THE INDIGENOUS GUT MICROBIOTA MEDIATES COLONIZATION RESISTANCE AGAINST *CLOSTRIDIUM DIFFICILE*

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Microbiology and Immunology)
in The University of Michigan
2012

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DEDICATION

I dedicate this work to my parents, Lloyd and Priscilla Hopkinson, for instilling in me the values of integrity and perseverance and to my husband, Dallas Reeves, for understanding and supporting me through this process.

ACKNOWLEDGEMENTS

I am grateful to the University of Michigan for the educational and training opportunity to complete my doctoral work. I am thankful to the Department of Microbiology and Immunology for seeing my potential and allowing me to matriculate in the program as an international student. I would like to thank my undergraduate mentor, Dr. Louis Levinger for introducing me to scientific research, a field that I have come to love and for his encouragement during my graduate school application process. I am thankful to the faculty and staff of the University of Michigan for their excellent teaching and support especially Michelle Melis who was instrumental in helping me to identify my thesis lab. I am thankful to my thesis advisor, Dr. Young for allowing me to join his laboratory team and for his help in pushing me to become a more critical thinker, his mentorship and recommendations. I would also like to thank Dr. Hanna, Dr. Eaton, Dr. Huffnagle and Dr. Bergin for their role as my graduate committee members and for their helpful and excellent suggestions. I owe thanks to the Young lab members both past and present (Judy Opp, Kathy Wozniak, Casey Theriot, Seth Walk, Nabeetha Nagalingam, Courtney Robinson, Mark Koenigsknedht, Christine Bassis, Adam Nelson…) for helpful discussions, editing and making the lab a fun and interesting place to work. I am grateful to the Schloss lab for their helpful discussions in sequence data analysis and the Martens, Arnoff and Huffnagle labs for their help in providing reagents and laboratory equipment use. Thanks to

everyone at the University of Michigan germ-free core facility including Sara Poe and Chriss Vowles for all their technical help with my germ-free experiments.

My parents and family have worked very hard and made many sacrifices for me to attend school in the United States. I thank them for believing in me and insisting the values of honesty, hard work and perseverance. I thank my aunt and uncle, Olive and Karran Singh, for their support while I was at York College. And to my husband, Dallas Reeves, I thank you for all your support, love and encouragement that you have given me throughout my graduate career. And to those that I forgot to thank, thank you.

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ABSTRACT

Clostridium difficile is a pathogen that causes nosocomial antibiotic-associated diarrhea and colitis. The indigenous gastrointestinal (GI) microbiota plays an important role in protecting the host against infection with *C. difficile*. Administration of antibiotics disrupts the GI microbiota thus allowing for *C. difficile* to colonize and cause disease. The overall goal of this project was to understand the relationship between antibiotic administration and the role of specific members of the indigenous GI microbiota in mediating colonization resistance against *C. difficile*. Mice were treated with two different antibiotic regimens to make them susceptible to experimental *C. difficile* infection (CDI). Clinical signs of disease such as weight loss, diarrhea and hunched posture were monitored and at necropsy, tissue was harvested for histopathologic and culture-independent analysis of the gut community. Results from these experiments demonstrate that antibiotic administration is associated with major shifts in the microbial GI community structure that predispose mice to CDI. Specifically, antibiotic-treated mice challenged with *C. difficile* strain VPI 10463 either developed rapidly lethal CDI or were stably colonized with mild disease. The GI microbiota of animals with mild disease was predominated by bacteria from the family Lachnospiraceae, resembling the baseline community, while the GI community of animals with severe disease was predominated by *Escherichia*

coli. To test the hypothesis that Lachnospiraceae was less permissive to *C. difficile* colonization than *E. coli*, both Lachnospiraceae and *E. coli* members were isolated from wild-type mice and tested in germ-free mice. Results from these experiments indicate that a single Lachnospiraceae isolate (D4), but not *E. coli,* partially restored colonization resistance against *C. difficile* and improved clinical CDI. Thus, understanding how members of the indigenous GI microbiota, specifically Lachnospiraceae, interfere with *C. difficile* colonization could lead to new modalities for prevention and treatment of this important infection.

Chapter 1

Introduction

1.1 Indigenous gastrointestinal (GI) microbiota

For almost a century it has been known that humans are inhabited by a highly dense and diverse microbial ecosystem. Only now are we beginning to understand the many roles that the indigenous GI microbiota play in human health, development and disease processes. Knowing the composition and function of this ecosystem is a very important step toward understanding the many roles of the indigenous GI microbiota.

The microbiota (microbiome) refers to the total community of microorganisms that reside on and within the host**.** It is estimated that bacterial cells out number human cells by a factor of ten due largely to the extremely high density of bacterial cells found in the human GI tract (typically 10^{11}–10^{12} microbes/ml of luminal content) (147). Although Bacteria predominate, Archaea and Eukarya are also represented. Acid, bile and pancreatic secretions hinder colonization of the stomach and proximal small intestine by most bacteria. However, bacterial density increases in the distal small intestine and more so in the large intestine (114).

The microbial ecosystem in the human GI tract serves many important

functions. It provides protection against pathogens, assists with nutrient processing, stimulates angiogenesis and also regulates host fat storage (96, 97, 151). In addition, many diseases in humans have suspected links to the GI microbiota, including stomach cancer (120), mucosa-associated lymphoid tissue lymphoma (94), inflammatory bowel disease (116, 146), and necrotizing enterocolitis (50). The intestinal microbiome has metabolic activity that is both adaptable and renewable (21). Through the production of short-chain fatty acids, resident bacteria positively influence intestinal epithelial cell differentiation and proliferation, and mediate other metabolic effects (147). Together, this complex metabolic activity recovers valuable energy and absorbable substrates for the host, and provides energy and nutrients for bacterial growth and proliferation.

1.2 Molecular methods used to analyze microbial communities

Previously, much of our knowledge on the microbial ecology of the GI tract was described through microbiological culturing techniques. Though it is still a useful tool, it is becoming increasing clear that there are serious limitations in the application of such techniques to analyze complex microbial communities. Many of the bacteria residing in the GI tract are fastidious and require specific growth conditions and as a result may be difficult to grow or culture (158). It is estimated that over 50% of the species present in the indigenous gut microbiota have not been previously cultured (184). Recently, the use of the highly conserved phylogenetically informative gene that encodes the 16S rRNA present in all bacteria has enabled the development of molecular techniques to characterize

the whole microbial community present at a specific time in the GI tract (Figure 1.1) (93). The key advantage of using the 16S rRNA gene is the presence of highly conserved and variable regions, for example, primers can be designed based on the conserved regions while the variable regions can be used to distinguish different types of bacteria (Figure 1.1) (7). Molecular techniques have allowed us to characterize microbial community structure and composition, diversity, monitor microbial community dynamics as well as track specific strains of bacteria.

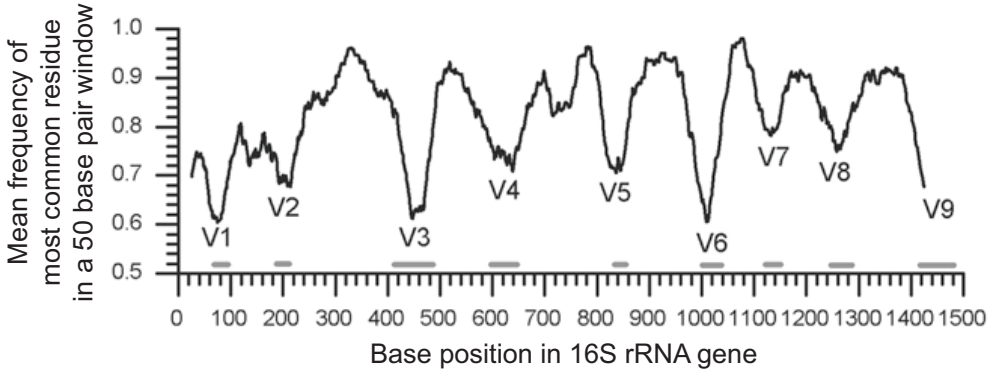

Figure 1.1 Variable regions of the 16S rRNA gene.The plot represents variable (V) and conserved regions of the 16S rRNA gene. The y-axis is the mean frequency within a window of 50 bases, moving one base at a time along the gene. Conserved regions on the graph are represented by the peaks. The locations of the hypervariable regions are labeled with gray bars on the x- axis defining these regions as V1 to V9 (From reference (7)).

Application of modern molecular techniques to study the gut microbial community is dependent upon the development of rapid and reliable techniques for identifying both culturable and non-culturable species. Characterization of microbial community structure and composition can be done using polymerase chain reaction (PCR) based techniques that target the 16S rRNA gene such as

16S rRNA gene clone libraries, pyrosequencing techniques, denaturing gradient gel electrophoresis (DGGE), terminal-restriction fragment length polymorphism analysis (T-RFLP) and quantitative PCR. Other common molecular techniques require the use of oligonucleotide probes specific for bacterial groups or species. These include dot blot hybridization, fluorescent in situ hybridization (FISH) and DNA microarray (phylochip) technology. Many of these techniques are summarized in Table 1.1.

