticin, is more potent against clinical *C. difficile* isolates(176). More

specific killing is seen with thuricin CD, a bacteriocin produced by *Bacillus thuringiensis*(177). In addition, *C. difficile* strains produce their own bacteriocins (termed diffocins) that selectively kill other *C. difficile* strains in a murine model with only moderate disruption of the microbiota(81, 82).

Table 1.1. Examples of bacteriocins that can be used to decrease colonization with *C. difficile* and vancomycin-resistant *Enterococcus* infections. [a]Bacteriocin encoded on a plasmid, then produced by the indicated bacterium.

Bacterial host	Bacteriocin produced	Known targets
Lactococcus lactis	Nisin	Many gram-positive bacteria, including *C. difficile* and various vancomycin-resistant *Enterococcus* strains(15, 39, 74, 127, 128, 152)
Lactococcus lactis	Lacticin	Many gram-positive bacteria, including *C. difficile* and various vancomycin-resistant *Enterococcus* strains(101, 176)
Bacillus thuringeinsis	Thuricin CD	*C. difficile*(177)
Bacillus cereus	Cerecidins	Many gram-positive bacteria; strong activity against vancomycin-resistant *E. faecalis*(232)
Clostridium difficile	Diffocins	*C. difficile*(81, 82)
Enterococcus faecalis[a]	Bac41 family	*E. faecalis*(122)
Enterococcus faecalis[a]	Bacteriocin 21	Multidrug-resistant *E. faecalis* and *E. faecium*(119)

Recent studies have identified and engineered more effective bacteriocins. The cerecidins produced by some strains of *Bacillus cereus* are four times more potent than nisin in killing the vancomycin-resistant *E. faecalis* strain V583(232). Introduction of the bacteriocin 21--producing plasmid into a commensal *E. faecalis* strain enabled it to outcompete resident vancomycin-resistant *E. faecalis* in the mouse gut without transferring the plasmid to other resident bacteria(119). Introduction of a plasmid

containing three bacteriocins and a receptor that recognizes an *E. faecalis*--secreted pheromone into an *L. lactis* strain resulted in highly specific killing of VRE in the gut(23).

1.4.6 Bacteriophages can selectively target drug-resistant bacteria with minimal disruption to commensal microbes

Bacteriophages have potential as therapeutic agents because they can selectively target pathogens while preserving commensal bacteria. Bacteriophages are also able to penetrate and kill bacteria in biofilms(113). Phage therapy for VRE infection has mainly been conducted in the in vitro context or with mouse models. One study reported that the endolysin produced by the *E. faecalis* bacteriophage IME-EF1 was able to rescue 80% of mice from a lethal sepsis challenge and mouse survival correlated with lower bacterial counts in the blood(255). Even greater reduction of mortality followed treatment with phage ENB6(21), and phage ø1 can rescue animals from a lethal challenge with *S. enterica* serovar Paratyphi B(33). The effectiveness of phage therapy is strain dependent, and there is no known bacteriophage that is effective against the majority of clinical VRE isolates(87, 164, 171, 225, 233).

Phage therapy can be more effective when multiple phages are combined: For example, a cocktail of five phages reduced *Shigella sonnei* burden by 1--2 logs in mice while maintaining commensal bacteria populations(145). Other groups have described the ability of *E. coli*--targeting phages to lower the pathogen burden using in vitro and in vivo models, reporting greater efficacy when the phage therapy was combined with antibiotic treatment or probiotic supplementation(49, 58, 67, 149). In humans, phages targeting *E. coli* infections appear to be safe but have not achieved improvements in diarrhea symptoms(35, 187, 188).

1.5. CONCLUSIONS

Inadvertent and substantial alteration of the human microbiota resulting from medical treatment began in earnest with the introduction of penicillin in 1928 and became more pronounced with the addition of broader-spectrum antibiotics, many of which achieve high levels in the intestinal lumen and kill obligate anaerobic commensal bacteria. Although loss of microbiota diversity, and obligate anaerobes in particular, has been known to increase susceptibility to intestinal infections for over 70 years(22, 97), our understanding of the mechanisms of microbiome-mediated colonization resistance is much more recent and has been greatly facilitated by next-generation nucleic acid sequencing platforms and metabolomic analyses. It is now increasingly appreciated that the microbiota and microbiome represent deep reservoirs for the discovery of novel potential therapeutics that can be developed to prevent and treat infections, including and perhaps especially those caused by the increasing number of highly antibiotic-resistant pathogens(38, 163, 240).

1.6 SUMMARY AND CHAPTER OUTLINE

Clostridium difficile is an important, yet incompletely understood human pathogen. Outcomes of infection are dependent on the host's immune function as well as the microbes populating the gut and individual strain type variability. The focus of this dissertation is to 1) determine the impact of *C. difficile*-targeting antibiotics on the risk of subsequent infections and 2) evaluate different *C. difficile* strains for variable virulence in the mouse model. In Chapter 2, we look at the impact of short-course antibiotic therapy on commensal microbiota populations, and how changes in these bacteria influence the risk of further infections with *C. difficile*, vancomycin-resistant *Enterococcus*, *E. coli*, and carbapenem-resistant *Klebsiella pneumonia*. In Chapter 3, we interrogate a small number of clinical *C. difficile* isolates for their differences in

tolerance to secondary bile acid, and demonstrate the relationship with this variable to disease burden in the mouse model. In Chapter 4, we expand the number of clinical isolates and use whole genome sequencing to identify specific gene groups that are associated with highly virulent strains, a methodology that has provided novel hypotheses for how *C. difficile* causes disease in the host.

Notes

Adapted from Microbiota-based therapies for *Clostridium difficile* and antibiotic-resistant enteric infections. Lewis BB and Pamer EG. Annu Rev Microbiol. 2017.

CHAPTER 2

LOSS OF MICROBIOTA-MEDIATED COLONIZATION RESISTANCE TO CLOSTRIDIUM DIFFICILE INFECTION IS GREATER FOLLOWING ORAL VANCOMYCIN AS COMPARED WITH METRONIDAZOLE *

2.1. INTRODUCTION

Clostridium difficile is an intestinal pathogen that causes a wide spectrum of disease in hospitalized patients, ranging from diarrhea to pseudomembranous colitis to potentially lethal toxic megacolon(14). The past fifteen years have seen a dramatic increase in the number of cases, with strains emerging that produce increased amounts of toxin and are resistant to fluoroquinolone antibiotics(95, 150). The Centers for Disease Control and Prevention classifies *C. difficile* as "Threat Level Urgent," a rating intended to call for "urgent and aggressive action" against this bacterium(38). Recent estimates by the CDC indicate that in the United States, *C. difficile* infections kill 14,000 individuals and lead to over $1 billion in medical costs each year. *C. difficile* infections are becoming more common outside hospitals, particularly among elderly individuals living in assisted care facilities but also in the general community(73, 166).

Antibiotic treatment is key to both the initiation and resolution of *C. difficile* infections (CDIs). One European study found that 92% of patients diagnosed with *C. difficile* reported antibiotic use during the preceding three months, with third

* B. Lewis, C. Buffie, R. Carter, I. Leiner, N. Touissant, L. Miller, A. Gobourne, L. Ling, and E. Pamer. J Infect Dis. 2015

generation cephalosporins and clindamycin correlating most strongly with the risk of subsequent CDI(17, 79, 199). Once CDIs have been diagnosed, standard protocols call for administration of more antibiotics: metronidazole for mild and moderate cases, and vancomycin for severe infection(167). Vancomycin yields a slightly higher cure rate in some patients, but concerns over cost and the emergence of vancomycin-resistant pathogens limit its use to patients with severe disease(65, 109, 253).

Growing concern over the rise in *C. difficile* infections has prompted some physicians to prescribe metronidazole prophylactically to high-risk patients(183). The Infectious Disease Society of America (IDSA) recommends prescribing vancomycin to patients with suspected severe or severe complicated *C. difficile* illness before infection has been confirmed; however, it is estimated that many clinicians start empiric therapy based on suspicion of mild or moderate disease, with questionable therapeutic benefit(186). These practices are concerning, as little is known about how metronidazole and vancomycin impact commensal bacteria and how they alter the host's susceptibility to other enteric pathogens commonly encountered in hospital settings(4). To address this problem, we treated mice with brief courses of metronidazole, vancomycin, or both in combination, and assessed the impact to native bacterial populations as well as colonization resistance to *C. difficile* spores. We then extended our investigation to other hospital-acquired infections, and asked if the same antibiotic regimens affected the microbiota's ability to suppress infections in mice challenged with vancomycin-resistant *Enterococcus*, Carbapenem-resistant *Klebsiella pneumoniae,* and *Escherichia coli*.

2.2. METHODS

2.2.1. Mouse husbandry. All experiments were performed with wild type female C57BL/6 mice, aged 6-8 weeks and purchased from Jackson Laboratories. The mice

were housed in the specific pathogen free facility at Memorial Sloan Kettering's Animal Resource Center, fed irradiated feed and provided with acidified water. BBL, CGB, and RC performed all mouse experiments and changed cages at least once per week. The experiments were performed in compliance with Memorial Sloan Kettering institutional guidelines and were approved by MSKCC's Institutional Animal Care and Use Committee (IACUC).

2.2.2. *C. difficile* susceptibility time course experiments. *C. difficile* susceptibility was assessed as described in Buffie et al. (29), with different antibiotic treatments: we used metronidazole (1.0 $g \cdot L^{-1}$, Sigma-Aldrich), vancomycin (1.0 $g \cdot L^{-1}$, NOVAPLUS), or 1.0 $g \cdot L^{-1}$ of both antibiotics. Mice were housed in groups of 5 during antibiotic treatment. At day 1, 3, 7-8, 14-15, and 21-22 after cessation of antibiotics, one mouse was removed from each group cage, transferred to an individual cage, and inoculated with 1000 spores of *C. difficile* strain VPI 10463 (ATCC 43255). Each experiment tested three groups of mice per antibiotic treatment, and experiments were repeated twice, with $n = 9$ for each time point per treatment group tested.

In one group of experiments, mice were administered antibiotics (metronidazole or metronidazole in combination with vancomycin) by oral gavage instead of the drinking water. 3.5mg of antibiotics was dissolved in 200µl of water and administered every day for three days. Twenty-four hours after the final dose corresponded to day 1 of the previous experiments, and mice were then challenged with *C. difficile* spores as described.

2.2.3. Quantitative *C. difficile* culture. *C. difficile* burden in mouse ceca twenty-four hours after infection was assessed as previously described(30).

2.2.4. Sample collection, DNA extraction, and quantification of 16S copy number density. Intestinal content samples were collected from mice on day -3, 1, 3, 7-8, 14-15, 21-22 (feces), and twenty-four hours after infection on day 2, 4, 8-9, 15-16, and 22-23 (colon). The samples were immediately flash frozen and DNA was extracted as described(224). Briefly, the frozen samples (approximately 100 mg) were suspended in 500 µL extraction buffer (200 mM Tris, pH 8.0/200 mM NaCl/20 M EDTA), 200 µL 20% SDS, 500 µL phenol:chloroform:isoamyl alcohol (24:24:1), and 500 µL 0.1-mm-diameter zirconia/silica beads (BioSpec Products). Bacterial cells were lysed with bead beating (BioSpec Products) for two minutes, and DNA was isolated with two rounds of phenol:chloroform:isoamyl alcohol extraction. Following extraction, the DNA was precipitated in ethanol and resuspended in 200 µL TE buffer with 100 µg/mL RNAse, and further purified with QIAmp mini spin columns (Qiagen).
The DNA extracted from fecal samples was then subjected to rtPCR of 16S RNA gene sequences. We used the broad range bacterial 16S primers 517F (5'-GCCAGCAGCCGCGCTAA -3') and 798R (5'- AGGGTATCTAATCCT -3') at 0.2 mM concentrations with the DyNAmo SYBR green qPCR kit (Finnzymes). Sample amplification was compared to standard curves to quantify 16S copy number. Cycling conditions were as follows: 95˚C for 15 minutes, then 40 cycles of 94˚C for 15 seconds, 52˚C for 30 seconds, and 72˚C for 30 seconds. The program finished with 95˚C for 15 minutes, 60˚C for 1 minute, and 95˚C for 15 minutes.

2.2.5. 16S ribosomal RNA gene amplification, multiparallel sequencing, and sequence analysis. Amplicons of the V4-V5 region 16S rRNA region were amplified and sequenced with the Illumina MiSeq platform as described previously(29). Sequences were analyzed using the mothur pipeline, version 1.33.3(189), as described previously(29). OTUs were classified with a modified Greengenes reference

database(53). OTU-based microbial diversity was estimated by calculating the inverse Simpson index and Bray Curtis dissimilarity index. Phylogenetic trees were inferred by using Clearcut(197) on the alignment created by mothur, and then unweighted UniFrac(143) was run on the resulting tree, followed by a principal coordinate analysis of the distance matrix.

2.2.6. VRE, KPC, and *E. coli* susceptibility time course experiments. Mice were treated with metronidazole (1.0 $g \cdot L^{-1}$), vancomycin (1.0 $g \cdot L^{-1}$), or no antibiotic for three days, then switched to untreated water. At day 1, 7, or 14 after stopping antibiotic treatment, mice were orally gavaged 50,000 colony forming units of vancomycin-resistant *Enterococcus faecium* (ATCC 700221), Carbapenem-resistant *Klebsiella pneumoniae* (KPC), or *E. coli* (KPC and *E. coli* were both isolated from blood cultures collected from patients at Memorial Sloan Kettering Cancer Center). All mice were singly housed after infection with the indicated pathogen. Twenty-four hours after infection, fecal samples were collected from the mice and the corresponding pathogen burden was enumerated by plating serial dilutions on selective agar plates (vancomycin-resistant *Enterococcus:* EnterococcoseI agar plates [Difco] with vancomycin [8µg/mL]; Carbapenem-resistant *Klebsiella pneumoniae:* Luria Broth with agar [Difco], carbenacillin [100µg/mL, LabScientific] and neomycin [50µg /mL, Sigma-Aldrich]; *E. coli*: Luria Broth with agar and neomycin [50µg /mL]).

2.2.7. Statistical analysis. Statistical analysis was conducted using the R (v. 3.1.1) and GraphPad Prism (v. 6.0c) software packages. Analysis of the number of 16S rRNA copies, inverse Simpson indices, and Bray-Curtis dissimilarity was performed using 2-way ANOVA with Bonferroni correction and Prism software. Analysis of

differential VRE, KPC, or *E. coli* burden was performed using a Kruskal-Wallis test with Dunn's correction. *P* values less than 0.05 were considered to be significant. For the principal coordinate analysis, the analysis of molecular variance (AMOVA) method(7) was used to compare samples that supported *C. difficile* growth (susceptible samples) to samples in which no *C. difficile* was detected (resistant samples).

2.3. RESULTS

2.3.1. Differential impact of metronidazole and vancomycin treatment on *C. difficile* susceptibility.

In the absence of pre-treatment with antibiotics, administration of *C. difficile* spores does not lead to infection or colonization of conventionally housed wild-type C57BL/6 mice(30, 42). To determine the impact of metronidazole and oral vancomycin on susceptibility to *C. difficile* infection, we treated mice with metronidazole, vancomycin, or metronidazole plus vancomycin in their drinking water for three days (1.0 $g \cdot L^{-1}$ for each). Each treatment protocol was tested in three replicate cages. After three days, antibiotic-containing water was replaced with untreated water for the remainder of the experiment (**Figure 2.1A**). At days 1, 3, 7-8, 14-15, and 21-22 after stopping antibiotic treatment, one mouse was removed from each cage at random and challenged with 1000 spores of *C. difficile* strain VPI 10463 by oral gavage. Each infected mouse was housed individually to prevent cross-contamination. Twenty-four hours after infection, the mice were euthanized and cecal and colonic contents were collected for assessment of *C. difficile* burden and sequencing, respectively. Each experiment tested three mice per antibiotic treatment and was conducted three separate times (*n=9* mice for each treatment and time point).

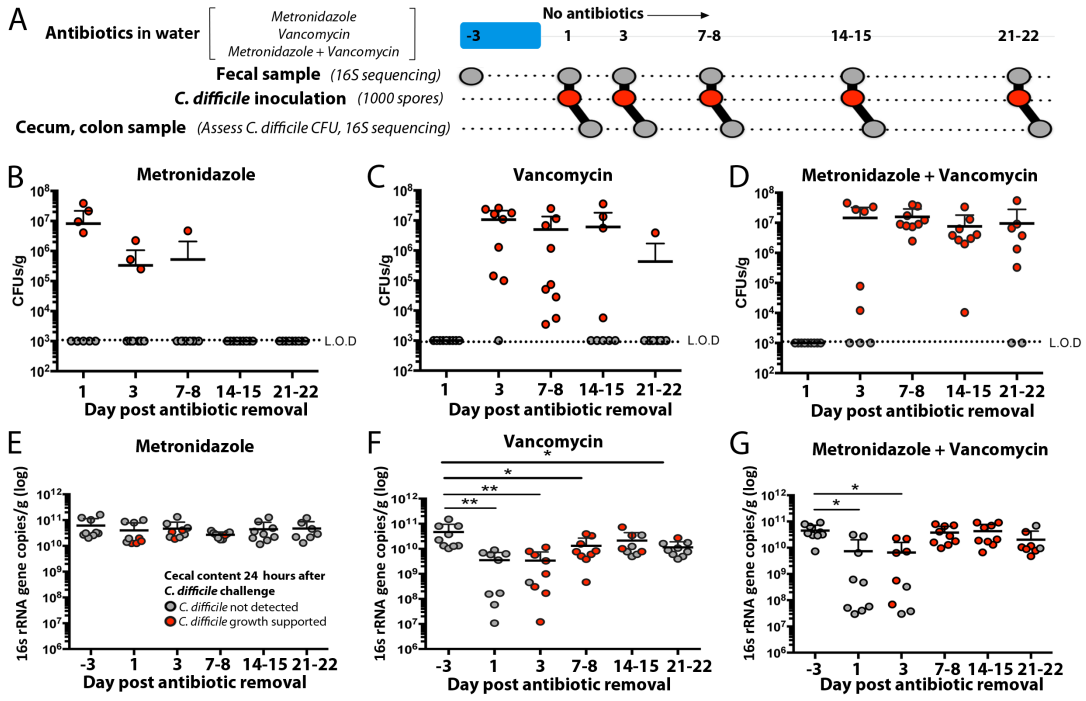


Figure 2.1. Exposure to metronidazole, vancomycin, or both increases susceptibility to *C. difficile* infection. (**A**) Summary of experimental protocol. (**B-D**) *C. difficile* burden in the ceca of mice twenty-four hours after infection. (**E-G**) Intestinal bacterial density of animals before and after antibiotic exposure, **P*<0.05, ***P*<0.01. Center values (mean), error bars (s.d.). *n* = 9 mice for all time points except metronidazole + vancomycin treated mice on day 21-22, where *n* = 8.

Of the mice treated with metronidazole alone, approximately half (55%) had no detectable *C. difficile* in their ceca when challenged 1 day following antibiotic removal (**Figure 2.1B**). When mice were challenged 3 and 7-8 days after stopping metronidazole treatment, the number of mice with undetectable *C. difficile* levels increased to 67% and 89% of mice, respectively. All mice had recovered colonization resistance two weeks after stopping metronidazole treatment.

In contrast, none of the mice treated with vancomycin had detectable *C. difficile* in their ceca one day after stopping treatment (**Figure 2.1C**). Vancomycin has been shown to persist in the lower intestine of mice and humans days after the last

dose has stopped, so it is likely that residual vancomycin killed germinating spores at this early time point(4). Indeed, by days 3 and 7-8, almost all mice become heavily colonized (89% of mice on day 3, 100% of mice on days 7-8). By the last time point studied, only one mouse had detectable *C. difficile* CFU in its cecum. The disruption in colonization resistance was more pronounced in mice treated with both metronidazole and vancomycin, 78% of which had ceca that supported *C. difficile* growth three weeks after stopping antibiotic treatment (**Figure 2.1D**).

To investigate the causes of the loss of colonization resistance to *C. difficile* infection, we first assessed changes in total bacterial density by quantitative RT-PCR (qPCR) targeted to bacterial 16S rRNA-encoding genes. We found that the density in metronidazole-treated mice remained relatively stable throughout the course of the experiment (**Figure 2.1E**), in contrast to the vancomycin-treated mice (**Figure 2.1F**) and metronidazole plus vancomycin-treated mice (**Figure 2.1G**). Bacterial density approached baseline levels by day 14-15 of the experiment. However, as many of the mice had not re-established colonization resistance to *C. difficile* at this time, the change in bacterial density did not fully explain changes in *C. difficile* colonization resistance (**Figure 2.1B-D**).

2.3.2. Prolonged impact of metronidazole and vancomycin treatment on intestinal bacterial populations.

We next expanded our investigation beyond density measures to examine changes in bacterial composition over time. DNA extracted from fecal samples was sequenced on the Illumina MiSeq platform and reads were analyzed using an in-house pipeline mainly derived from the mothur software(189). We found that before antibiotic treatment was initiated, fecal samples were composed of a rich community of bacterial OTUs spanning multiple taxa, including the Bacteroidales class S24.7 as

well as many members of the Lactobacillus and Clostridium genera, among others (**Figure 2.2A-C**, day -3 time point).

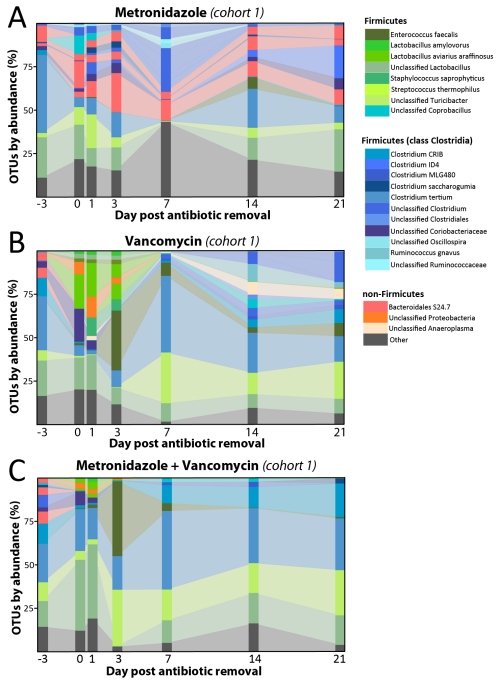


Figure 2.2. Exposure to metronidazole, vancomycin, or both disrupts commensal bacterial species found in the lower intestine. Fecal samples were collected from mice before antibiotic treatment (day -3) and at the indicated times following cessation of antibiotics. Samples were assessed for bacterial operational taxonomic units (OTUs) as described in the Methods section. Each stacked bar represents the mean microbiota composition of three independently-housed mice.

Animals treated with metronidazole alone experienced relatively transient disruptions in their fecal microbial communities, and returned to a state similar to pre-treatment composition by 1-2 weeks after stopping treatment (**Figure 2.2A**). In contrast, animals treated with vancomycin or metronidazole plus vancomycin experienced a profound shift in their microbiota composition, with taxa such as Bacteroidales S24-7 falling permanently below the limit of detection (**Figure 2.2B-C**). In parallel, bacterial OTUs present at low or undetectable levels before antibiotics

expanded greatly, including members of the genus *Enterococcus*, unclassified Proteobacteria, and novel members of Lactobacillus and Clostridium.

Repeating these experiments with new cohorts of mice revealed that the expanding bacterial populations were highly dependent on the initial commensal bacteria present in the mice, and that different mouse cohorts (all wild-type C57BL/6 mice obtained from the Jackson Labs) were colonized with different communities (**Supplementary Figure 2.1**). In three separate experiments performed with mice obtained at least one month apart, vancomycin treatment led to the expansion of primarily *Lactobacillus aviarius* and *Enterococcus faecalis* (**Figure 2.2B**, cohort 1), *Klebsiella oxytoca* (**Figure 2.3C**, cohort 3), or *Klebsiella oxytoca* and *Akkermansia muciniphilia* (**Figure 2.3D**, cohort 4).

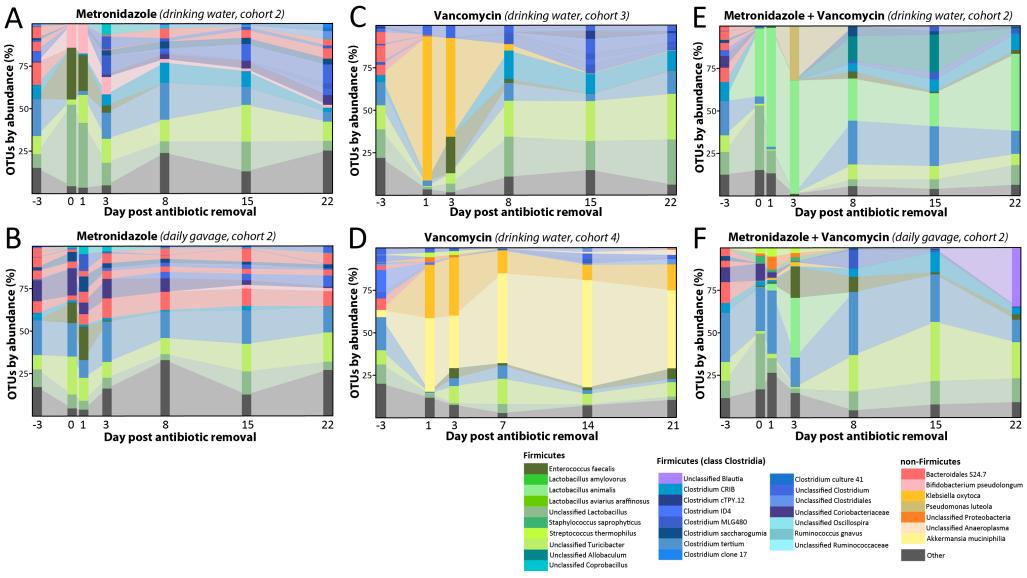
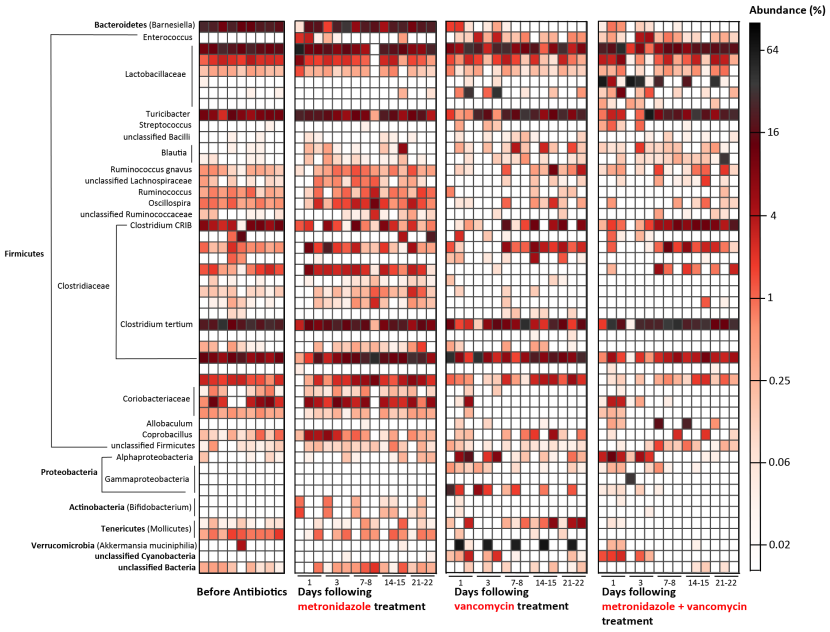


Figure 2.3. Impact of starting microbiota and route of antibiotic administration on resulting changes in bacterial composition. The experiment represented by Figure 2 (mouse cohort #1) was repeated with different cohorts of wild-type C57BL/6 Jackson mice. Mice from cohort 2 were subdivided into two additional treatment groups, with one group receiving antibiotics dissolved in water (**A**, **E**), and another group receiving antibiotics by daily oral gavage (**B**, **F**). Each stacked bar represents an average of three mice per time point per treatment group (exception: panel **F** at time point 22 days, where $n = 2$ mice).