Technique	16S rRNA based?	Taxonomic resolution/ sensitivity	Advantages	Disadvantages
Culture based	No	Moderate	(I) Have the organism	(I) Many GI bacteria are difficult to culture (II) Labor intensive
16S rRNA clone library (Sanger sequencing)	Yes	Very good	(I) Large portion of the 16S gene is sequenced which maximizes the taxonomic resolution	(I) Expensive (II) Underestimation of phylogenetic diversity
454 pyro-sequencing	Yes	Good	(I) Higher through-put (II) More sensitive (III) Multiple samples can be analyzed in a single sequence run (IV) No cloning bias introduced (V) Less susceptible to PCR bias	(I) Shorter sequence reads so less robust taxonomic resolution (II) Error rate per nucleotide is high
DGGE	Yes	Poor	(I) Rapid (II) Fingerprints provide a good basis to compare communities from various treatment groups (III) Bands of interest can be excised and sequenced	(I) Short PCR product so less taxonomic information (II) Reproducibility between gels is difficult
TRFLP	Yes	Poor	(I) Fingerprints provide a good basis to compare communities (II) Multiple restriction enzymes can be utilized for greater resolution (III) Reproducible	(I) Limited taxonomic resolution (II) One phylotype can represent more than one species
DNA microarrays	Yes	Very good	(I) Useful for screening (II) Fast and easy to use (III) Clinical applications	(I) Detection limited by the sequences contained on the chip (II) Cross-hybridization issues
FISH	Yes	Good	(I) Target specific bacterial groups of interest (II) Flexible scope: probes can target individual bacterial species or bacterial groups (III) Direct enumeration of bacteria-16S copy number is not an issue	(I) Can't identify new bacterial groups (II) No a community wide survey (III) Reference strains are required to validate results (IV) Microscope work can be time- consuming
qPCR	Yes	Good	(I) Target specific bacterial groups of interest (II) Flexible scope: primers can be designed to target groups or individual species	(I) Reference strains required (II) 16S copy number varies (III) Can't identify new bacterial groups

Table 1.1 Summary of techniques used to study the GI microbial community.

1.2.1 Characterizing microbial community structure and composition

A number of methods can be used to generate data that examines microbial community structure and composition. The techniques that will be discussed are denaturing gradient gel electrophoresis, 16S rRNA gene clone libraries and pyrosequencing.

Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) allows the separation of amplified DNA fragments of similar size based on the extent of sequence divergence between different PCR products (111). A single PCR reaction is carried out on whole community DNA and partial 16S rDNA sequences. Sequences are then amplified from the different bacterial species present. DNA fragments of different sequences have varying melting temperatures. Fragments of the same size may be separated on gels that melt double-stranded DNA during electrophoresis, using a temperature or chemical denaturant gradient. DGGE has the potential to determine the identity of bacterial species present in complex microbial consortia without the need for prior sequence information. Thus, it provides a powerful tool in initial characterization of both culturable and non-culturable microbial communities in a specified system.

16S rRNA gene clone libraries

This method involves the use of classical DNA Sanger sequencing. The 16S rRNA encoding gene from bacterial species found in an experimental sample of interest is first amplified by PCR using broad range primers (Figure 1.2). The amplified 16S rRNA sample is purified and cloned into a vector then

transformed into competent cells. These clones are further screened with vector specific primers to ensure correct band lengths of the 16S rRNA which are then sequenced. Usually a clone library will consist of only 96 clones, which is a small number in comparison to the number of sequence reads obtained by newer methods such as pyrosequencing. However one advantage is that the sequenced 16S rRNA read lengths are long ranging from approximately 500 base pairs to full length which can be used to identify more accurately the organism from which the 16S rRNA gene was derived. Some bias does exist when constructing 16S rRNA clone libraries by PCR. These include differences in the specificity of polymerases, inhibition of the reaction by interfering substances, differential PCR amplification and PCR artifacts (e.g. chimeric structures and formation of deletion mutants) (173).

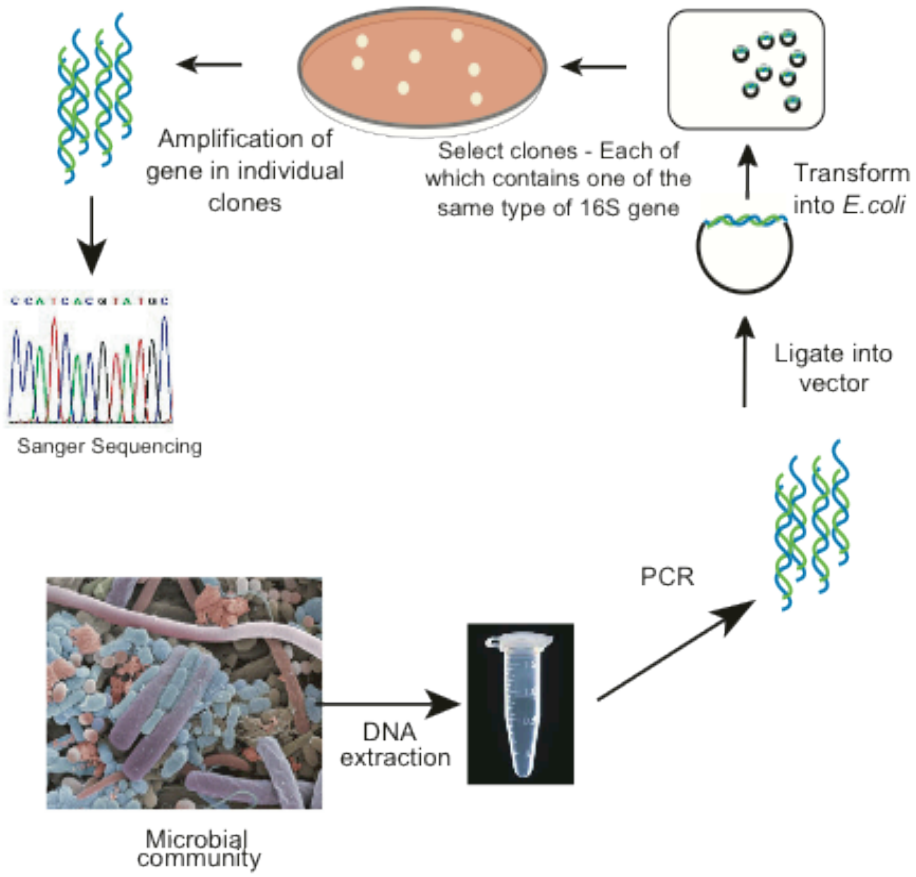

Figure 1.2 Schematic overview of 16S rRNA clone library construction. Total DNA is extracted from intestinal tissue and 16S rRNA is amplified. 16S amplicons are then ligated into a vector then transformed into competent *E. coli* cells. These clones are screened to ensure they contain only a single 16S rRNA gene, cultured, DNA extracted and then further amplified prior to sequencing.

Pyrosequencing

Pyrosequencing is a new method that was developed as an alternative to classical DNA Sanger sequencing. It is highly quantitative, fast and inexpensive and has many applications in DNA sequencing, genotyping, single nucleotide polymorphism analysis, allele quantification and whole-genome sequencing. Depending on the platform used, read lengths can be variable (113). Analysis of the 16S rRNA gene by the pyrosequencing technique involves four main stages:

first, target DNA is amplified using PCR; second, double-stranded DNA is converted to single-stranded DNA templates; third, oligonucleotide primers are hybridized to a complementary sequence of interest and, finally, the pyrosequencing reaction itself where a reaction mixture of enzymes and substrates catalyzes the synthesis of complementary nucleotides. Pyrosequencing is now increasingly used for bacterial detection, identification and typing. Pyrosequencing can also be partially or fully automated, thus enabling the high-throughput analysis of samples (113). Pyrosequencing has been applied to a wide range of microbial communities and variable (V) regions of the 16S rRNA gene, such as V6 in deep-sea vents microbial communities (41); V1, V2, V6 and V3 in human (4, 43) and macaque GI tract (108); as well as V9 in soil-derived microbial DNA (136).

1.2.2 Analyzing sequence data

An important part of microbial community analysis is the classification of sequences into a taxonomic framework. Many methods have been used with significant differences in classification results depending on the underlying algorithms and parameters used. Once sequence data is generated there are many ways in which the data can be analyzed. In this thesis 16S rRNA data was analyzed using a operational taxonomic unit (OTU; defined by sequence-based phylogenetic distance) approach which involves the use of a bioinformatic program such as mothur (**http://www.mothur.org**) (145). OTUs were binned according to 97% sequence similarity and then analyzed in two ways.

Phylogenetic names were assigned to each OTU by comparing representative sequences from each OTU against known 16S rRNA sequences in a database such as the Ribosomal Database Project (RDP) (**http://rdp.cme.msu.edu/**) (29). RDP has been widely used for the classification of 16S rRNA-encoding genes. This pipeline processes sequences and clusters them based on similarity to sequences in the RDP database (29). In addition to RDP, SILVA (**http://www.arb-silva.de/**) and greengenes (**http://greengenes.lbl.gov/cgi-bin/nph-index.cgi**) also provide comprehensive 16S rRNA gene databases to which 16S rRNA gene data can be aligned (42, 128). The second way in which OTU data was analyzed in this thesis was by examining community structure as displayed on a dendrogram. The beta diversity measure Morisita-Horn was used to calculate the level of community structure dissimilarity between different microbial communities (145).