Many patients report that metronidazole has an unpleasant metallic taste, which could impact how much treated water the mice drink during the three days of antibiotic administration. We therefore gavaged mice every day for three days with metronidazole or metronidazole plus vancomycin (using doses that corresponded to the measured daily intake of water in untreated mice) and then challenged them with *C. difficile* spores as described previously. While the mice administered antibiotics via oral gavage appeared to preserve slightly more diversity as measured by the inverse Simpson and Bray-Curtis indices, this trend was not statistically significant (**Figure 2.3B**, **F**, and **Supplementary Figure 2.2**). These results indicate that the timing of antibiotic administration has less of an impact on bacterial shifts than the initial microbial populations present in the mice before treatment begins. A summary of the effects of antibiotic treatments on microbial composition and diversity is shown in **Figure 2.4 and Supplementary Figure 2.3**.

Figure 2.4. Summary of effects of metronidazole and vancomycin on fecal microbial populations. Sequences from fecal samples were binned into operational taxonomic units and abundance of the fifty most highly represented OTUs was plotted based on percentage of total sequences. Each horizontal bar represents one OTU. Each vertical bar represents the mean abundance of individual OTUs within one cohort of mice at the specified time point and treatment group (*n=3* for each vertical bar).



2.3.3. Correlation of antibiotic treatment-mediated changes in commensal bacterial populations with *C. difficile* colonization resistance.

We next asked if the observed shifts in commensal microbial populations following antibiotic treatment corresponded with susceptibility to *C. difficile* spore challenge. Twenty-four hours after infection, colonic content was collected from mice, sequenced, and analyzed as described previously. Mice treated with metronidazole alone were found to maintain a relatively stable microbiota, which corresponded with the rapid recovery of resistance to *C. difficile* (**Figure 2.5A**). In contrast, the colons of vancomycin- and metronidazole plus vancomycin-treated mice were found to contain severely disrupted microbiota, which correlated with delays in returning to a colonization-resistant state (**Figure 2.5B** and **2.5C**, respectively). Examination of microbial communities with principal coordinate analysis demonstrated that, in the case of vancomycin (**Figure 2.5E**) and metronidazole plus vancomycin treatment (**Figure 2.5F**), colon samples from mice that were unable to suppress *C. difficile* growth (susceptible mice) clustered separately from the samples in which *C. difficile* growth was undetected twenty-four hours after spore challenge (resistant mice) (analysis of molecular variance F-statistic = 2.38, $p<0.001$ in vancomycin-treated mice; in metronidazole plus vancomycin-treated mice, F-statistic = 3.11, $p<0.001$). These data demonstrate that not only do *C. difficile*-targeting antibiotics significantly alter intestinal microbial communities, but that these disrupted communities are then more likely to support *C. difficile* expansion.

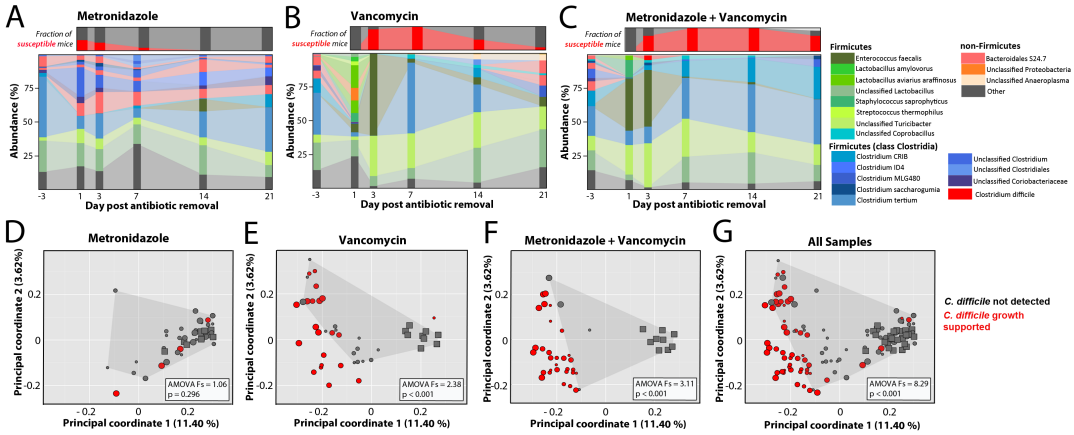


Figure 2.5. Antibiotic-induced disruptions of microbial communities contribute to *C. difficile* susceptibility. Colon samples were collected from mice twenty-four hours after *C. difficile* infection and assessed for abundance of individual bacterial operational taxonomic units (**A-C**, large panels). Each stacked bar represents the mean microbiota composition of three independently-housed mice from cohort 1. Small panels in **A-C** represent the fraction of mice found susceptible to *C. difficile* twenty-four hours after infection in all cohorts (red bar), $n = 9$ mice per time point. (**D-G**) Principal coordinate analysis of colon samples from all cohorts twenty-four hours after infection. Squares represent pre-antibiotic samples and circles represent post-antibiotic treatment samples. The size of circles represents the time point of each post-treatment sample, with large circles representing earliest time points. Analysis of molecular variance F statistic used to compare samples in which *C. difficile* was not detected (grey points bounded by shaded region) to samples that supported *C. difficile* growth (red points).

2.3.4. Impact of metronidazole and vancomycin treatment on susceptibility to infection with other nosocomial pathogens.

We next asked if the observed shifts in microbial composition following metronidazole or vancomycin treatment also contribute to heightened risk of colonization with other nosocomial infections. Of growing concern in many hospitals are vancomycin-resistant *Enterococcus* (VRE) species, carbapenem-resistant *Klebsiella pneumoniae* (KPC), and *Escherichia coli* infections(38). We therefore treated a cohort of wild-type C57BL/6 Jackson mice with metronidazole or

vancomycin for three days followed by challenge with approximately 50,000 colony-forming units of VRE, KPC, or *E. coli* at 1, 7, or 14 days following antibiotic cessation (**Figure 2.6**). In all cases we saw a similar pattern of intestinal colonization: First, the untreated cohort was colonized at very low densities (or not at all) throughout the time course. Second, the metronidazole cohort displayed moderate bacterial burden at early time points, then recovered colonization resistance resembling the control mice. Finally, the vancomycin cohort remained highly susceptible to colonization throughout the duration of the experiment. Only in the VRE-infected group did the vancomycin-treated mice display a trend towards recovery of colonization resistance two weeks after antibiotic removal (**Figure 2.6A**).

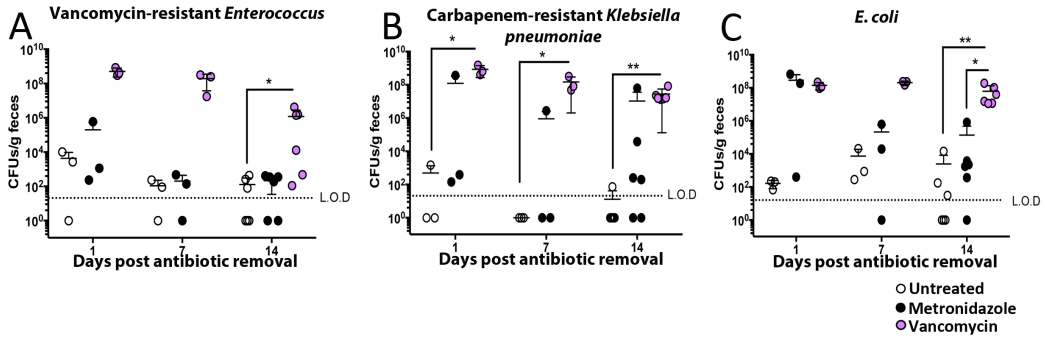


Figure 2.6. Exposure to metronidazole and vancomycin impacts colonization ability of vancomycin-resistant *Enterococcus*, Carbapenem-resistant *Klebsiella pneumoniae*, and *E. coli*. Mice were treated with indicated antibiotics for three days and then allowed to recover. After stopping antibiotics, mice were challenged with approximately 50,000 colony-forming units of vancomycin-resistant *Enterococcus* (**A**), Carbapenem-resistant *Klebsiella pneumoniae* (**B**), or *E. coli* (**C**). Fecal pellets were collected from mice 24 hours after infection and assessed for the corresponding pathogen burden. **P*<0.05, ***P*<0.01. Center values (mean), error bars (s.d.) $n = 3$ (day 1, day 7) or $n = 6$ (day 14).

2.4. DISCUSSION

The high incidence of *C. difficile* infection in select, highly vulnerable patient populations has led to the consideration of using metronidazole or vancomycin prophylactically(160, 186). Although not recommended by the Infectious Disease Society of America, clinicians caring for highly immunocompromised patients undergoing cancer treatment or organ or stem cell transplantation are increasingly administering these antibiotics to prevent *C. difficile* infections. Both metronidazole and vancomycin are known to effectively treat active infections, but their impact on commensal bacterial populations has not been extensively studied. We found that metronidazole treatment disrupts the microbiota initially, but the effect is transient and mice are able to regain colonization resistance to *C. difficile* relatively quickly. In contrast, vancomycin treatment causes severe shifts in commensal microbial species that correspond with prolonged susceptibility to *C. difficile*, an effect that is magnified when vancomycin is administered with metronidazole concurrently. The continued disruption of commensal bacterial species can help explain high relapse rate associated with *C. difficile* disease, which most commonly occur within a few weeks of successful treatment(9).

In addition to recurrent *C. difficile* episodes, a disordered commensal microbiota also leaves patients susceptible to other nosocomial infections. Of chief concern in hospitals are infections with vancomycin-resistance *Enterococcus* species, Carbapenem-resistant Enterobacteriaceae like *Klebsiella pneumoniae*, and *E. coli.* These pathogens are found with increased prevalence in hospital settings and many have acquired resistance to remaining available antibiotics(38). We found that in addition to its effect of prolonged susceptibility to *C. difficile* infection, brief treatment with vancomycin opens a niche in the intestinal environment that allows heavy

colonization with VRE, KPC, and *E. coli*. Metronidazole treatment, however, has a more transient effect. When prescribing metronidazole or vancomycin for active *C. difficile* infection (or as empiric therapy for suspected cases), clinicians must take into account the collateral damage to protective bacterial species and take steps to prevent patient exposure to nosocomial pathogens long after treatment has been completed. As an alternative, Fidaxomicin is a recently FDA-approved antibiotic for the treatment of *C. difficile* infections that, in contrast to vancomycin and metronidazole, has been demonstrated to have a narrower spectrum of activity against obligate anaerobes of the colon(71, 142). Given this specificity, it is likely that Fidaxomicin administration would have less of an impact on colonization resistance, but this remains an area that requires further study.

More broadly speaking, our experiments reveal an important challenge regarding any investigation of commensal bacterial populations. Concordant with previous studies(223), we discovered that wild-type mice purchased from the same vendor harbored different microbes depending on the date and barrier facility from which they were obtained (**Supplementary Figure 2.1**). The differences in starting commensal species were magnified after conditioning antibiotic treatments (**Figure 2.3C-D**). This finding highlights the need to actively monitor the microbiota in laboratory animals even before starting experimental treatments.

While vancomycin treatment is known to improve *C. difficile* symptoms, studies in mice demonstrate that it also increases recurrence rates when compared to metronidazole treatment(235). Our experiments were specifically designed to study the impact of these two agents on the normal microbiota and their relative ability to destroy colonization resistance. Our findings demonstrate that oral vancomycin and, to a lesser extent metronidazole, leave the host far more vulnerable to infection with *C. difficile* but also to additional antibiotic-resistant bacterial species. Another

antibiotic occasionally used to treat *C. difficile* infections (tigecycline) has been shown to disrupt colonization resistance in a similar manner(16). In clinical circumstances where the risk of exposure to and acquisition of *C. difficile* spores is high, the short-term potential benefits of antibiotic prophylaxis with either metronidazole or vancomycin needs to be weighed against the increased long-term risk for infection with *C. difficile*, VRE, Carbapenem-resistant Enterobacteriaceae, and *E. coli*.

CHAPTER 3

BILE ACID SENSITIVITY AND IN VIVO VIRULENCE OF CLINICAL CLOSTRIDIUM DIFFICILE ISOLATES *

3.1. INTRODUCTION

Clostridium difficile is an anaerobic, spore-forming bacterium that causes an increasing number of antibiotic-associated intestinal infections in the hospital and community setting(95, 150, 180). Exposure to *C. difficile* spores leads to a range of outcomes, from asymptomatic carriage to pseudomembranous colitis with life-threatening toxic megacolon(14). Individuals most at risk for severe infection are those over 65 years of age with recent exposure to oral antibiotics and weakened immune systems(73, 166). In addition, virulent *C. difficile* strains exhibit significant genetic variability, and new strains are continuing to be discovered(138, 173, 208). *C. difficile* toxin production(86, 141), sporulation(151, 230), flagella structure(210), and germination efficiency(154)(34) have all been proposed to contribute to virulence, yet the bacterial phenotypes that influence disease outcome in the host remain incompletely defined. However, it is known that bile salts play an important role in the germination and growth of *C. difficile* in the mammalian host(96, 203–205, 237, 242).

Bile acids are amphipathic molecules produced by the liver and released by the gallbladder in response to food entering the intestine. They serve a critical role in the absorption of dietary fats and are increasingly appreciated as modulators of microbial

* B. Lewis, R. Carter, and E. Pamer. Anaerobe 2016.

populations in the gut. Humans synthesize their own primary bile acids(179, 185), which can then be dehydroxylated by a subset of commensal bacteria to form secondary bile acids (primarily deoxycholic acid and lithocholic acid). The relative concentrations of bile acids are especially important in *C. difficile* infections. The primary bile acid taurocholic acid induces germination of metabolically latent spores(203, 243), while secondary bile acids inhibit the growth of vegetative, toxin-producing cells(29, 77, 203, 216). Only a select group of bacteria produce the enzymes necessary to dehydroxylate primary bile acids, and they are sensitive to killing by many commonly prescribed antibiotics(179, 215). Recent work in our lab and others has shown that antibiotic treatment in mice shifts the bile acid pool towards a predominance of primary bile acids, and that the restoration of secondary bile acids to pre-antibiotic levels is associated with resistance to *C. difficile* infections(29, 118, 216). However, this work has been conducted with a limited number of *C. difficile* strains. It remains to be determined if clinically relevant isolates exhibit heterogeneity in response to secondary bile acids, and if that heterogeneity associates with observed differences in virulence with a murine model of infection.

3.2. RESULTS AND METHODS

To address this question, we tested fecal samples obtained from patients undergoing allogeneic hematopoietic stem cell transplantation for the presence of *C. difficile* organisms by a PCR-based assay for *C. difficile* toxin B. While some patients had been diagnosed with *C. difficile*, other patients had not been diagnosed with *C. difficile* and thus were asymptomatic carriers(116). We isolated individual *C. difficile* colonies from the infected stool and typed them using multi-locus sequence typing (MLST)(91). Of the 21 isolates collected, 16 were members of clade 1 (including

sequence types 2,6,12,42,46,58,107), two were members of clade 2 (sequence type 1, also classified as ribotype 027), and 1 each was a member of clade 4 and 5 (STs 39 and 11, respectively)(91, 105). We prepared purified spore suspensions for each clinical isolate according to published protocols(202).

For the *in vivo* experiments, wild-type C57BL/6 mice (Jackson labs) were rendered susceptible to *C. difficile* by replacing their drinking water with antibiotic-containing water for three days. We used a cocktail of three antibiotics: metronidazole, ($0.25gL^{-1}$, Sigma-Aldrich), neomycin ($0.25\ g{\cdot}L^{-1}$, Sigma-Aldrich), and vancomycin ($0.25gL^{-1}$, NOVA-PLUS). Two days after stopping antibiotic water, mice were additionally treated with 200 μg clindamycin (Sigma-Aldrich) via intraperitoneal injection. We inoculated mice 24 hours later with 100 spores of individual clinical isolates via oral gavage, infecting five mice with each isolate. After infection, mice were monitored daily for indicators of morbidity and given a disease score(2). Additionally, fecal pellets were collected 1, 2, 7, and 14 days after infection and quantified for *C. difficile* burden(30).

We found that infection with *C. difficile* clinical isolates caused a range of disease severity in mice (**Figure 3.1A-B**). The most virulent strains were members of clade 2 (MLST1/ribotype 027) and clade 5 (MLST11, also classified as ribotype 078) (**Figure 3.1A**). These strains approached and/or surpassed the disease score of our reference strain, *VPI10463*, a notoriously virulent strain(151, 217). Members of clade 4 (1 isolate) and clade 1 (16 isolates) had heterogeneous disease scores but consistently produced lower morbidity than *VPI10463* (**Figure 3.1A** and **B**, respectively). The differences in observed morbidity could not be attributed to differences in colonization ability of the clinical isolates, as fecal samples from all infected mice harbored in excess of 10^6 colony forming units per gram two days after

infection (**Figure 3.1C**), and mice remained heavily infected throughout the two-week experiment (data not shown).

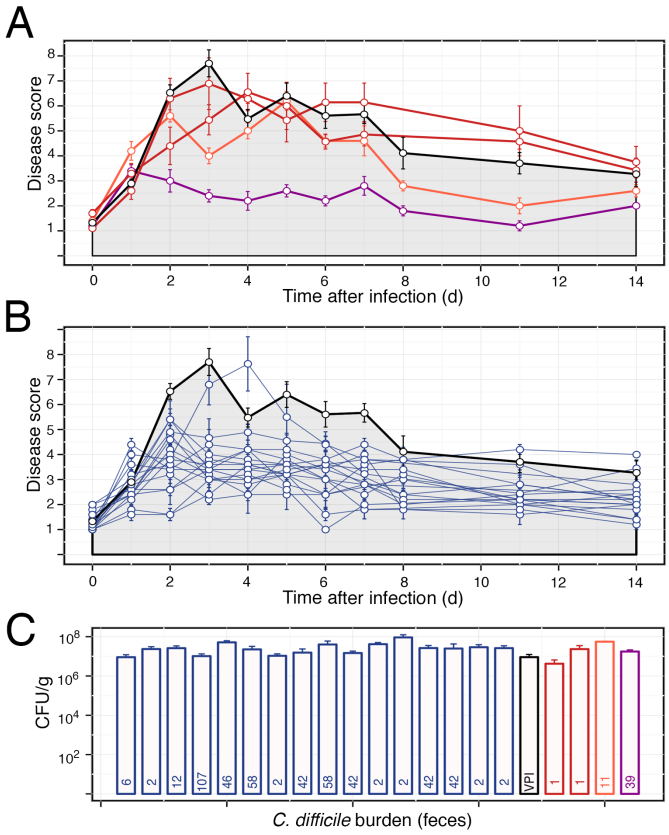
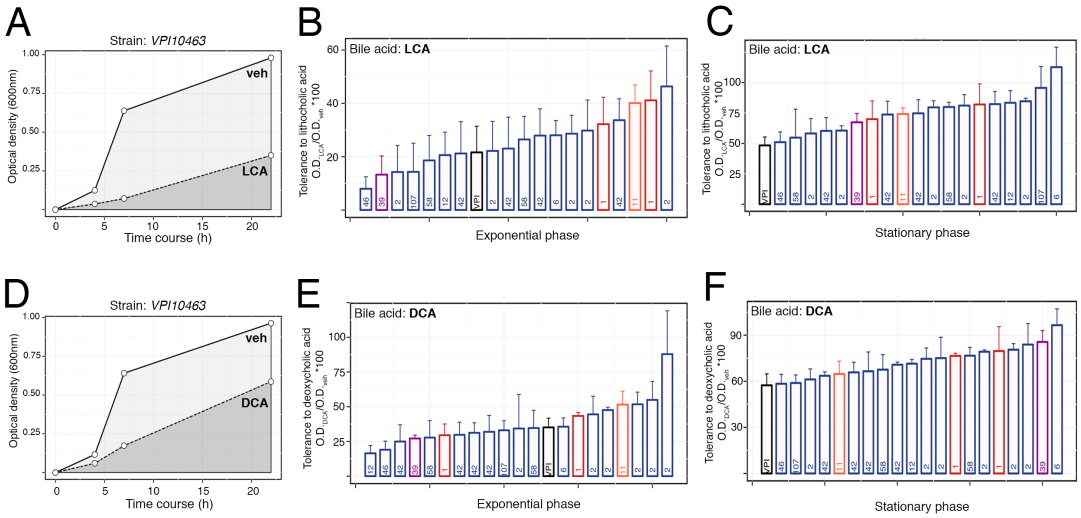


Figure 3.1. *In vivo* disease scores and bacterial burden of *C. difficile* clinical isolates. (**A**) Disease scores for wild type C57BL/6 mice infected with *C. difficile* isolates identified as MLST1/Clade 2 (red, two isolates), MLST39/Clade 4 (purple), and MLST11/Clade 5 (orange) as compared with the reference strain, *VPI10463* (black). (**B**) Disease scores for wild type mice infected with *C. difficile* isolates identified as members of Clade 1 (blue, various MLST types) as compared with *VPI10463* (black). (**C**). *C. difficile* burden as measured from mouse fecal content two days after infection. Numbers at the base of the bars indicate MLST classification. *n* = 5 mice for each clinical isolate tested, data shown are mean +/- SEM. d = day; CFU/g = colony forming units per gram of feces.

We next investigated the impact of secondary bile acids on the growth kinetics of the isolates in an *in vitro* setting. Anaerobic culturing at 37˚C was performed and growth was monitored by measurement of optical density of each clinical isolate over a 22-hour period. Each isolate was grown under three treatment conditions: (1) vehicle (brain-heart infusion media supplemented with yeast extract and cysteine), (2) vehicle with 0.01% lithocholic acid, and (3) vehicle with 0.01% deoxycholic acid (BHI and yeast extract from BD Biosciences, others from Sigma-Aldrich). We repeated each experiment twice, for a total of three trials.

Figure 3.2 reports the growth curves of the laboratory reference strain *VPI10463* in response to treatment with lithocholic acid (LCA, **Figure 3.2A**) and deoxycholic acid (DCA, **Figure 3.2D**). In addition, each of the examined clinical isolates was inhibited by LCA and DCA, although to varying degrees (see **Figure 3.2B** and **2E** for growth comparison during the exponential phase, and **Figure 3.2C** and **2F** for growth comparison after cultures reached the stationary phase). In some cases, variation in bile acid tolerance was seen even in strains classified as the same MLST group (see **Figure 3.2B**, MLST 2's, and **Figure 3.2E**, MLST 1's as examples).

Figure 3.2. *In vitro* growth suppression of *C. difficile* clinical isolates by the secondary bile acids lithocholic acid and deoxycholic acid. (**A,D**) Growth kinetics of *C. difficile* strain *VPI10463* in the absence (solid line, bounded by light gray region) or presence (dotted line, bounded by dark gray region) of lithocholic acid (**A**) and deoxycholic acid (**D**), as measured in the change in optical density. (**B-C**) Tolerance of each *C. difficile* clinical isolate to administration of lithocholic acid and (**E-F**) deoxycholic acid at indicated time points. Numbers at the base of the bars indicate MLST classification. All experiments were conducted three times, data shown are mean +/- SEM.



Because each of the clinical isolates was unique in terms of secondary bile acid tolerance and murine *in vivo* disease score, we next explored the relationship between these two variables (**Figure 3.3**). We found that at two days after infection, the clinical isolates rated most virulent exhibited a trend towards increased lithocholic acid resistance ($p = 0.108$, **Figure 3.3A**, orange points). This trend achieved statistical significance with the clinical isolates that caused morbidity beyond the window of acute infection. *C. difficile* isolates with the highest disease score one and two weeks after infection were also the isolates most tolerant to LCA exposure *in vitro* ($p = 0.017$ at week 1 (blue points), $p = 0.037$ at week 2 (gray points)). In contrast, we did not find a significant correlation between host disease scores and tolerance to deoxycholic acid (**Figure 3.3B**).

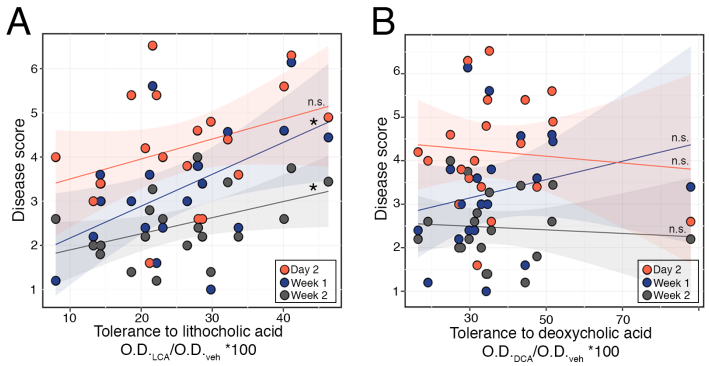


Figure 3.3. Comparison of secondary bile acid tolerance and *in vivo* disease score of clinical *C. difficile* isolates. Tolerance to (**A**) lithocholic acid or (**B**) deoxycholic acid during exponential growth was compared to the mean *in vivo* disease scores recorded two days (orange), one week (blue), and two weeks (gray) after infection. We compared bile acid tolerance to disease score at each time point by calculating the linear regression (solid lines, with 95% confidence interval in the corresponding shaded region), *p<0.05. ns = not significant. O.D. = optical density (600 nm).

3.3. DISCUSSION

Antibiotics of various classes disrupt the normal balance of bile acids in the mouse intestine, decreasing the relative concentrations of secondary bile acids as they increase susceptibility to *C. difficile* infections(29, 84, 216). Administration of a cocktail of bacteria that contained a species capable of converting primary bile acids to their secondary counterparts (*Clostridium scindens*) is sufficient to increase resistance to *C. difficile* challenge. The link between antibiotic administration, secondary bile acid depletion, and increased susceptibility to *C. difficile* infections has been well documented in the literature(29, 118, 216, 244); however, the impact of strain type on bile acid tolerance and *in vivo* virulence has remained largely unexplored.

Our results demonstrate that for a small cohort of *C. difficile* clinical isolates, *in vitro* tolerance to lithocholic acid administration is associated with higher disease scores in the mouse model. The association is stronger in later stages of the infection (one and two weeks after spore administration), during which time the microbiota is recovering from antibiotic treatment and secondary bile acids are returning to their pre-treatment levels(216, 244). In contrast, no association was found for *in vivo* disease scores and deoxycholic acid tolerance. Little is known about the response of *Clostridial* species to bile acid stress; studies with other gram positive organisms suggest strain variation in bile acid sensitivity is common(41, 156). Further investigation into the mechanism of LCA and DCA tolerance may help explain the observations presented in this study.

Growth in the presence of toxic metabolites is just one way an individual *C. difficile* strain can cause disease in the host; other contributing factors include toxin production, germination efficiency, sporulation efficiency, and flagellar structure(34, 86, 141, 151, 154, 210, 230). Future studies will test the hypothesis that bile acid tolerance acts in combination with other virulence mechanisms. For example, a

relative increase in lithocholic acid tolerance may extend a given *C. difficile* strain's ability to produce toxin and allow it to cause persistent disease in the host. Our study is limited both by the small sample size of clinical isolates used and by their restricted phylogenetic diversity (most are members of clade 1). Additional relationships between secondary bile acid tolerance and *in vivo* virulence could be elucidated with a larger, more diverse cohort of clinical isolates.

CHAPTER 4

PATHOGENICITY LOCUS, CORE GENOME AND ACCESSORY GENE CONTRIBUTIONS TO CLOSTRIDIUM DIFFICILE VIRULENCE *

4.1. INTRODUCTION

Clostridium difficile is an opportunistic, Gram-positive pathogen and the etiologic agent of most cases of healthcare associated intestinal infections(134). *C. difficile* exploits intestinal dysbiosis to induce profuse inflammatory diarrhea and colitis, which, in extreme cases, can be lethal(38). Although *C. difficile* has been recognized as an important pathogen since the 1970s(83), the past fifteen years have seen a rise in the number and severity of infections(95). This is partially attributed to the emergence of a strain referred to as multilocus sequence type 1 (MLST 1) and ribotype 027 (R027)(150). Today, *C. difficile* is recognized as an urgent threat to the healthcare system, with an estimated half million infections per year in the US and at least one billion dollars in excess medical costs(38, 134).