1.2.3 Other techniques used to enumerate microbial communities

Oligonucleotide probes specific for groups of bacteria or bacterial species may be designed using the phylogenetic information present in 16S rRNA sequence databases.

Dot blot hybridization

Dot blot hybridization involves extraction of total 16S rRNA genes from the sample, binding of total rRNA to a membrane and hybridizing the bound rRNA with labeled probes of varying specificity. Using probes for selected groups of bacteria and universal probes designed to hybridize with 16S rRNA from all

bacteria, an estimate of the contribution of selected groups of bacteria to the total 16S rRNA pool may be achieved by comparing the intensity of reporter molecules. Bacteria differ in ribosome content depending on their metabolic activity and species. The ratio of bound group specific probe to total bound probe is an estimate of bacterial numbers present but this may not correlate directly with microbial numbers in situ. Dot blot hybridization has been used to study the rumen microbiota and to monitor the important human colonic phylogenetic groups in the fecal microbiota of infants (44, 150)

DNA Microarrays

This is a powerful tool that is designed for high throughput screening of human GI communities. The first DNA microarray contained probes that were designed to detect members of the GI microbiota based on the Agilent platform (118, 119). These probes targeted up to 359 microbial species and up to 316 new OTUs during human microbial ecology studies. More recently, Paliy and colleagues developed a more sensitive microarray representing approximately 775 species using the Affymetrix GeneChip platform (117). This chip detected and quantified differences in the gut microbiota of healthy individuals and also detected bacterial DNA present in minute amounts (0.00025%) of the total community DNA (117). However, there are some biases concerning detection limits and hybridization that need to be addressed before these microarray chips can become commercially available.

Fluorescent in situ hybridization (FISH)

Fluorescent in situ hybridization (FISH) uses oligo-nucleotide probes that

target 16S rRNA gene sequences which then allows the enumeration and visualization of whole bacterial cells in situ within GI and fecal samples (3, 30, 190). Genotypic probes that target the predominant components of the gut microbiota are usually tagged with fluorescent markers where changes in fecal or the intestinal bacterial population may be quantified using fluorescence microscopy. FISH is a truly quantitative technique as intact bacterial cells can be counted directly without extraction or amplification of nucleotide sequences. The major advantage of FISH is that bacterial populations may be enumerated in a culture-independent manner in environmental samples. Similarly, the technique allows visualization of target bacterial cells in situ.

Quantitative polymerase chain reaction

Quantitative or Real-time PCR (qPCR) is a variation on the polymerase chain reaction (PCR) where the quantity of nucleic acid can be measured. This method involves the use of probes or primers that can be designed to detect and quantify specific bacteria or bacterial communities. During normal PCR, DNA is amplified exponentially during a temperature dependent cycle using DNA polymerase. Only the end point product is retrieved for measurement. With qPCR, measurements are taken continuously during the amplification run correspondingly with the plotting of amplification curves. A fluorescent probe is used to detect amplification sits on the gene of interest. As the polymerase tracks down the DNA strand it causes the quencher to be released from the probe resulting in the fluorescence of the reporter dye. Time points can be visualized as soon as DNA is detected and is associated with the concentration of the target

DNA (98). Data calculation requires the use of a reference gene which allows for the normalization of the results and is usually a gene that is present in single copy numbers in the sample tissue.

1.3 Colonization Resistance

A major function of the human GI microbiota is colonization resistance. Colonization resistance refers to the ability of the microbial community to resist invasion by exogenous pathogenic organisms (57, 132). Since it is difficult to study colonization resistance in humans, one strategy that has been used to study colonization resistance is to disrupt the microbial community in animals by using antibiotics followed by challenge with a specific pathogen. In a previous study, it was shown that when mice were treated with either high doses of ciprofloxacin and levofloxacin or low doses of gatifloxacin and moxifloxacin, the level of *C. difficile* colonization in the mouse cecum increased compared to saline-treated controls (1) suggesting that the antibiotics altered the resident gut microbiota which allowed *C. difficile* to colonize. In an earlier study, Van der Waaij and colleagues demonstrated that after mice were treated with antibiotics there was a reduction in the density of cecal microbiota. In turn, this was responsible for a loss of colonization resistance to three experimentally introduced invaders (*Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*). However, colonization resistance returned over time as the indigenous microbiota recovered (170).

1.3.1 Mechanisms of colonization resistance

Some mechanisms that have been proposed to explain pathogen colonization suggest that the normal intestinal microbiota provides a natural barrier that is capable of preventing the establishment of pathogenic bacteria (89, 115, 187). Studies have proposed inhibitory mechanisms to explain what changes might be occurring in the GI tract. These include changes in redox potential and pH (64, 176), production of inhibitory compounds such as short-chain fatty acids (SCFAs), hydrogen sulphide production, bacteriocins (53, 64, 104, 137, 153), competition for nutrients (53) and competition for binding sites on the epithelium layer of the GI tract (161, 176). The GI microbiota can also influence colonization resistance through modulation of host immune responses. For instance, lipopolysaccharide (LPS) produced by Gram- negative bacteria is recognized by the immune system and in one study of vancomycin resistant Enterococcus (VRE), the administration of LPS to mice was shown to up-regulate an antimicrobial peptide, RegIIIγ which targets Gram-positive bacteria such as VRE and inhibited its colonization in mice (27). The host itself can also play a role in preventing pathogen virulence. For example, a study performed by Savidge and colleagues demonstrated that *C. difficile* toxins, which are necessary for virulence and disease, were changed chemically by S-nitrosylation by the infected host. This process inhibited cleavage of both TcdA and TcdB thereby preventing cell entry and attenuated *C. difficile* virulence (143).

The mechanisms which enable pathogens such as C. *difficile* to colonize the GI tract of humans are not very clear (137). Studies performed by Freter and

colleagues suggested several important factors in the gut that were necessary for controlling the bacterial ecosystem, principally- competition for adhesion sites to the gut wall and limiting nutrition (52). The notion of competition for adhesion sites was developed from a number of studies performed in the past. Itoh and colleagues observed that when germ-free mice were colonized with a number of anaerobic Clostridia prior to *C. difficile* challenge, *C. difficile* was eliminated from the GI tract of the animals. However, a similar effect was not observed when aerobic bacteria was used (69). Other studies have shown that anaerobic Clostridia in mice were able to associate closely with the mucosal layers of the cecum and colon (81, 142). When the ceca of conventional hamsters were subjected to scanning electron microscopy, spiral shaped organisms were observed at the opening of crypts in the cecum but these organisms were not observed in hamsters treated with clindamycin that had CDI (100). In addition, Savage and colleagues demonstrated that after the administration of antibiotics via oral gavage, the bacterial layers in the mucus and on the epithelial surfaces of the murine cecum and colon disappeared (141). A similar effect was also observed during dietary and environmental stress (159). These studies provide some evidence that occupancy of a specific niche by anaerobic organisms might be important for colonization resistance against *C. difficile*.

A number of Lactobacilli species have been explored in vitro to examine whether they can inhibit *C. difficile* growth or toxin production. These studies demonstrate that only specific strains of Lactobacilli are capable of inhibiting *C. difficile.* It was speculated that the inhibition observed was associated with the

production of hydrogen peroxide, lactic acid, bacteriocins or the release of a bioactive compounds which inhibit cytotoxin production (11, 112). However many of these studies were performed using in vitro models which might not be a true representation of what occurs in vivo.

Because of the complexity of studying the microbiota, in this thesis I will examine the roles of individual members of the normal indigenous gut microbiota in mediating colonization resistance against *C. difficile* in germ-free mice.

1.3.2 Short-chain fatty acid (SCFA) production in the GI tract

Many researchers believe that SCFAs play a role in colonization resistance (35, 52, 137, 153). Bacterial fermentation of complex polysaccharides is an important component of the human digestive process (104). Fermentation of dietary fiber typically results in SCFAs such as acetic, propionic, butyric, hexanoic and valeraic acids being produced as end products. Acetate, propioniate and butyrate are normally found in concentrations of 90 to 120 mM and are rapidly absorbed in the colon (37, 104).

Fermentation is the process by which small amounts of energy is derived from the oxidation of organic compounds, such as carbohydrates, and involves the use of an endogenous electron acceptor which is also an organic compound (36). The types of carbohydrates used are primarily from plant cells such as cellulose, pectin, starches, dextran and soluble carbohydrates (18) and the principle end products are usually SCFAs (36). Bacteria can undergo different types of fermentation and in return produce different end products. During

fermentation, pyruvate is metabolized to various compounds. For example, homo-lactic fermentation is the production of lactic acid from pyruvate; alcoholic fermentation is the conversion of pyruvate into ethanol and carbon dioxide; and hetero-lactic fermentation is the production of lactic acid as well as other acids and alcohols.