C. difficile produces two potent cytotoxins, toxin A and toxin B, which disrupt the actin cytoskeleton of intestinal epithelial cells(85). The toxins damage epithelial barrier integrity and lead to inflammatory cell influx and translocation of commensal bacteria. However, not all patients with dysbiosis develop colitis upon exposure to *C. difficile* spores, with some patients becoming asymptomatically colonized or

* B. Lewis, R.Carter, L. Ling, I. Leiner, Y. Taur, M. Kamboj, E. Dubberke, J. Xavier, and E. Pamer. mBio. 2017 (submitted)

experiencing only self-limited episodes of diarrhea. For example, one study in a long-term care facility found that 51% of residents were colonized with *C. difficile* despite a lack of apparent clinical illness(180). A more recent prospective study of patients receiving bone marrow transplants reported an asymptomatic carriage rate of 27%(116). It is not clear what distinguishes asymptomatic patients from those who experience clinical infection, as the colonizing strains are frequently toxin producers and members of epidemic lineages.

Previous work has highlighted the importance of a healthy gut microbiota and intact immune system to prevent and recover from *C. difficile* challenge(2, 29, 30, 106, 118, 136, 215). In contrast, it has been more difficult to identify *C. difficile* strains that are consistently associated with severe disease versus mild diarrhea or asymptomatic colonization. Patients with *C. difficile* infection have heterogeneous comorbidities, variable states of immune function, and generally have received diverse combinations of microbiota-disrupting antibiotics(116, 123, 140, 199, 218, 246). In these complex clinical settings it has been difficult to distinguish the role of bacterial strain differences in the severity of colitis from the contributions of a patient's co-morbidities.

To control for human host variability, we have used a mouse model of *C. difficile* infection and disease progression. Mice share many of the key aspects of *C. difficile*-associated disease with humans, including antibiotic-induced susceptibility(30, 42, 136, 217), inflammatory cell recruitment to the colonic mucosa(106), and chronic carriage(125). In contrast to patient populations, however, experimental mice used in our studies are genetically identical, harbor microbiota that is compositionally similar and undergo identical antibiotic pre-treatment prior to infection with precisely quantified inocula of *C. difficile* spores. We obtained 33 phylogenetically diverse *C. difficile* isolates from patients hospitalized at Memorial

Sloan Kettering Cancer Center and Washington University St. Louis-area hospitals, introduced them to susceptible mice, and monitored them for signs of symptomatic disease. We then performed a series of in vitro assays and in silico whole genome sequence analyses to determine which bacterial phenotypes and genetic variants were associated with virulence. We found that that clinical isolates belonging to *C. difficile*'s clade 2 (particularly, the MLST 1/R027 strain) resulted in higher lethality in mice, but that these differences virulence were not attributable to the amount of toxin production or differences in toxin gene sequence. Strains with highly similar core genome sequences were found to cause a range of disease severity in the host, suggesting that in vivo pathology is, at least in part, attributable to differences in non-core, accessory gene representation.

4.2. RESULTS

4.2.1. Classification of clinical isolates reveals a range of phylogenetic diversity and human disease severity.

We obtained a diverse array of *C. difficile* clinical isolates from patients hospitalized at Memorial Sloan Kettering Cancer Center (MSKCC, *n*=21) and Washington University-St. Louis area hospitals (*n*=12) and classified them by multi-locus sequence typing (MLST)(57, 91). Of the 33 isolates examined, 24 belonged to *C. difficile*'s clade 1 (with MLST 2 and 42 most prevalent), 6 were classified as clade 2 (5 of which were R027/MLST 1), 1 was classified as clade 4 (MLST 39), and 2 were classified as clade 5 (both MLST 11).

Clinical isolates obtained from Washington University-St. Louis area hospitals were assigned human virulence scores as described previously(256). Briefly, severe disease was recorded if the patient had clinically significant diarrhea with a white

blood cell count of ≥ 15,000 cells/mm^3 and/or serum creatinine ≥ 1.5 times the premorbid level at the time of *C. difficile* diagnosis. Clinical isolates obtained from MSKCC could not be similarly scored because samples were usually collected from bone marrow transplant patients, whose disease process results in low white blood cell counts that do not rise after *C. difficile* infection. MSKCC patients were classified as "*C. difficile* severe" if they had serum creatinine ≥ 1.5 times the premorbid level and had confirmed colitis as evidenced by imaging.

4.2.2. Clinical isolates produce a range of disease severity and mortality outcomes in mice.

Wild-type C57BL/6 mice were rendered susceptible to *C. difficile* infections by providing them with a cocktail of several antibiotics (see **Figure 4.1A** and *Methods* section). Twenty-four hours after antibiotic cessation (designated day 0), the mice were orally gavaged with 100 spores from a single *C. difficile* clinical isolate, and then monitored for the next two weeks for measures of disease burden and *C. difficile* colonization. We found that maximum weight loss and peak disease score occurred between days 2-4 post infection. Each clinical isolate was therefore assigned an "acute disease score," which corresponded with the average disease score on day 3 after infection (**Figure 4.1B**).

The majority of the mice infected with the clinical isolates were able to recover from the infection (**Figure 4.1C**). For example, only 2/24 strains from clade 1, and none of the three strains from clade 4 and 5 demonstrated virulence sufficient to kill mice. In contrast, mice infected with clinical isolates belonging to clade 2 experienced significant mortality (4/6 strains killed at least some of the mice in the cohort). Following mice for two weeks after infection reveals that clade 2 strains caused more severe and more prolonged morbidity when compared to the other clinical isolates

(**Figure 4.1D**). A chi-squared test of independence revealed that mouse survival is likely dependent on *C. difficile* clade ($p = 0.012$). High acute disease scores were correlated with poor survival outcomes during the course of the experiment ($p<0.01$ as assessed by linear regression).

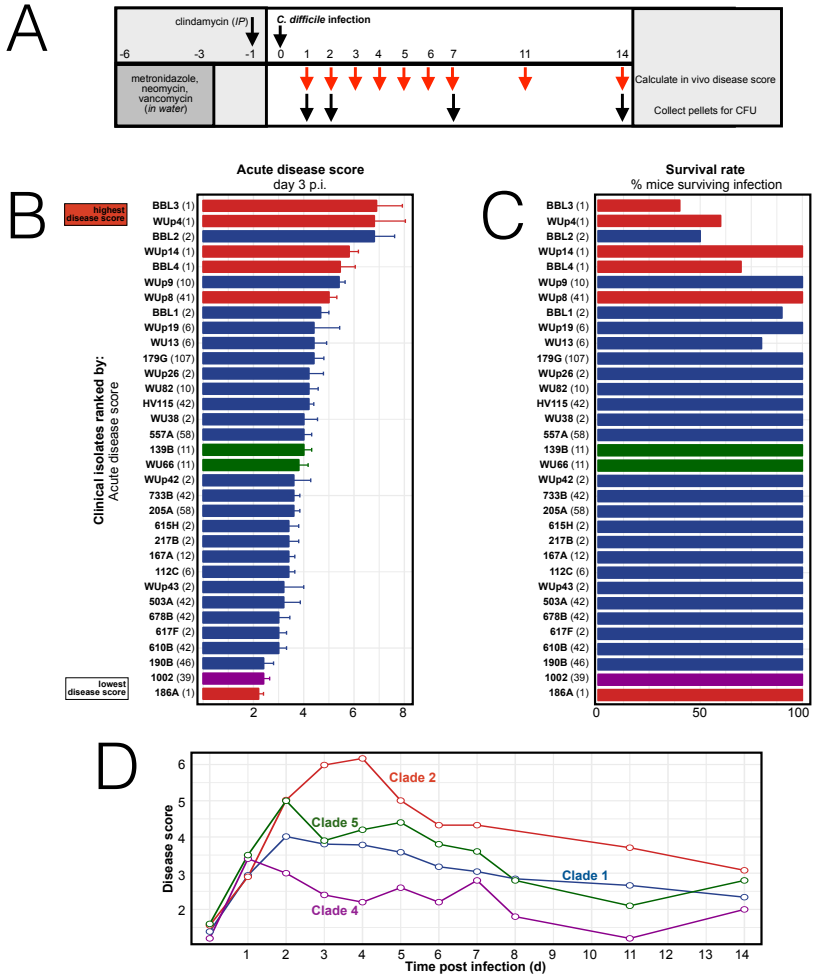


Figure 4.1. In vivo disease burden of *C. difficile* clinical isolates in the mouse model. A. Summary of experimental protocol. **B.** Average acute disease score taken three days post infection for each of the clinical isolates, n = 5-10 mice per isolate. **C.** Survival rate in the two weeks following infection. Clinical isolates are labeled by their unique strain identifier (**bold**) followed by MLST type in parentheses. Error bars indicate standard error. **D.** Disease score following infection. Disease scores for each clinical isolate were averaged by clade. Clade is identified by color: blue = clade 1, red = clade 2, purple = clade 4, green = clade 5.

4.2.3. Clinical isolates that are ranked as "severe" in mice represent a subset of the "severe" isolates in humans.

The mouse model, as it allows the experimenter to control host genetic factors and microbiota composition, provides useful information about bacteria-specific variations in virulence. However, the model becomes more advantageous if it can be shown to replicate some aspects of human disease. To address this point, we first divided the clinical isolates into two categories based on their disease burden in mice: "severe" strains killed at least one mouse in the cohort, and "non-severe" strains did not result in increased mortality. We then compared the murine disease severity to the severity reported in the original human host (**Figure 4.2**). We found that none of the human, non-severe isolates caused severe disease in mice, and that a subset of the human, severe isolates was similarly severe in mice. Therefore, characteristics of highly virulent *C. difficile* strains as identified in the mouse model are likely important virulence factors in the human host. A summary of the clinical isolates' disease severity in humans and mice can be found in **Table 4.1**.

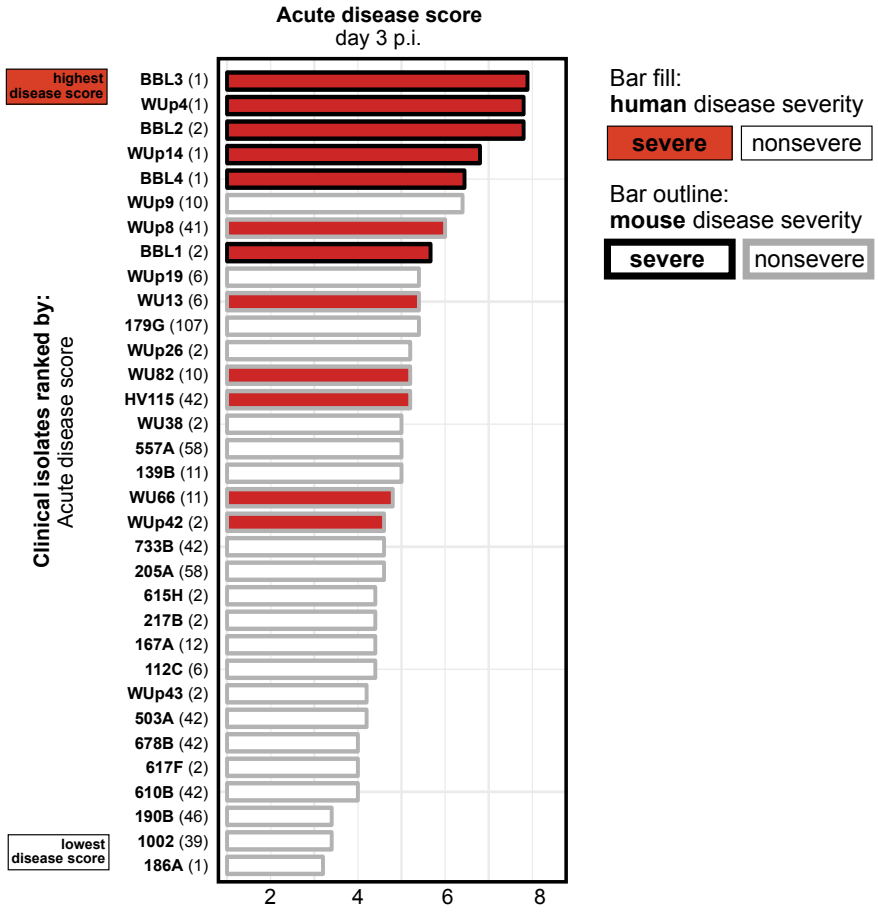


Figure 4.2. Comparison of *C. difficile* clinical isolate disease severity in humans and mice. Clinical isolates are ranked by their average acute disease score: bottom strains have the lowest acute disease score, top strains have the highest acute disease score. Bar fill color indicates if the clinical isolate was identified as severe (red) or non-severe (white) in humans. Bar outline indicates if the clinical isolate was identified as severe (black) or non-severe (grey) in mice. Clinical isolates are labeled by their unique strain identifier (**bold**) followed by their MLST type in parentheses.

Table 4.1. Classification of *C. difficile* clinical isolates by clade, MLST, and disease severity. *Clinical isolate 217B used as the reference strain for pathogenicity locus and core genome analysis.