Fermentation takes place throughout the GI tract of all animals, but the intensity of fermentation depends on the number of bacteria present which are generally the highest in the large intestine (18). Thus, the large intestine is quantitatively the most important site of fermentation in humans. Many bacteria prefer to ferment carbohydrates than protein and therefore saccharolytic bacterial fermentation occurs predominantly in the proximal colon. On the other hand, if the supply of fermentable carbohydrates is depleted, proteolytic fermentation will occur in the distal colon (36). The latter is considered less favorable for the host because potentially toxic metabolites are formed such as ammonia, sulphur containing compounds, indoles and phenols (59). The quantity and proportions of volatile fatty acids produced by colonic bacteria are determined by the amount and type of substrate fermented. For instance, diet can change the metabolic activities of bacteria and as a result diet can influence the quantity and types of fermentation end products produced (18).

1.3.3 Functions of short-chain fatty acids in the GI tract

Dietary fiber is the major source of energy to support microbial populations in the GI tract (10). The chemical properties of dietary fiber as well as its

fermentation in the intestine have an important role in keeping the balance between communities of resident microbes and pathogens (110). SCFAs are the major contributors of energy from fermentation to the host and are rapidly consumed by the enterocyte (10, 133). Butyrate in particular is rapidly consumed by colonic enterocytes (139). The concentration of SCFA in the intestines may reduce the pH. A more acidic pH may inhibit the proliferation of some pathogens such as *E. coli* (110), *C. difficile* (104) and *Salmonella* sp. (34).

In addition, SCFAs have a trophic effect in the intestine, increasing the enterocyte turnover rate (134, 160). All of these described effects of SCFAs in digestive physiology help in maintaining the integrity of the intestinal mucosa, which acts as a barrier to protect against colonization by pathogenic bacteria (103). Butyrate also exhibits diverse regulatory functions on cell growth and differentiation, ion transport, and immunity in the intestinal epithelium (60, 80, 165, 171). Gram-negative foodborne pathogens, such as *Salmonella typhimurium* and enterohemorrhagic *E. coli*, alter virulence gene expression in response to butyrate, highlighting an important role for butyrate in host-pathogen interactions in the GI tract. SCFAs may also ameliorate diarrhea and prevent dehydration by promoting reabsorption of water and sodium in the large intestine (135).

Many studies theorize that increased concentrations of SCFAs causes a reduction in pH which is capable of limiting *C. difficile* growth and toxin production (104). Some studies have looked both in vitro and in vivo at the effect of butyrate to inhibit *C. difficile* growth and toxin production. However, many of

these studies are contradictory (137, 153). One study measured the physiological levels of various SCFAs in hamster ceca and translated these physiological concentrations in vitro to test the ability of each SCFA to inhibit *C. difficile* growth. This study demonstrated that in vitro inhibition of *C. difficile* by all SCFAs was correlated with pH and the concentrations of SCFAs in the cecum (137). They also determined that only butyric acid reached a concentration in the hamster ceca that was inhibitory to *C. difficile* growth in vitro (137). On the other hand, germ-free mice di-associated with *C. butyricum* (a butyrate producer) and *C. difficile* did not change the levels of *C. difficile* colonization even though there was a 20-fold increase in the concentration of butyric acid compared to *C. difficile* mono-associated mice (153). Although these studies on SCFAs are contradictory, both still conclude that SCFAs may play a role in preventing *C. difficile* colonization but other mechanisms are also involved.

1.4 Effect of antibiotics on the GI microbiota

Since the development of penicillin, antibiotic therapy has been used for the treatment of many infectious diseases. However, antibiotic therapy can affect not only the targeted pathogen but also the normal indigenous microbiota of the host. The impact on non-targeted microbial populations depends on the particular antibiotic used, its mode of action, and degree of resistance in the particular community (71). Antibiotics vary in their spectrum of activity and thus can be used to selectively target bacterial populations that inhabit mucosal surfaces. Many studies have been aimed at characterizing antibiotic-induced changes in

the intestinal microbiota and the impact on intestinal colonization by pathogens such as vancomycin resistant Enterococcus (VRE) (167), *Helicobacter pylori* (70), and *C. difficile* (178). Antibiotic treatment can decrease the density and dramatically alters the community structure of the intestinal microbiota (132). Other studies have also examined the ability of the murine gut microbial community to recover following antibiotic treatment. For instance, Antonopoulos and colleagues treated mice with different antibiotic regimens. The gut community of mice that were treated with a cocktail of amoxicillin, bismuth, and metronidazole returned to baseline following a two-week recovery period. On the other hand, cefoperazone treatment resulted in prolonged alteration of the gut community structure after a six-week recovery period (5).

Certain antibiotics are specifically active against anaerobic bacteria that are dominant in the human intestinal microbiota. Anaerobes play an important role in maintaining gut homeostasis by producing extensive amounts of SCFAs (144). Therefore, treatment with antibiotics that select against important groups of anaerobic bacteria can have substantial consequences for the resultant functional stability of the microbiota. One example is clindamycin, a relatively broad-spectrum antibiotic that primarily targets anaerobic bacteria. Clindamycin is excreted in bile and concentrations can be high in feces. It has been shown to have a large negative impact on the intestinal microbiota which can lead to colonization by pathogens such as *C. difficile* (14, 16).

1.5 *Clostridium difficile*

Clostridium difficile is a Gram-positive, spore-forming rod that belongs to the family Clostridiaceae and the genus *Clostridium*. It is a motile bacterium that is variably aerotolerant (2, 179). *C. difficile* was first isolated in 1935 from stool samples of newborn children and was named *Bacillus difficilis* because it was difficult to culture (58). Vegetative cells of *C. difficile* are typically larger than other bacterial cells measuring 3 - 16.9 µm in length, 0.5 -1.9 µm in width and producing sub-terminal spores (61) that are highly resistant to most standard forms of sterilization and disinfection. *C. difficile* is a heterotrophic organism with an optimal growth temperature of 37°C, most strains are motile and possess peritrichous flagella. Colonies of *C. difficile* following 48 hours incubation in anaerobic conditions at 37°C are typically large, flat and slightly grey in color. *C. difficile* also has a distinctive odor primarily due to the production of iso-valeric acid, iso-caproic acid and p-cresol, which are the products of various metabolic pathways within the organism (95).

C. difficile was initially identified as a commensal organism of the digestive tract in young infants (58). It was not until 1977 that a clostridial toxin was isolated from patients with pseudomembranous colitis (PMC) (88). In 1978 *C. difficile* was identified as the causative agent of antibiotic-associated pseudomembranous colitis (PMC) and was acknowledged as a human pathogen (17, 55, 89).

1.5.1 *C. difficile* virulence factors

Clostridium species produce many protein toxins that contribute to their virulence (73). It has been shown that some strains of *C. difficile* possess flagella (the components of flagella FliC (flagellin) and FliD (flagellar cap protein)) (163) which are involved in cell adhesion while other strains lacking flagella are unable to adhere to cells in vitro (162). Capsules have also been observed in some strains of *C. difficile* which may provide evasion from the host immune system (38). The surface layer proteins of *C. difficile* have been proposed to have immunoreactive properties (8) and are also involved in adhesion to host cells (25). Other cell surface factors reported to have adhesive properties include fibronectin binding proteins (63), Cwp66 (177) and the heat shock protein GroEL (62). It has also been reported that some of these proteins also stimulate an immune response (122).

Toxin A (TcdA) and Toxin B (TcdB)

C. difficile produces two major protein exotoxins; Toxin A (TcdA) and Toxin B (TcdB) that are high molecular weight glucosyltransferases (308kDa and 270kDa). Some *C. difficile* strains produce both TcdA and TcdB, while some *C. difficile* strains do not produce any toxin. Non-toxin producing strains of *C. difficile* are not associated with disease (78). *C. difficile* strains that are TcdA-/B+ have been identified. However, to date no TcdA+/B- strains have been identified.

Toxin A and B are encoded by the genes *tcdA* and *tcdB* respectively, which reside on the 19.6kb pathogenicity locus (PaLoc) in addition to the genes *tcdC, tcdD* and *tcdR* (83). The genes *tcdC* and *tcdD* are the respective negative and

positive regulators of the toxin genes (68). There is also growing evidence which suggest that another gene, *tcdE,* located outside of the PaLoc is responsible for holin function and facilitate the release of the toxins from the cell (157).

Research into the action of both toxins has been difficult since *C. difficile* is hard to genetically manipulate. Until recently, the virulence of strains that only produce TcdA could not be investigated because they are not found naturally in nature (102). New methodologies to genetically manipulate *C. difficile* have recently allowed novel studies to be carried out that investigate the independent action of both toxins in a hamster model. In contrast to earlier work, these results have suggested that TcdB is essential for virulence while genetically altered strains that only produce TcdA markedly lose the ability to cause disease (102). Such evidence also conflicts with earlier work which suggested that TcdA and TcdB work synergistically (84, 101). Thus, some controversy about which toxin is more important still remains.

Both TcdA and TcdB are produced during the late log and stationary phases of *C. difficile* growth (175) allowing cells to become established within the host gut before toxin production begins. Toxins are taken up by host cells through receptor-mediated endocytosis (166). The receptors for both toxins differ, with the receptor for TcdA better characterized than that for TcdB. The receptor for TcdA is the disaccharide Galß1- 4GlcNac found on I, X and Y blood antigens that are expressed on several types of cells such as intestinal epithelial cells (166). The receptor for TcdB has not yet been identified but its ability to infiltrate a variety of cells suggests a common receptor (175). When both the

toxin and receptor are internalized, the endosome enclosing them is acidified. This allows the toxin to undergo structural transformations upon which the active portions of the toxin (catalytic domain) are released into the cytosol (46, 56). Both toxins exert their effect on cells by glycosylating the Rho family of proteins (Rho, Rac, Cdc 42); proteins which are essential for many processes within the cell including regulation of the actin cytoskeleton, disruption of tight junctions and parts of the cell cycle (46, 56).

Glycosylation of the Rho GTPases leads to inactivation and inhibition of their regulatory activity within the cell, most notably leading to de-polymerization of the actin cytoskeleton and rounding of the cells and ultimately apoptosis. Cell rounding also leads to the disruption of tight junctions due to both the loss in the structure of the actin cytoskeleton but also because Rho proteins also regulate tight junctions. The loss of tight junctions then leads to increased permeability causing the diarrhea that is characteristic of *C. difficile* infection (CDI) (127).

In addition to TcdA and TcdB, some strains of *C. difficile* also produce a binary toxin (CDT) that has been identified as an actin-specific ADP-ribosyltransferase. This toxin is similar to other clostridial iota toxins which act specifically on actin within the cell (125). The role of the binary toxin in CDI is still unknown at present but this toxin has been shown to have a cytopathic effect on Vero cells (African green monkey kidney epithelial cells) in vitro (123). Not all strains of *C. difficile* produce CDT suggesting that this toxin is not essential to the virulence of the organism. The production of binary toxin is most frequently seen alongside TcdA and TcdB and is produced primarily by the PCR ribotype 027

strains (26). However, to date no studies have identified an association between CDT and disease phenotype.

C. difficile spore production

The ability of *C. difficile* to produce highly resilient endospores enables effective transmission and survival within an environment (24). Spores also allow the organism to persist within the gut despite antibiotic treatment thereby providing a type of resistance. The nosocomial transmission of *C. difficile* can be largely attributed to the ingestion of spores that have been picked up from contaminated surfaces or through aerial transmission (168). Spore formation allows *C. difficile* to spread efficiently (91). Sporulation occurs when vegetative cells of *C. difficile* are exposed to conditions that are not favorable such as nutritional deprivation (149). In such environments a spore is formed within the mother cell. This ensures the preservation of the strain until conditions are such that the spore will be stimulated to germinate into its vegetative cell state where it can produce toxin and cause disease.

Spores of *C. difficile* germinate in the presence of certain bile salts which are found in the small intestine of humans (182). Therefore, it is likely that this is the site where germination occurs. There are several bile salts that induce the germination of *C. difficile* spores, however taurocholate is the most effective and well documented; glycine and thioglycolate also act as co-germinants (149, 180).

1.6 *Clostridium difficile* infection (CDI)

Signs of CDI can range in severity from mild to moderate diarrhea or colitis,

sometimes accompanied by abdominal pain, fever, nausea, lethargy and dehydration to severe pseudomembranous colitis, sepsis, toxic megacolon and even death. In uncomplicated cases, CDI can often be resolved by discontinuation of the offending antibiotic, and rehydration therapy if required. In more serious cases of CDI, antibiotics such as metronidazole or vancomycin may be required to eliminate *C. difficile* from the gut.

The asymptomatic carriage of *C. difficile* in adults is reported to be due to previous infection (131), prior hospitalization (12) and also possible carriage of non-toxin producing isolates (40). The high rates of asymptomatic carriage amongst neonates are believed to be due to the immaturity of gut receptors to which *C. difficile* toxins can bind (183). It is well documented that the infant gut microbiota is different from adults (118, 169). Therefore it is thought that the maturation of the healthy gut microbiota bolsters colonization resistance in infants thereby eradicating *C. difficile* prior to receptor maturity (45). The significant difference in the reported carriage rates of *C. difficile* between infant and adult populations indicates that colonization resistance provided by the indigenous GI microbiota are influential in CDI outcome and may be an important factor in determining asymptomatic carriage. Asymptomatic carriage can be due to non-toxigenic strains or toxin producing strains but any pathogenic effect may be inhibited by the presence of an intact, undisturbed gut microbiota.

1.6.1 Clinical manifestations of *C. difficile* infection

Antibiotic-associated diarrhea (AAD)

AAD is a common complication of antibiotic use (20). It is described as unexplained episodes of diarrhea that begin during or up to two months following cessation of antibiotic therapy (47). Infectious AAD results from the disruption of the normal indigenous gut microbiota, and overgrowth of opportunistic pathogenic bacteria. Overgrowth of *C. difficile* is the predominant cause of infectious AAD. However, a large proportion of AAD cases are not due to infection and are often the result of varied physiological responses to antibiotics within the gut (66).

Pseudomembranous colitis

Pseudomembranous colitis (PMC) is primarily caused by *C. difficile*. Clindamycin was implicated as the cause of PMC in 1974 (164). PMC only occurs in 10% of AAD cases (106) but *C. difficile* is implicated in over 90% of these PMC cases. Symptoms of PMC include profuse watery diarrhea and severe abdominal pain, often accompanied by fever, swelling and tenderness of the abdomen (78). Endoscopic examination of the colon reveals the presence of yellow pseudomembranous plaques. Histopathologically, these lesions consist of dead mucosal cells, mucus, fibrin and neutrophils with the extent of plaque formation often correlating with the severity disease (78).

Perforation

This condition occurs in approximately 1-3% of all cases of CDI and is associated with mortality. Patients experience severe abdominal pain and distension, nausea, fever and tachycardia. It is defined as the complete penetration of the GI wall resulting in the release of intestinal contents into the

abdominal cavity. This may result in peritonitis (infection of the abdominal cavity). Surgical intervention is often required to prevent further complications and death (78).

Toxic megacolon

Toxic megacolon is a condition whereby the colon rapidly dilates. The dilation of the colon also causes abdominal distension and tenderness with fever. Toxic megacolon is a rare but life-threatening complication of CDI associated with a high risk of perforation, sepsis and shock. Treatment of toxic megacolon is usually through surgery by performing either a partial or total colectomy. Steroids can also be administered to reduce inflammation and dilation (9).

1.7 *C. difficile* recurrence and re-infection

Recurrent CDI is a complication that occurs in approximately 7-35% of patients after the initial resolution of infection (13, 47). It typically occurs 1 to 2 weeks after completion of antimicrobial therapy for CDI, but may take up to three months to develop (74). It is not clear why recurrence of CDI is so high in comparison to other infections. However some studies have identified several important risk factors for the development of recurrent CDI, including inadequate antibody response to toxin A, persistent disruption of colonic microbiota, being older than 65 years, prolonged hospital stays, severe illness, use of antibiotics other than *C. difficile* therapy during or after an episode of CDI, and use of immunosuppressive medications (109). Recurrence is often associated with

treatment failure where *C. difficile* has not been successfully eradicated from the GI tract. Following cessation of antibiotic therapy, the patient again becomes symptomatic due to the same strain. It has been suggested that the retention of spores within the gut that are unaffected by antibiotic therapy are likely to be a contributing factor (78, 105).

Another explanation for the high recurrence rates associated with CDI is the possibility that the observed recurrence is due to re-infection. Re-infection with the same or different strain of *C. difficile* is likely to occur while a patient is still recovering from a previous episode of CDI. Studies that have investigated recurrent CDI have reported high levels of re-infection (13, 181). It can take up to three months for the GI tract microbiota to become properly re-established resulting in the patient becoming vulnerable to infection for a prolonged period of time (105).

1.8 Immune response in *C. difficile* infection

An individual's immune response to *C. difficile* can influence the extent and severity of disease (77). Individuals that are immune-compromised are at an increased risk of CDI; however more subtle differences between hosts can also influence the symptoms that a person may experience. Studies have demonstrated that higher antibody levels towards the toxin can provide protection against both symptomatic infection and recurrence of infection in patients who have previously suffered from an episode of CDI (6, 79, 86, 87). This has provided the basis for using immunoglobulin therapy as a treatment option (140).

It has also been shown that an alternative genotype in the IL-8 gene may be associated with predisposing patients to CDI (72).

1.9 *C. difficile* infection treatment options

Antibiotics

Metronidazole and vancomycin are two antibiotics that are commonly used for the treatment of patients with CDI. Metronidazole displays bactericidal activity towards both protozoa and many anaerobic bacteria. Metronidazole is now the preferred treatment in the majority of CDI cases as it is more cost effective and selective than vancomycin (85). The selective activity of metronidazole is attributed to a unique metabolic pathway found only in protozoal and anaerobic cells. When metronidazole diffuses into a cell with a low redox potential, ferredoxin donates electrons to the nitro group present on metronidazole. The reduction of the nitro group allows the drug to take on its active form generating compounds that interfere with nucleic acid synthesis and ultimately leading to cell death. Metronidazole is most effective when administered orally and is almost completely absorbed. It has also been suggested that metronidazole is equally effective when administered intravenously and can even achieve higher therapeutic levels (22).