Strain	Source	Clade	MLST	Disease severity (human)	Disease severity (mouse)	Acute disease score
BBL3	MSK	2	1	severe	severe	6.9
WUp4	WashU	2	1	severe	severe	6.8
BBL2	MSK	1	2	severe	severe	6.8
WUp14	WashU	2	1	severe	severe	5.8
BBL4	MSK	2	1	severe	severe	5.4
WUp9	WashU	1	10	non-severe	non-severe	5.4
WUp8	WashU	2	41	severe	non-severe	5.0
BBL1	MSK	1	2	severe	severe	4.7
WUp19	WashU	1	6	non-severe	non-severe	4.4
WU13	WashU	1	6	severe	non-severe	4.4
179G	MSK	1	107	non-severe	non-severe	4.4
WUp26	WashU	1	2	non-severe	non-severe	4.2
WU82	WashU	1	10	severe	non-severe	4.2
HV115	MSK	1	42	severe	non-severe	4.2
WU38	WashU	1	2	non-severe	non-severe	4.0
557A	MSK	1	58	non-severe	non-severe	4.0
139B	MSK	5	11	non-severe	non-severe	4.0
WU66	WashU	5	11	severe	non-severe	3.8
WUp42	WashU	1	2	severe	non-severe	3.6
733B	MSK	1	42	non-severe	non-severe	3.6
205A	MSK	1	58	non-severe	non-severe	3.6
615H	MSK	1	2	non-severe	non-severe	3.4
*217B**	MSK	1	2	non-severe	non-severe	3.4
167A	MSK	1	12	non-severe	non-severe	3.4
112C	MSK	1	6	non-severe	non-severe	3.4
WUp43	WashU	1	2	non-severe	non-severe	3.2
503A	MSK	1	42	non-severe	non-severe	3.2
678B	MSK	1	42	non-severe	non-severe	3.0
617F	MSK	1	2	non-severe	non-severe	3.0
610B	MSK	1	42	non-severe	non-severe	3.0
190B	MSK	1	46	non-severe	non-severe	2.4
1002	MSK	4	39	non-severe	non-severe	2.4
186A	MSK	2	1	non-severe	non-severe	2.0

4.2.4. Ex vivo and in vitro experiments explain some, but not all, of the observed differences in acute disease scores.

One potential explanation for the observed range in disease severity is that the *C. difficile* clinical isolates colonize the mice with variable efficiency. To test this, we collected fecal pellets from mice on day 2, 7, and 14 post infection and quantified *C. difficile* burden(30). By day 2, all mice were colonized in excess of 4.0×10^6 colony forming units (CFU) per gram of stool (**Figure 4.3A**), and they remained colonized for the duration of the experiment (data not shown). Slight variations in *C. difficile* burden did not correlate with differences in acute disease score (p=0.21, **Figure 4.3D**).

We next examined each clinical isolate for its production of toxin in an ex vivo setting. One commonly cited explanation for the increased virulence of the MLST 1/R027 epidemic strain is that it produces increased toxin when compared to pre-epidemic controls; however, initial studies measured toxin in an in vitro setting that may not accurately represent the infectious environment(141, 150). We instead measured toxin titer directly from fecal samples collected from mice 2 days after infection by using a functional, cell-based assay(30). Despite colonizing the mice to similar levels, the clinical isolates produced a wide range of toxin during acute infection (**Figure 4.3B**). Strains producing more toxins did not reliably cause greater acute disease (p=0.29, **Figure 4.3E**).

Finally, recent studies from our lab and others have examined the relationship between secondary bile acid concentrations and the ability of *C. difficile* to germinate into metabolically active vegetative cells(29, 118, 137, 216, 244). In an in vitro setting, the clinical isolates exhibited a broad range of tolerance to the secondary bile acid lithocholic acid (LCA) (**Figure 4.3C**). Isolates from the phylogenetically diverse clade 1 were variable in terms of their ability to grow in the presence of LCA. Of note, high virulence clade 2 isolates grew better in the presence of LCA than the lone low-

virulence clade 2 isolate, 186A, and overall the strains with increased lithocholic acid tolerance had higher acute disease scores (p=0.0096, **Figure 4.3F**).

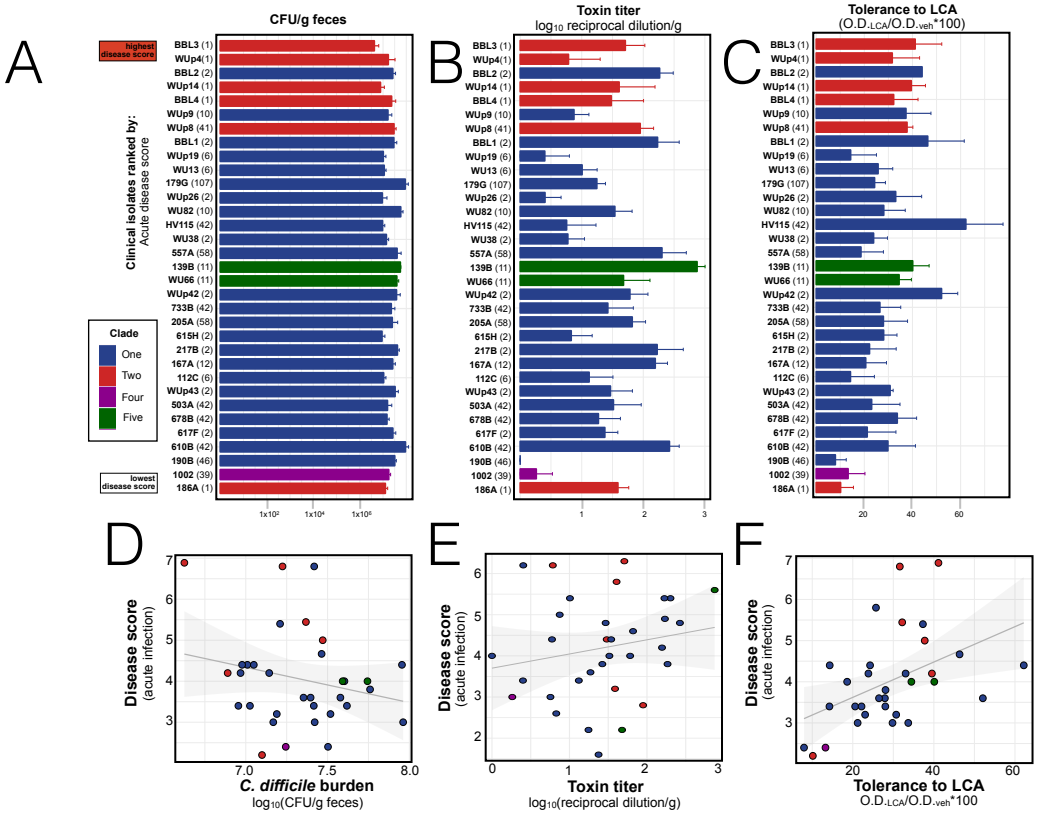


Figure 4.3. Ex vivo and in vitro assessment of putative virulence factors of *C. difficile* clinical isolates. A. *C. difficile* burden in the feces of mice two days after infection, n = 5-10 mice per isolate. **B.** Toxin titer in the feces of mice two days after infection, n = 5-10 mice per isolate. **C.** Tolerance of each clinical isolate to administration of 0.01% lithocholic acid, n = 3 experimental trials per isolate. **A-C.** Clinical isolates are ranked by their average acute disease score: bottom strains have the lowest acute disease score, top strains have the highest acute disease score. They are labeled by their unique strain identifier (**bold**) followed by their MLST type in parentheses. Error bars indicate standard error. **D-F.** Comparison of ex vivo and in vitro data to corresponding acute disease score in each isolate. Solid lines represent the linear regression, with 95% confidence interval in the corresponding shaded region. C.F.U. = colony forming units, O.D. = optical density (600 nm).

4.2.5. Examination of the pathogenicity locus reveals clade-based variability in toxin sequences.

We next moved to a sequence-based approach to identify genomic regions associated with acute virulence score in the mouse model. We first investigated the *C. difficile*'s pathogenicity locus (PaLoc). The PaLoc is a 19.6 kb genetic locus that contains the genes encoding toxin A and B (*tcdA* and *tcdB*, respectively) as well as three small open reading frames that encode putative regulatory elements (*tcdR/tcdD*, *tcdE*, and *tcdC*)(43). Variants of the PaLoc genes have been reported to explain strain hyper virulence in the human host(141).

Using a clade 1, MLST 2 strain (217B) as our reference or comparison strain, we mapped single nucleotide variants (SNPs) across the PaLoc and then constructed a tree based on these variants (**Figure 4.4A**). We then assigned colors to the tree leaves based on acute disease scores. The tree revealed that the PaLoc sequences tend to cluster based on the clinical isolate's clade, and to a lesser degree, MLST type. While the high-virulence clade 2 isolates clustered together on the tree, other high virulence strains mapped to more distant branches (see BBL2, WUp9). Plotting PaLoc variants linearly and arranging each strain based on its acute virulence rank can also visualize this finding (**Figure 4.4B**). These two analyses demonstrate that no one variant pattern of the PaLoc is consistently associated with high acute disease scores; high virulence strains were found to have PaLoc sequences that belong to at least three distinct sequence patterns (see BBL3, WUp9, and WUp8 as examples).

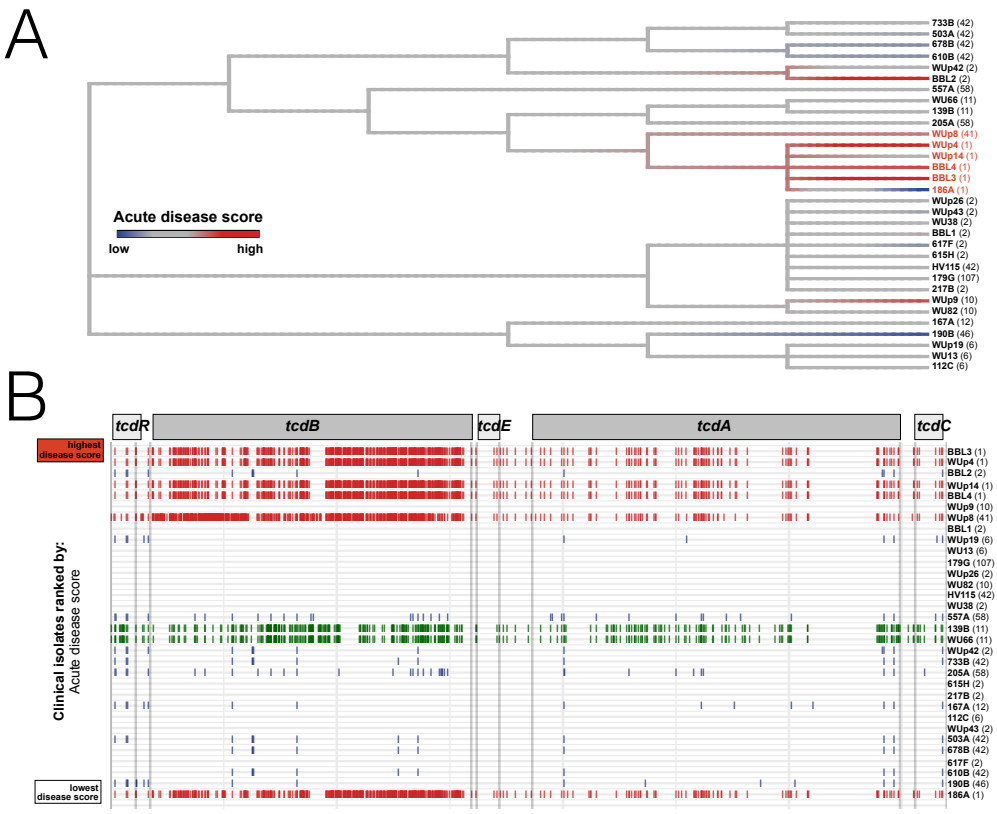


Figure 4.4. Comparative analysis of *C. difficile* pathogenicity loci. A. Phylogenetic tree of pathogenicity locus sequences with each clinical isolate. Tree leaves are colored by relative acute disease scores: blue = low virulence, red = high virulence. Clinical isolates are labeled by their unique strain identifier (**bold**) followed by MLST type in parentheses, and clade 2 isolates are labeled with red text. **B.** Single nucleotide variant differences of pathogenicity locus sequences as compared to the reference strain 217B (MLST 2). Each variant is indicated by a small vertical line. Clinical isolates are arranged by their acute disease score, with low virulence strains on the bottom and high virulence strains at the top of the figure.

4.2.6. Examination of the core genome reveals distinct sub-clusters of high virulence strains.

Following examination of the pathogenicity locus, we inferred the phylogeny of our clinical isolates by comparing the sequences of their shared, or core genome. Comparison of all open reading frames (ORFs) across the isolates yielded

identification of the core genome. The gene content within a single isolate averaged 3,868 ORFs (range: 3,602 to 4,206 ORFs), and the shared genome was composed of 1459 ORFs. The sequences of the core genes were then compared in an analysis similar to that of the pathogenicity locus, again using strain 217B as the reference. No two clinical isolates had identical core genome sequences; WUp26 was most closely related to the reference strain with 87 variants in the core genome.

The phylogenetic tree assembled using core gene sequences revealed distinct clustering of the clinical isolates based on their previously identified clade (**Figure 4.5**). The isolates also tended to cluster among their MLST group, with MLST 2 and MLST 42 demonstrating the most heterogeneity. Unlike the other MLST groups, MLST 2 and MLST 42 did not fall into discrete lineages. Assigning color to the tree leaves according to acute disease score (**Figure 4.5**, left) or mortality rate (**Figure 4.5**, right) revealed a high-virulence cluster within MLST 1/R027 isolates. In addition, the core genome analysis identified strains whose high virulence is not immediately attributable to their position on the tree (e.g., BBL2, WUp9).

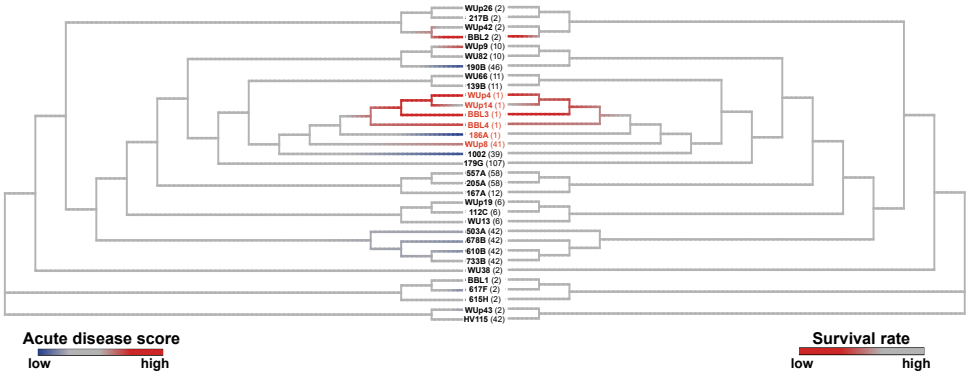


Figure 4.5. Core genome phylogeny of *C. difficile* clinical isolates. Phylogenetic tree of core genome sequences with each clinical isolate. Tree leaves are colored by acute disease score (left) or survival rate (right). Clinical isolates are labeled by their unique strain identifier (**bold**) followed by MLST type in parentheses, and clade 2 isolates are labeled with red text.