Vancomycin is a potent glycopeptide antibiotic used in the treatment of severe Gram-positive infections. Vancomycin has a bactericidal effect on cells by inhibiting the synthesis of the peptidoglycan cell wall and is administered intravenously for the majority of infections. However, this can lead to side effects

and problems relating to toxicity. Vancomycin is a large hydrophilic molecule and does not transfer across the intestinal wall effectively, thus treatment of CDI requires oral administration in order to establish the high therapeutic concentrations needed in the GI tract. In recent years the use of vancomycin for the treatment of CDI has increased although concerns regarding the acquisition of vancomycin resistance by other organisms in the gut still exist (85). Vancomycin is commonly used in cases of multiple CDI recurrence, pregnancy, allergy and unresponsiveness to metronidazole (126).

Recently a new drug, Fidaxomicin was approved by the Federal Food and Drug Association (FDA) for the treatment of CDI. It belongs to the macrolide class of antibiotics and has narrow spectrum activity against Gram-positive Clostridia. It is a RNA polymerase inhibitor with bactericidal activity against *C. difficile*. Clinical trials suggest that this drug causes minimal disruption of the indigenous gut microbiota resulting in the maintenance of the normal physiological environment of the colon which suggests that the incidence of recurrence might be decreased (99).

Probiotics

Probiotics are live microorganisms which have a beneficial effect on the intestinal balance of the host when ingested (54). The role of probiotics as both a prophylactic and treatment option in CDI has been greatly debated and their effectiveness has been highly variable in many studies (92, 107, 188). Common probiotics used in humans are Lactic acid bacteria, *Bifidobacteria* and *Saccharomyces boulardii.* Hickson and colleagues performed a randomized,

placebo-controlled study examining the efficacy of preventing AAD and CDI in 135 hospitalized patients receiving antibiotics. This study demonstrated that consumption of a probiotic drink containing *Lactobacillus casei, Lactobacillus bulgaricus*, and *Streptococcus thermophilus* led to a lower incidence of AAD and CDI (65). Other studies that have investigated the effectiveness of probiotics in either the prevention or treatment of CDI have failed to provide any strong evidence for their use (124).

Immune therapies

The *C. difficile* vaccine (ACAM C.diff) is a toxoid vaccine currently in Phase II clinical trials in the UK (85). If the vaccine proves to be effective it has been proposed for administration to high-risk patient groups. In current clinical trials, the vaccine is being administered to individuals experiencing their first episode of CDI with the hope that the vaccine will prevent recurrence. The vaccine contains toxoid A and toxoid B which simulate an immune response to both toxins through the production of serum IgG antitoxin A and serum IgG antitoxin B antibodies (82).

Fecal therapy

The aim of fecal transplant therapy is to re-colonize the GI tract with a population of indigenous bacteria similar to those present in the patient prior to infection. Fecal transplant therapy is not widely available or an approved therapy. In a review by Van Nood and colleagues, it was reported to be extremely effective in both the treatment and the prevention of recurrences of CDI (172). These studies provide evidence for the important role of the gut microbiota in

colonization resistance against *C. difficile.*

1.10 Animal models of *C. difficile* infection

Several animal models such as hamsters, hares, guinea pigs, germ-free mice and piglets, rats and conventional mice have been used to study CDI. In the past, the most commonly used model to study *C. difficile* pathogenesis was the Syrian golden hamster (15, 49, 148). Although the results of in vivo CDI studies are dependent on the particular strain of *C. difficile* that is used in an experiment, many studies tend to highlight common findings (156). First, the mortality of untreated *C. difficile* diarrhea in hamsters is close to 100%. Secondly, all antimicrobial agents that have shown treatment efficacy in vivo can also be used to precipitate the disease in hamsters. Lastly, while both vancomycin and metronidazole protect infected hamsters during therapy, once therapy is discontinued the organism and cytotoxin becomes detectable in the stool and the animals succumb within 2 to 9 days (15, 49). When strict animal housing or handling methods have been employed in these experiments the mortality rates drop. This suggests that these animals are still susceptible following vancomycin treatment and that conventionally housed hamsters re-acquire the organism in the form of spores present in their environment (49).

C. difficile infection in the hamster can also represent a model of human infant asymptomatic infection. Infant hamsters are susceptible to *C. difficile* colonization at an early age but this susceptibility is lost with the establishment of the normal indigenous microbiota (138). After two weeks of age *C. difficile* infection is completely dependent on exposure to antimicrobial agents (187). The

primary site of infection in antibiotic-treated hamsters is the cecum, an organ in rodents that is proportionately much larger than in humans, but which serves functions similar to that of the human colon (138).

The hamster model has been used for three decades to study CDI therapy and mechanisms of disease. However, the hamster model has some limitations. For instance, the model represents a lethal course of disease (48) when toxigenic *C. difficile* strains are used. This does not represent the usual course and spectrum of CDI observed in adults which is usually a gradual onset with varying levels of severity. There is also a lack of both molecular tools and immunologic reagents for hamsters when compared to mice and other rodents (28).

Apart from the hamster model, guinea pigs have been used to study various aspects of *C. difficile* pathogenesis. Xia and colleagues used guinea pigs to examine whether the intestinal actions of *C. difficile* toxin A have a neurally mediated component. They found that toxin A affected the electrical behavior of the neuronal cell bodies (189). Although these researchers described the guinea pig as a model, the method used involved the removal of segments of the guinea pig small intestine which were then mounted into recording chambers to measure the effects of *C. difficile* toxin A.

In neonatal pigs, *C. difficile* has become a common cause of enteritis (19, 39). Steele and colleagues demonstrated that germ-free piglets were consistently colonized with *C. difficile* following oral gavage (152). Piglets challenged with a non-toxigenic strain of *C. difficile* did not develop signs of CDI. These

researchers also demonstrated that depending on the *C. difficile* challenge dose and the age of the piglet, it resulted in the induction of either acute and severe CDI or mild chronic disease. Infected piglets seem to mimic many of the key features of CDI in humans and as a result could be used investigate *C. difficile* strain severity (152).

1.11 Murine CDI models

Previously, conventional rodents such as mice or rats were not readily susceptible to CDI (89, 115, 137), but studies using germ-free mice and rats have shown that these animals can be colonized by the organism and develop intestinal pathology (115, 154, 186). Germ-free rodents offer a well-defined model to study the pathogenicity of *C. difficile* because it is possible to study the interaction of *C. difficile* and its toxins with the host without any influence of other bacteria. In this model, animals are infected by toxinogenic *C. difficile* strains and develop lethal cecitis (32, 115, 186). Germ-free animals also develop pseudomembranes in the colon as observed with CDI in humans. Depending on the strain of *C. difficile* used in an experiment there may be varying degrees of severity. Some studies have shown that germ-free mice may remain colonized with a low toxin producing strain *C. difficile* for as long as 30 days with chronic inflammation (115, 185) while others have demonstrated that germ-free mice succumb to the infection when high toxin producing strains of *C. difficile* are used (32, 33).

Other studies have tried to conventionalize germ-free mice by colonizing

their GI tract with microbiota from humans, hamsters or wild-type mice to study factors that prevent *C. difficile* colonization (32, 153, 185, 186). For instance when the hamster microbiota is introduced into germ-free mice, their usually large cecum is reduced to conventional size, *C. difficile* colonization is suppressed and the hypocellularity that is normally characteristic of the small bowel of germ-free mice is corrected (186).

Germ-free mice also provide a model in which to study the role of the indigenous microbiota in contributing to colonization resistance against *C. difficile*. In this thesis I have utilized this model to examine the ability of specific indigenous gut bacteria to limit *C. difficile* colonization and disease severity. However there are limitations with this model. Germ-free animal models are far more expensive and less amenable to experimental use than conventional mice and rats. Also the fact that no other organisms are present makes the model less like the situation in humans. Germ-free experiments using aged mice have been performed as a model of elderly infection with *C. difficile* in humans. One study found that aged (7-9 month old) germ-free C57BL/6 mice were susceptible to severe CDI and provided a useful model to elucidate the host immune response to acute CDI (121).

When conventional mice are infected directly with *C. difficile* they are not readily susceptible to CDI. It is only after their normal indigenous microbiota is disrupted by antibiotic use do they become susceptible to infection (28, 67, 130). A murine model of *C. difficile* infection was reported where pre-treatment of mice with multiple antibiotics rendered mice susceptible to *C. difficile* colonization and

the development of colitis (28). Results from this model closely mimicked important aspects of human infection including varied disease severity, response to antibiotic treatment directed against *C. difficile* and the development of recurrence. In this model, mice were pre-treated with a cocktail of five antibiotics (colistin, kanamycin, gentamicin, metronidazole and vancomycin) administered in drinking water for three days, followed by a two-day period of time without antibiotics (28). Animals then received a single intra-peritoneal dose of clindamycin followed by the administration of *C. difficile* via oral gavage one day later. Only animals that received both the preconditioning antibiotic cocktail and a dose of clindamycin were susceptible to disease (28, 130).