4.2.7. Analysis of the accessory genome reveals gene groups that are associated with in vivo disease scores.

Excluded from the previous analysis is the accessory genome, the genes not universally shared among the various *C. difficile* isolates. The accessory genome, also known as the flexible or dispensable genome, contains genes non-essential for bacterial growth but often includes factors that provide a given strain with a survival advantage in specific environments (e.g., antibiotic resistance or resistance to detergents)(104). We identified the accessory genome in our *C. difficile* clinical isolates and assembled a large matrix that denoted the presence or absence of each ORF in every strain. This matrix was then simplified by aggregating the ORFs with identical gene presence/absence patterns in all strains. By aggregating the ORFs this way, the accessory genome matrix was reduced in size to 1,156 unique "ORF groups." Next, we used generalized linear regression to identify which ORF groups were associated with acute disease score (**Figure 4.6A**).

In contrast to the core genome and pathogenicity locus analysis, which groups all R027/MLST 1 isolates together despite differences in disease score (**Figure 4.4A** and **Figure 4.5**), examination of the accessory genome demonstrates that the high virulence MLST 1 strains (BBL3, WUp4, WUp14, and BBL1) all contain ORF groups that are absent from the lone low virulence MLST 1 strain (186A) (**Figure 4.6B**). These ORF groups were found to contain a diverse group of genes including transcription-associated proteins, cell surface proteins, and many genes of unknown function (**Table 4.2**).

The accessory genome also helps discriminate between clade 1 isolates with disparate disease scores. This can also be seen with two closely related MLST 2 strains, WUp42 (intermediate virulence) and BBL2 (high virulence). These isolates are highly similar based on pathogenicity locus sequence (**Figure 4.4A,B**) and core

genome sequence (**Figure 4.5**), but accessory genome analysis reveals strain BBL2 contains ORF groups that match other high virulence strains irrespective of MLST (**Figure 4.6C, Table 4.2**).

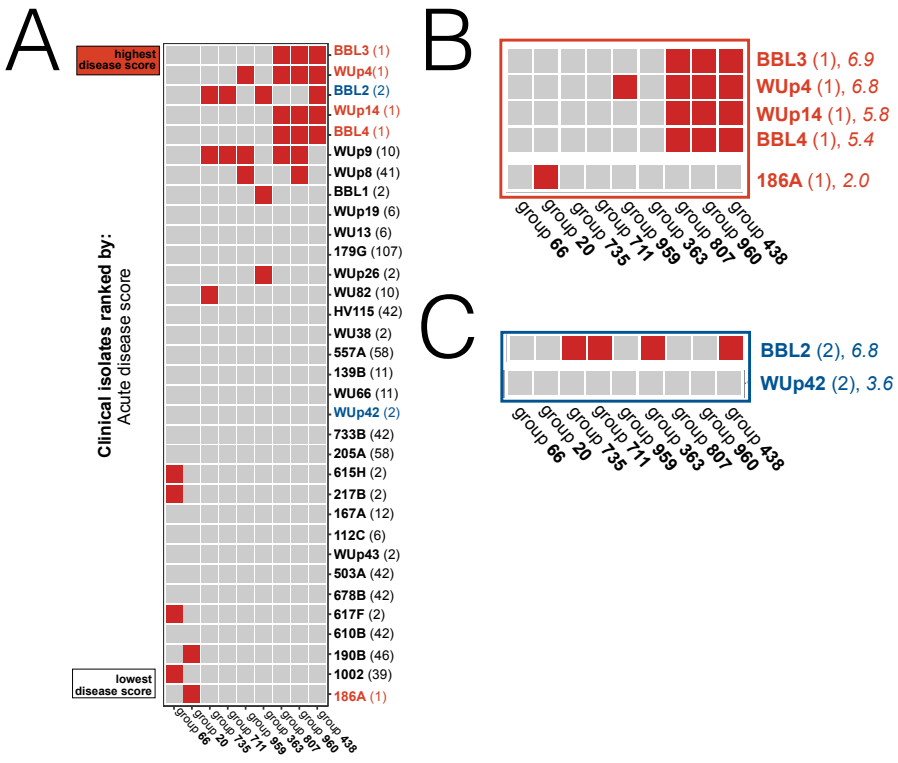


Figure 4.6. Top discriminant features in the accessory genome from elastic net regression. A-C. Presence (red) or absence (grey) of the indicated ORF groups within each *C. difficile* clinical isolate. Clinical isolates are labeled by their unique strain identifier (**bold**) followed by MLST type in parentheses, and in **B** and **C**, acute disease score is written in italics. **A.** Overall elastic net results. **B.** Comparison of low virulence (186A) and high virulence MLST 1 strains. **C.** Comparison of intermediate (WUp42) and high virulence (BBL2) MLST 2 strains.

Table 4.2. Top discriminant features in the accessory genome according to the elastic net regression. Each ORF group from the elastic net regression was examined for the genes within the group, and the putative functions for each gene are reported.

ORF group	ORF(s) identified	Function/Annotation	Associated with high or low virulence
20	*group_8636*	hypothetical protein	low
	group_8649	hypothetical protein	
66	*group_1800*	hemolysin XhiA family protein	low/intermediate
363	*group_1744*	DNA packaging protein	high
438	*group_695*	Fis family transcriptional regulator	high
711	*group_9089*	hypothetical protein/phage protein	high
	group_9090	hypothetical protein	
	group_9091	transcriptional regulator	
	group_9122	hypothetical protein	
	group_9123	hypothetical protein	
	group_9124	hypothetical protein	
735	*group_9160*	Unknown	high
807	*rep*	DNA helicase	high
	recF_1	DNA recombinase	
	group_3254	hypothetical protein	
	group_531	helicase	
	group_894	ATPase AAA	
	group_9165	single-stranded DNA-binding protein	
	group_9166	hypothetical protein	
	group_9167	conjugal transfer protein	
	group_9168	hypothetical protein	
	group_9169	cell surface protein	
	group_9170	DNA topoisomerase III	
	group_9171	DNA binding protein	
	group_9172	hypothetical protein	
	group_9173	transcriptional regulator	
	group_9175	transposase	
	group_9176	endonuclease	
	group_9177	hypothetical protein	
	group_9178	conjugal transfer protein	
959	*group_14507*	transposase	high
960	*group_35*	ATP/GTP binding protein	high
	iap_3	Lysozyme-like family protein	

4.3. DISCUSSION

C. difficile was first identified as a benign organism colonizing the gastrointestinal tract of infants(93); paradoxically, later investigations isolated it as the etiologic agent of pseudomembranous colitis in adults(83). The mechanisms by which bacteria can cause severe disease in one individual but silently colonize another are multifactorial and involve host factors, microbiota composition, and *C. difficile* strain differences. Differences in the host (age, gender, comorbid conditions) and microbiota composition are beyond the scope of this investigation. Instead, we believe we have developed a model that can reliably distinguish between low virulence and high virulence *C. difficile* strains in an in vivo setting.

Previous work investigating virulence factors in epidemic lineages such as MLST 1/R027 has been frustrated by conflicting data(5, 198, 234). For example, the increased toxin production reported in MLST 1 and MLST 11 isolates was initially attributed to a deletion in the gene *tcdC*, a putative negative regulator of *tcdA* and *tcdB* transcription(48, 88, 103, 141). However, subsequent investigators did not see increased toxin production when they artificially introduced the same *tcdC* mutation in a non-epidemic *C. difficile* strain(13, 36). Another gene within the pathogenicity locus, *tcdE,* has a similarly contentious role in virulence: some groups note that its similarity to phage holins suggest that the protein allows for increased toxin release(90), while others found that toxin release was unaffected when TcdE was functionally inactivated(157). What's more, it now clear that heterogeneity exists within the notoriously hypervirulent MLST 1 group, perhaps explaining some of the variability in published reports(31).

A major hurdle facing previous investigations of *C. difficile* strain type and virulence has been working with the human host: the typical patient suffering from *C. difficile* colitis has endured a variety of insults to her health, including antibiotic

treatment, chemotherapy, or other medical interventions. In this heterogeneous population, it is perhaps unsurprising that identification of genotypic correlates to phenotype has proven challenging(256). Our mouse model addresses this problem by controlling host and microbiota factors while testing a variety of clinically relevant *C. difficile* strains for differences in virulence. Mice share many of the key aspects of *C. difficile*-associated disease with humans, including antibiotic-induced susceptibility(30, 42, 217), inflammatory cell recruitment(106), and asymptomatic carriage(125). We find that the most virulent strains in mice were also virulent in humans. Concurrently, we find that some of the isolates that were obtained from severely ill human patients do not similarly cause severe disease in our murine model. While this may be due to physiological discrepancies between the two hosts, it may also reflect the fact that clinically ill patients are suffering from multiple diseases at the time of *C. difficile* diagnosis, an avenue not pursued by this investigation.

Of the 33 clinical isolates tested, we found that the majority belonged to *C. difficile* clade 1 and were relatively low virulence in the mouse model. The fact that these strains caused limited disease in mice despite high levels of colonization and intact toxin production is perhaps surprising but indicates that the host immune response is sufficient to overcome infection. In fact, mice that lack a functional innate immune response have been shown to suffer from increased mortality regardless of the infecting strain(2, 106, 117).

In comparison to the clade 1 isolates, we found that clade 2 (specifically MLST 1/R027) were more virulent in the mice. This finding in our model corroborates and strengthens reports that infections with MLST 1/R027 strains have increased mortality in humans(150). However, while much of the literature suggests that the increased virulence of MLST 1/R027 can be attributed to increased toxin production, we found only a modest association of ex vivo toxin levels and acute disease burden.

This finding was further supported when we examined the pathogenicity loci of all clinical isolates; both high- and low-virulence MLST 1 isolates shared identical PaLoc variants that differed substantially from the PaLoc sequences found in other *C. difficile* clades. Therefore, neither toxin production nor toxin sequence is sufficient to explain the observed variability in our in vivo infections.

Our study is limited by the relatively small number of clinical isolates and by their distribution across the *C. difficile* phylogeny. However, other investigations into bacterial genotype-phenotype correlations suggest that sample sizes as low as 30 isolates are sufficient for studies such as ours(68). In addition, the relatively low representation of non-clade 1 isolates makes it difficult to determine the specific virulence determinants in strains belonging to clades 4 and 5. Isolates from clade 2 were more virulent in our mouse model but underrepresented in the total population, which reflects the relative incidence of MLST 1 infections in our hospital (Memorial Sloan Kettering Cancer Center) in recent years(112).

The in vitro phenotype most strongly associated with in vivo disease scores was tolerance to the secondary bile salt lithocholic acid. This finding expands on previous work that *C. difficile* strains capable of growing in the presence of secondary bile salts are more likely to cause prolonged morbidity(137). It has been established elsewhere that *C. difficile* germination and growth is dependent on favorable concentrations of primary and secondary bile acids; antibiotic treatment removes commensal species with the enzymes necessary to generate inhibitory secondary bile acids from germination-promoting primary bile acids and thus provides an opportunity for *C. difficile* colonization(244). Transfer of a consortium of commensal bacteria, including one species capable of converting primary bile acids to secondary bile acids, is sufficient to ameliorate *C. difficile*-induced morbidity in mice(29). However, there is limited data examining the variability of secondary bile acid sensitivity across

different *C. difficile* strain types. We hypothesize that secondary bile acid tolerance provides a growth advantage to *C. difficile* in the lower intestine, and that this growth advantage could be exploited by the bacterium to cause more severe disease. However, the mechanism by which lithocholic acid tolerance is linked to in vivo disease severity remains to be explored.

While ex vivo and in vitro experiments provide valuable information regarding the phenotypic variability of the clinical isolates, studies are limited by *a priori* hypotheses of virulence determinants. We therefore moved to a top-down approach to the investigation and performed whole genome sequencing of each clinical isolate. Annotation of each strain allowed us to divide the analysis into two groups, the core genome (shared by all strains in the dataset) and the accessory genome (present in only a subset of the strains). The core genome was able to group the clinical isolates based on their previously determined MLST type, with MLST 2 and MLST 42 demonstrating the most heterogeneity. Although the lone low-virulence MLST 1 isolate (186A) clustered separately from the high virulence MLST 1 isolates, its core genome was more closely related to clade 2 than any of the other clinical isolates.

When we examined the accessory genome of our clinical isolate dataset, we were able to find genetic differences that associated with acute disease score more than overall phylogeny. Specifically, the low-virulence 186A is missing a number of putative genes that were found in the high virulence MLST 1 strains as well as high virulence strains belonging to clade 1. The functions of many of the genes in this group remain to be explored but include DNA-associated proteins (*rep*, *recF*) and membrane proteins (*iap*). Future studies are needed to confirm the roles of these genes with respect to in vivo virulence, but analyses such as these suggest that whole genome sequencing in general, and the accessory genome in particular, can help identify virulence determinants in closely related bacterial pathogens.

4.4. MATERIALS AND METHODS

4.4.1. *C. difficile* clinical isolate collection and classification. Fecal samples were collected from patients previously identified as colonized or infected with *C. difficile* and plated anaerobically onto brain-heart-infusion agar plates supplemented with yeast, cysteine, and the antibiotics cycloserine and cefoxitin (BHI and yeast extract from BD Biosciences, others from Sigma-Aldrich). Individual colonies that were able to grow in the presence of these antibiotics and that had the characteristic phenotype of *C. difficile* were selected and isolated, then typed using multi-locus sequence typing (MLST)(96).

4.4.2. Mouse husbandry. All experiments were performed with wild-type female C57BL/6 mice, aged 6-8 weeks and purchased from the Jackson Laboratories. Mice were housed in the specific pathogen-free facility at Memorial Sloan Kettering's Animal Resource Center, fed irradiated feed, and provided with acidified water. Two of us (B.B.L. and R.A.C.) performed all mouse experiments, including replenishing food as needed and changing cages at least once per week. The experiments were performed in compliance with Memorial Sloan Kettering's institutional guidelines and were approved by its Institutional Animal Care and Use Committee.

4.4.3. In vivo (murine) virulence assessment of clinical isolates. *C. difficile* spores for each clinical isolate were prepared as previously described(214). Mice were rendered susceptible to infection by pre-treating them with a cocktail of antibiotics: metronidazole (0.25 g L^{-1}; Sigma-Aldrich), vancomycin (0.25 g L^{-1}; NOVA-PLUS), and neomycin (0.25 g L^{-1}; Sigma-Aldrich) were added to the drinking water for three days, after which it was replaced with untreated water. Two days after antibiotic

cessation, mice were injected into the peritoneal cavity with 200μg clindamycin (Sigma-Aldrich). The next day (designated day 0), the mice were each infected with 100 spores of a given *C. difficile* clinical isolate via oral gavage. Mice were housed in groups of five, and the cages were mixed prior to *C. difficile* infection to help control for inter-cage variability in microbiota composition (all mice within each cage were infected with the same strain). Each clinical isolate was tested in 5-10 mice.

Mice were monitored every day for the first week and every 3-4 days during the second week after infection for measures of disease severity as described previously(2). Briefly, measures of weight loss, surface body temperature, diarrhea severity, and phenotypic morbidity were scored and combined to produce a total disease score. In addition, on days 1, 2, 7, and 14 post infection, fecal pellets were collected for quantification of *C. difficile* burden(34).

4.4.4. Quantitative *C. difficile* culture and toxin A and B quantification. Toxin titer in fecal samples on day 2 post infection was assessed via a functional, cell-based assay as previously reported(34).

4.4.5. In vitro assessment of lithocholic acid tolerance. The effect of lithocholic acid on *C. difficile* clinical isolate growth was measured in an in vitro setting as previously described(144). Briefly, pure cultures of each isolate were incubated anaerobically at 37°C and growth was monitored by measurement of optical density (600nm) over a 22hr period. Each isolate was grown under two treatment conditions: (1) vehicle (brain-heart infusion media supplemented with yeast extract and cysteine), or (2) vehicle with 0.01% lithocholic acid. We repeated each experiment twice, for a total of three trials. Tolerance to LCA treatment was calculated at the exponential phase of culture growth.

4.4.6. Whole genome sequencing, assembly, and annotation of clinical isolates. Single clones of *C. difficile* clinical isolates were grown anaerobically overnight at 37°C in brain heart infusion media supplemented with yeast and cysteine. DNA was extracted using phenol-chloroform extraction with bead beating and purified with a Qiagen QiaAmp kit. Purified DNA was sheared using a Covaris ultrasonicator and prepared for Illumina sequencing with a Kapa library preparation kit with Illumina TruSeq adaptors to create 300 x 300-bp nonoverlapping paired-end reads(33).

Quality control of raw sequence reads was performed using Trimmomatic version 0.35(26). Trimmed reads were assembled into contigs using the short read assembler SPAdes (v.3.6.1)(15), and secondary scaffolding was performed with AlignGraph using published *C. difficile* reference sequences to guide the assembly(16). Quality assessment of finished assemblies was performed using QUAST (v.4.4)(97). The assemblies were then annotated with putative open reading frames using Prokka (v.1.12)(206), and the core, accessory, and pan genomes were identified with Roary (v.3.7.0)(168). The core genome alignment was performed using Parsnp (v.1.2) (Harvest suite)(232) using strain 217B as the reference.

4.4.7. Pathogenicity locus analysis. The pathogenicity locus was identified in the clinical isolates using Prokka annotations(206) as well as BLAST alignments(7). The PaLoc alignment was calculated using Parsnp (Harvest suite)(232) using strain 217B as the reference.

4.4.8. Comparing phylogeny to phenotype outcomes. The phylogenetic trees calculated by Parsnp for the pathogenicity locus variants and core genome variants were mapped to phenotypic outcomes (acute disease score, survival) using the phytools(189) and ape(172) packages in R (version 3.3.2)(183). Phylogenetic signal

between phenotypic outcomes and the phylogeny was assessed using the phylosignal(119) package in R, and Moran's *I* index was noted. Because $p > 0.05$ for both the pathogenicity locus and core genome, we could not reject the null hypotheses for any of the phylogeny-trait pairs, and we did not perform any phylogenetic signal corrections.

4.4.9. Accessory genome analysis. The accessory genome was identified with Roary based on sequence annotations provided by Prokka. The accessory genome was compiled into a matrix that defined the presence (1) or absence (0) of each open reading frame in all clinical isolates. This matrix was then consolidated so that ORFs with the same presence/absence pattern in each isolate was aggregated into "ORF groups." The glmnet package(82) in R was then used to fit a generalized linear model to determine which ORF groups were able to predict the acute disease scores of the clinical isolates.

4.4.10. Statistical analysis. A chi-squared test of independence was run to test if mouse survival was likely dependent on *C. difficile* clade. The test was run twice: comparing clade 2 survival against all non-clade 2 strains or clade 2 vs. clade 1 alone. Linear regression was used to assess if acute disease score was associated with survival rates in mice. Linear regression was also used to compare if the ex vivo and in vitro phenotypes were associated with acute disease scores. Statistical significance was determined by a cutoff of $p < 0.05$.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

The mammalian gastrointestinal tract is a complex environment where microbial species interact with each other and with host cells. During homeostasis, a diverse population of commensal bacteria is sufficient to protect against challenges from bacterial pathogens like vancomycin-resistant *Enterococcus*, multidrug resistant *Enterobacteriaceae*, and *C. difficile*. However, common medications such as antibiotics have broadly destructive effects on commensal populations and leave the host susceptible to a variety of infections. The work presented here outlines the lasting impacts of antibiotic therapy on host defenses to infection. In addition, we investigate some of the interspecies variability of one important pathogen, *C. difficile*, and determine which bacterial properties contribute to virulence in the susceptible host.

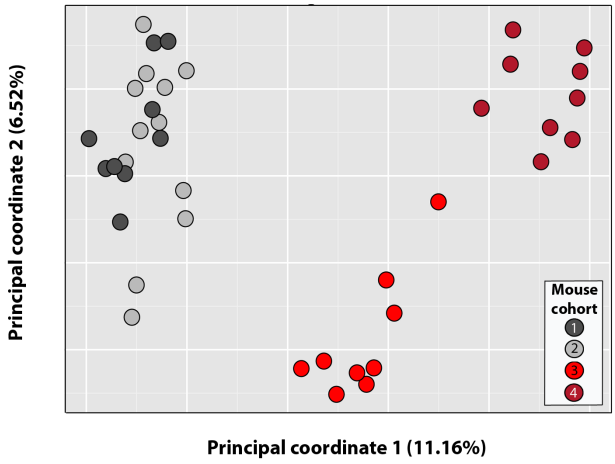
In Chapter 2 we showed that metronidazole and vancomycin, both antibiotics prescribed for *C. difficile* infections, have broad and long-lasting impacts on the colonic microbial populations of mice. Whereas naïve mice are naturally resistant to challenge with *C. difficile* bacteria, antibiotic-treated mice become colonized to high levels within 24 hours after spore inoculation. This disruption of protective commensal species is profound and may last as long as two weeks following antibiotic cessation. In the meantime, animals are susceptible to infections with a variety of hospital-associated and antibiotic resistant pathogens. More work needs to be undertaken in order to determine methods by which pre-antibiotic homeostasis can be restored.

While the second chapter of this work investigated the role of the microbiota in protection against pathogens like *C. difficile*, we were also curious about the degree of inter-species variability of one gastrointestinal pathogen. In Chapter 3, we obtained a group of clinical *C. difficile* isolates and studied them in a controlled mouse model. We knew from previous work in our lab and others that one of the major microbiota-based mechanisms of protection from infection is the production of secondary bile acids, amphipathic molecules that are toxic to vegetative *C. difficile* cells. We found that different *C. difficile* clinical isolates were highly variable in terms of their ability to grow in the presence of secondary bile acids like lithocholic acid and deoxycholic acid. In addition, the ability to tolerate application of these bile acids was a feature of higher virulence strains.

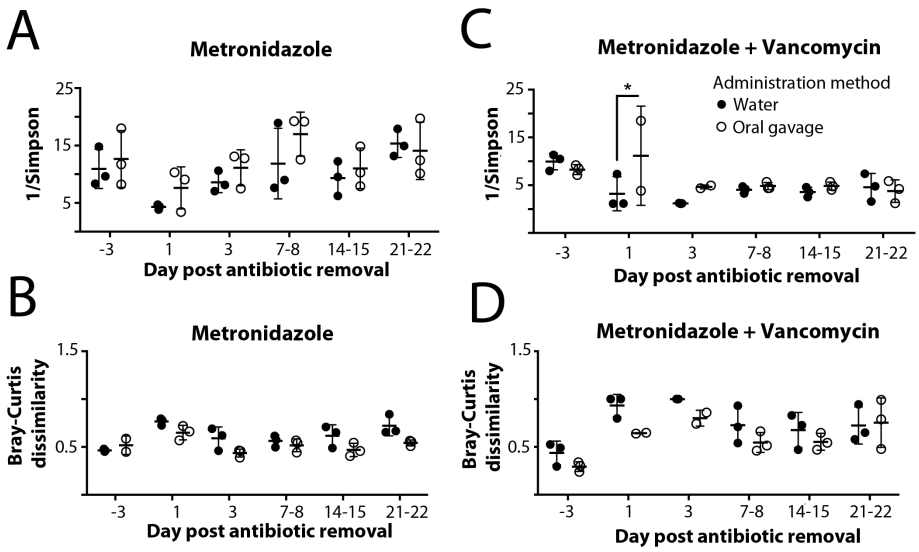
Our investigations of *C. difficile* clinical isolates was expanded further in Chapter 4, where we increased our strain repertoire and tested them for a variety of different functions, including toxin production. Surprisingly, the ability to produce high levels of toxin did not reliably lead to more severe infections in our model. Instead, we were able to use whole genome sequencing to identify groups of genes that were present only in a subset of high-virulence strains. Future work is needed to confirm the negative impact of these gene groups. Experiments such as these provide novel ways to discover virulence factors in pathogens like *C. difficile*.

APPENDIX 1

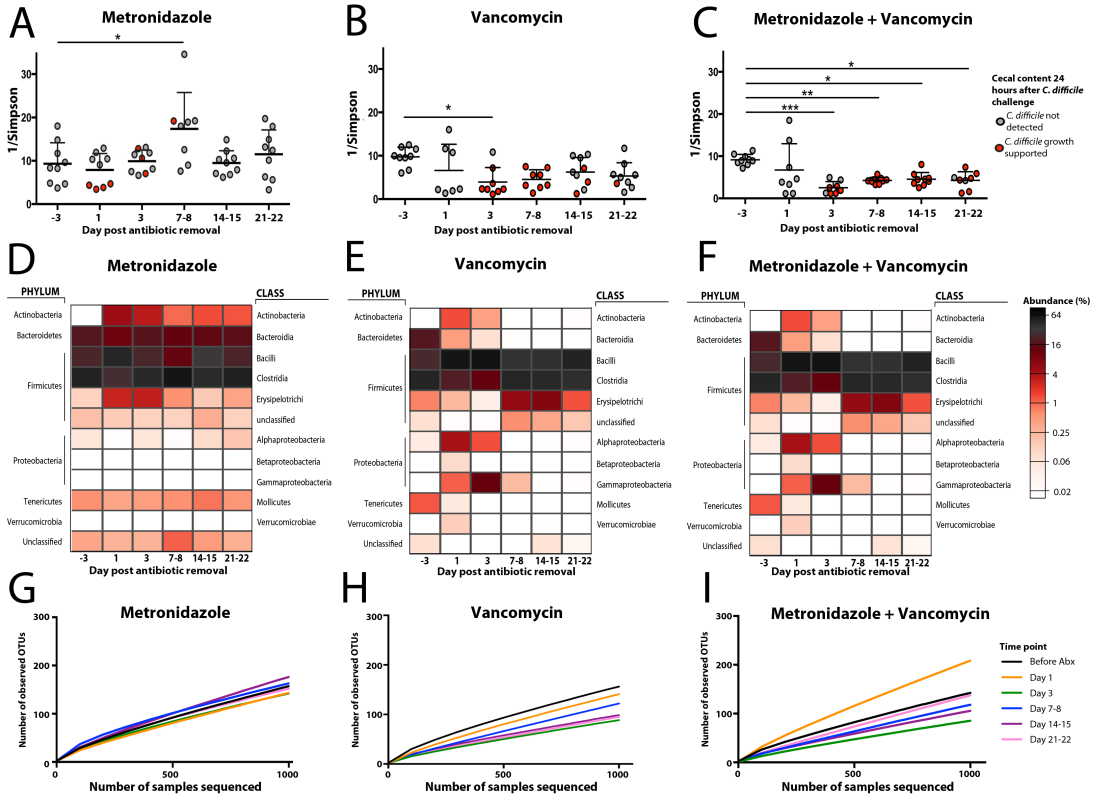
SUPPLEMENTARY FIGURES FOR CHAPTER 2



Supplemental Figure 2.1. Commensal microbiota of wild-type C57BL/6 Jackson mice is not uniform across time. Principal coordinate analysis of fecal sample obtained from mice upon arrival from Jackson laboratories, prior to antibiotic treatment. Each cohort was purchased at least one month apart. Cohorts 1 and 2 were purchased from the same barrier facility at Jackson laboratories, room MP-14.



Supplemental Figure 2.2. Effect of antibiotic administration route on alpha and beta diversity. (**A, C**) Inverse Simpson values from fecal samples following antibiotic treatment in mice administered antibiotics in their drinking water (closed circles) or by daily oral gavage (open circles). (**B, D**) Bray-Curtis dissimilarity indices from the same fecal samples. Bray-Curtis values given in reference to the same pre-antibiotic sample. **P*<0.05, center values (mean), error bars (s.d.). *n* = 3 mice for all time points.



Supplemental Figure 2.3. Effect of antibiotic treatment on alpha and beta diversity. (**A-C**) Inverse Simpson values in fecal samples following antibiotic treatment. Center values (mean), error bars (s.d.). **P*<0.05, ***P*<0.01, ****P*<0.001. (**D-F**) Operational taxonomic units from all mouse cohorts were binned at the phylum and class level and abundance of these taxa were tracked over time. Each rectangle represents the mean abundance of the specified taxon at the indicated time for the treatment group, $n = 9$ at each time point. (**G-I**) Rarefaction analysis of fecal samples in mice treated with the indicated antibiotics, $n = 9$ at each time point.

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