Other mouse models of CDI have since been published. Some researchers have adapted the five-antibiotic with clindamycin model to study CDI recurrence – a continuing problem with many recovering *C. difficile* infected patients (155). In the recurrence model, mice surviving the first round of pre-treatment with the five-antibiotic cocktail and clindamycin followed by *C. difficile* challenge were allowed to recover for 30 days. Afterwards, these mice were retreated with the five-antibiotic mixture and clindamycin and re-challenged with *C. difficile* (155). These types of studies make it possible to examine the host immune system in response to *C. difficile* infection. Jump and colleagues utilized a different mouse model to assess the effect of the antibiotic tigecycline on the establishment of in vivo colonization by using spores from a non-toxigenic *C. difficile* strain (75). In that study CD-1 mice were given subcutaneous tigecycline, clindamycin, or both antibiotics for 5 days. On day two of antibiotic treatment,

mice were administered *C. difficile* spores by oral gavage. Results from this model showed that mice given clindamycin had high levels of *C. difficile* in feces compared to mice given tigecycline (75).

Many patients on immunosuppressive therapy develop *C. difficile* infections in hospitals. As a result researchers have developed a mouse model to study the effect of immunosuppressive drugs on CDI development (76). In this mouse model Balb-C mice are treated for 7 days with cyclosporine then challenged with a toxigenic *C. difficile* strain. All mice were colonized for 7 days or throughout the duration of the experiment, however the level of histopathologic involvement was moderate (76).

CDI primarily occurs following the ingestion of spores from the environment. Individuals infected with *C. difficile* usually shed spores back into the environment which contributes to its perpetual spread. Lawley and colleagues established a mouse model where they examined the super shedding state as well as aspects of innate immune signaling (90). In this model C57BL/6 mice were treated with neomycin for 24 hours prior to oral gavage with *C. difficile.* They demonstrated that neomycin pre-treatment was not necessary to establish a carrier state. Clinically, treatment with antibiotics was the primary risk factor for CDI (174). To determine the effect of antibiotic treatment on the dynamics of *C. difficile* shedding, carrier mice were treated with clindamycin and by three days after antibiotic treatment, an increase in *C. difficile* spore shedding was observed. These investigators also examined the dynamics in host-to-host transmission. Naive (uninfected) mice were housed with either carrier mice or

super shedder mice after which the naïve mice were removed and treated with clindamycin for four days. Results demonstrated that super shedder mice promoted efficient host-to-host transmission while the carrier state did not (90).

While some studies require multiple antibiotics for mice to become susceptible to CDI, Buffie and colleagues recently demonstrated that mice treated with clindamycin only and then challenged with *C. difficile* VPI 10463 spores developed rapid onset of clinical CDI signs, had high *C. difficile* colonization and resulted in 50% mortality (23).

1.12 Interactions between members of the GI microbiota and *C. difficile*

Many in vitro and in vivo studies have investigated interactions between *C. difficile* and other bacterial species. Fitzpatrick and colleagues used the five-antibiotic and clindamycin mouse model established by Chen and colleagues (28) to examine the usefulness of a spore forming strain of *Bacillus coagulans* to improve clinical signs of CDI in mice. In this study mice were inoculated daily with *B. coagulans,* before and during the antibiotic cocktail regimen prior to *C. difficile* challenge. These researchers reported prolonged survival and improvement in CDI signs such as diarrhea and histopathology in the colon (51).

Over the years a number of in vitro studies have identified organisms that are capable of inhibiting *C. difficile* growth, toxin production or the ability of *C. difficile* to adhere to colonic epithelial cells. These studies have mainly focused on a number of different *Lactobacillus* species, yeast or *E. coli* species. For instance, studies have shown that *Lactobacillus delbrueckii* can inhibit the

cytotoxic effects and adhesion of *C. difficile* to Caco-2 cells (a human colonic epithelial cell line) (11). Additionally, another study investigated the antagonistic activity of 50 intestinal *Lactobacillus* species against 23 pathogenic *C. difficile* strains and found 22 strains that were antagonistic to all or some *C. difficile* strains tested. These investigators attributed this result to increased hydrogen peroxide and lactic acid production by the *Lactobacillus* strains (112).

Bifidobacteria are said to have beneficial effects in humans. Hopkins and colleagues investigated whether four different Bifidobacteria strains in combination could inhibit *C. difficile* growth in a chemostat model and found no antagonistic effect against *C. difficile* (67). Another study utilized the cyclosporine-induced *C. difficile* infection model in mice to examine the effects of *Lactobacillus acidophilus* in limiting CDI severity. This study demonstrated that *L. acidophilus* significantly reduced the level of inflammation and prolonged survival but did not significantly inhibit *C. difficile* growth (76).

Many germ-free mouse models have previously been used to investigate the antagonistic effect of individual or groups of bacteria on colonization resistance against *C. difficile*. Itoh and colleagues inoculated germ-free Balb/C mice with either whole or treated feces in which only Clostridia survived prior to *C. difficile* challenge and noted a significant decrease in *C. difficile* growth (69). Other studies have di-associated germ-free mice with non-pathogenic *E. coli* and *C. difficile* and observed a decrease in *C. difficile* growth (185). The addition of hamster cecal contents to these di-associated mice further eliminated *C. difficile* from the mouse gut (185).

Similarly, other studies have colonized germ-free mice with bacteria from human feces, rats and hamster cecal contents and observed resistance to *C. difficile* colonization (129, 153). Other di-association studies have evaluated neonatal strains of *E. coli* and *Bifidobacterium bifidum* from humans to inhibit *C. difficile* growth and found that these strains significantly reduced levels of *C. difficile* cytotoxin but not *C. difficile* growth (31). No protection or *C. difficile* growth and cytotoxin inhibition were observed when germ-free mice were di-associated with *Streptococcus faecalis, Eubacterium* species or *Bacteroides* species (31). Su and colleagues di-associated germ-free mice with a butyrate producer, *Clostridium butyricum* and found no inhibitory effect on levels of *C. difficile* growth. However other clinical factors were not evaluated in this model (153).

1.13 Summary

The indigenous gut microbiota plays an important role in protecting the host against infection with *C. difficile*. Administration of antibiotics disrupts the gut microbiota allowing *C. difficile* to colonize and cause disease (1, 28, 178). Work performed in this thesis was aimed at examining the effect of antibiotic administration in contributing to *C. difficile* susceptibility and the role of specific members of the indigenous GI microbiota in contributing to colonization resistance against *C. difficile*. The goals of this thesis were: (i) to study the effect of the administration of different antibiotic regimens including (a) the five-antibiotic cocktail with clindamycin (28), and (b) cefoperazone on the indigenous

GI microbiota; (ii) to identify changes within the GI microbial communities after antibiotic treatment that predispose wild-type mice to *C. difficile* infection; (iii) to isolate specific members of the murine indigenous GI microbiota that were possibly associated with *C. difficile* colonization resistance or susceptibility after antibiotic treatment (Lachnospiraceae and *E. coli*); (iv) to determine the direct effect of Lachnospiraceae and *E. coli* in conferring colonization resistance or susceptibility to *C. difficile* and disease development in germ-free mice.

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Chapter 2

The interplay between microbiome dynamics and pathogen dynamics in a murine model of *Clostridium difficile* infection

2.1 Introduction

The GI tract of mammals is inhabited by a complex microbial community that plays a crucial role in maintaining gut homeostasis (13, 34). The GI tract microbiota performs a number of beneficial metabolic functions (69) and also aids in the normal development of the mucosal epithelium and maturation of the mucosal immune system (20-22, 58). The indigenous microbiota protects the host from colonization by potentially pathogenic organisms, a function that is termed colonization resistance (61). It has been hypothesized that following the successful colonization by a pathogen, the ultimate pathology depends on the interplay between the host, pathogen and the indigenous microbiota (57). Thus, the resident microbiota can potentially modulate the outcomes of any pathogen/host interaction.

C. difficile is a Gram-positive, toxin-producing bacterium first described in 1935 as a commensal organism in the fecal microbiota of healthy newborn infants (17). It is currently the most common cause of health care-associated diarrhea and colitis and is responsible for significant morbidity and increased

health-care cost (12). *Clostridium difficile* infection (CDI) is associated with the use of broad-spectrum antibiotic therapy, increasing patient age, and hospitalization (13). In recent years, the appearance of an epidemic strain (BI/NAP1/027) with potentially increased virulence has prompted renewed interest in the pathogenesis and epidemiology of this bacterium (37, 47). Additionally, it appears that the overall incidence of *C. difficile* infection has been increasing (27).

As *C. difficile* is not normally a significant component of the GI tract microbiota of adult humans, it is proposed that the indigenous gut microbiota is important in mediating colonization resistance against this pathogenic bacterium (44, 64). According to this hypothesis, disruption of the indigenous gut microbiota by the administration of antibiotics results in a decrease in colonization resistance. Furthermore, recurrent CDI appears to occur in the setting where the indigenous microbiota is sufficiently disturbed so that colonization resistance cannot be restored even after cessation of the inciting antibiotics and completion of specific treatment directed against *C. difficile* (35). We have demonstrated that patients with recurrent *C. difficile* infection have decreased diversity of the indigenous gut microbiota which may reflect a corresponding defect in colonization resistance (7).

A number of animal models have been developed to facilitate the study of *C. difficile* pathogenesis**.** The hamster model has been used extensively and it was in this host that Koch's postulates were fulfilled for *C. difficile* as the causative agent of antibiotic-associated colitis (3). In this model colitis develops

after exposure to clindamycin and subsequent *C. difficile* challenge. However, the resulting disease is severe and lethal within three days after initial infection. This does not represent the usual course and spectrum of CDI in humans, which can range from asymptomatic to severe colitis (27). Furthermore, the limited availability of reagents to study host responses in hamsters has dampened the usefulness of this model. Germ-free mice challenged with *C. difficile* also develop intestinal disease but this model precludes an examination of the role of indigenous microbiota in mediating colonization resistance (41, 45, 65). Thus, the available animal models have limited studies of *C. difficile* pathogenesis.

It has been reported that treatment of mice with various antibiotics can render the animals susceptible to *C. difficile* colonization (30). In some cases this can lead to the development of colitis (8, 23). In this present study, I utilized antibiotic-treated mice to demonstrate that altering the community structure of the indigenous gut microbiota is associated with both the loss of colonization resistance against *C. difficile* and differences in the severity of disease. Our results indicate that a better understanding of the role of the indigenous microbiota in CDI could lead to novel and improved mechanisms for prevention and treatment.

2.2 Methods

2.2.1 Ethics statement

All animal protocols used during the conduction of these experiments were reviewed and approved by the University Committee on Use and Care of Animals

of the University of Michigan, Ann Arbor (protocol number 10212). The protocol was reviewed following guidelines for the care and use of laboratory animals set by the Office of Laboratory Animal Welfare, United States Department of Health and Human Services.

2.2.2 Animals and housing

The infection studies were performed with wild-type C57BL/6 mice from a breeding colony established using animals purchased from Jackson Laboratories. Mice were housed with autoclaved food, bedding and water. Cage changes were performed in a laminar flow hood. Animals experienced a cycle of 12 hr of light and 12 hr of darkness.

2.2.3 *C. difficile* growth conditions

The reference strain of *C. difficile*, strain VP1 10463 (ATCC 43255) was obtained and cultured on brain heart infusion agar containing 5% cysteine. An anaerobic environment was maintained at all times using an anaerobic chamber (Coy Industries). An incubation temperature of 37°C was used for growth. *C. difficile* suspensions for animal challenge were prepared by inoculating a single colony of *C. difficile* from a culture plate into brain heart infusion (BHI) broth, containing 5% cysteine, and allowing for overnight growth. Cells were harvested by centrifugation (5,000 rpm for 15 min) and washed three times with pre-reduced PBS, pH 7.4. Bacterial enumeration was done to ensure that the correct dose of *C. difficile* vegetative cells was reached at the time of challenge.

2.2.4 Antibiotic administration and infection with *C. difficile*

Mice were divided into treatment groups consisting of 5 to 8 animals that were six to eight weeks old. An antibiotic mixture of kanamycin (0.4 mg/mL), gentamicin (0.035 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL), and vancomycin (0.045 mg/mL) was prepared in sterile drinking water (8). Antibiotics were purchased from Sigma-Aldrich (cat# K1377, G1914, C4461, M1547, V2002, C5269, and C4292). The antibiotic cocktail was administered for 3 days then the animals were switched to regular autoclaved drinking water for 2 days. All mice in each experiment were housed under the same conditions and were fed standard autoclaved chow. A single dose of clindamycin (10 mg/kg) was administered intraperitoneally one day before *C. difficile* challenge. Cefoperazone (0.5mg/ml) was prepared in sterile drinking water and administered for 10 days. The cefoperazone drinking water was replaced with a fresh supply every 48 hours for the duration of cefoperazone administration. Animals were then switched to regular autoclaved drinking water for 2 days. A single dose of clindamycin (10 mg/kg) was administered intraperitoneally one day before *C. difficile* challenge. Some animals were allowed to recover for 6 weeks after cefoperazone treatment then a single dose of clindamycin was administered prior to *C. difficile* infection. Animals were infected by oral gavage with 1×10^5 CFU of *C. difficile* strain VPI 10463. Animals were monitored daily for signs of disease such as diarrhea, hunched posture and weight loss.

2.2.5 Necropsy and histological procedures

Mice were euthanized by CO_2 asphyxiation. The tip of the cecum of each mouse was removed, halved, and rinsed in phosphate-buffered saline to remove luminal contents. Approximately 5 mm of proximal colon and terminal ileal tissue and luminal contents were collected from each animal. All samples were snap frozen in liquid nitrogen and stored at -80°C. The remaining cecum, colon and ileal tissue were placed intact into histology tissue cassettes and stored in 10% buffered formalin for 24 hours then transferred to 70% ethyl alcohol (70). Tissue cassettes were then processed, paraffin embedded and then sectioned. Haematoxylin and eosin stained slides were prepared for histologic examination (McClinchey Histology Lab Inc.).

2.2.6 Histopathologic examination

Histological slides were coded, randomized, and scored in a blinded manner by a board-certified veterinary pathologist. A scoring system was adapted from a previously published method (8, 26). Edema, cellular infiltration and epithelial damage in each tissue (colon, cecum, ileum) were scored from 0-4 according to the following defined criteria: Edema scores – 0: no edema; 1: mild edema with minimal (<2x) multifocal submucosal expansion; 2: moderate edema with moderate (2-3x) multifocal sub-mucosal expansion; 3: severe edema with severe (>3x) multifocal sub-mucosal expansion; and 4: same as score 3 with diffuse sub-mucosal expansion. Cellular infiltration scores were graded as follows: 0: no inflammation; 1: minimal multifocal neutrophilic inflammation; 2: moderate

multifocal neutrophilic inflammation (greater submucosal involvement); 3: severe multifocal to coalescing neutrophilic inflammation (greater submucosal +/- mural involvement; and 4: same as score 3 with abscesses or extensive mural involvement. Epithelial damage was scored as follows: 0: no epithelial changes; 1: minimal multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); 2: moderate multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); 3: severe multifocal epithelial damage (same as above) +/- pseudomembrane (intraluminal neutrophils, sloughed epithelium in a fibrinous matrix); and 4: same as score 3 with significant pseudomembrane or epithelial ulceration (focal complete loss of epithelium).

2.2.7 DNA extraction

Total DNA from fecal and tissue samples was extracted using the MagNA Pure DNA isolation protocol (Roche, cat# 03730964001). Samples were placed in a Ultra Clean fecal bead tube (MoBio) to which 500 µl of MagNa Pure bacterial lysis buffer (Roche) was added. Samples were bead beaten for 1 min with a mini bead beater (Biospec), digested with proteinase K, incubated at 65°C, bead beaten for 1 min and then was heat inactivated at 95°C. Samples were placed in the MagNa Pure (Roche) and the MagNa Pure nucleic isolation kit protocol for bacterial DNA was followed as recommended by the manufacturer.

2.2.8 Construction of 16S ribosomal rRNA-encoding gene clone libraries

The community structure of infected and uninfected mice was analyzed by the construction of 16S rRNA gene clone libraries (42, 43). PCR targeting bacterial 16S rRNA genes using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (55) was performed on each extracted DNA sample. PCR was performed using Illustra Pure Taq Ready-To-Go PCR beads (GE Healthcare, cat# 27955901). Reaction mixtures were set up with 100 ng of template DNA, 20 pmol of each primer, and water to a total volume of 25 µl. The reaction mixtures were subjected to amplification in a DNA thermal cycler (Eppendorf Mastercycler gradient) with the following cycling conditions: initial denaturation at 94°C for 2 min followed by 20 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 45 sec, and extension at 72°C for 1.5 min. A final extension at 72°C for 10 min was performed. Control amplifications with sterile water were included in each amplification reaction and never gave visible amplicons. Amplicons were purified using a kit Illustra MicroSpin Column (GE Healthcare, cat# 27514001) according to the recommendations of the manufacturer. The purified PCR products were ligated into a plasmid vector (pCR 2.1; Invitrogen).

2.2.9 DNA sequencing and analysis

Plasmid purification and DNA sequence determination of 96 randomly selected clones from each library were performed by the DNA Sequencing Core facilities at the University of Michigan. Each clone was sequenced with a single primer

(8F) that typically yielded 750 bases of readable sequence. Sequences with numerous ambiguous base calls or with fewer than 350 total bases were excluded from further analysis. Sequences were analyzed for the formation of chimeras using the Chimera Check program from the Ribosomal Database Project (9). Potential chimeric sequences were excluded from additional analysis. Sequences were also aligned to a phylogenetically diverse collection of 16S rRNA gene sequences using the RDP Classifier (9). Partial 16S rRNA sequences were initially analyzed using mother (54) to calculate pair wise Morisita-Horn distances which was exported to Mega4 (28) software package and then UPGMA analysis was used to create dendrograms.

2.2.10 Quantitative PCR

Quantitative PCRs were used to assay the quantity of rRNA operons in the DNA samples relative to a single-copy host gene (mouse tumor necrosis factor alpha [TNFa]) as detailed in Antonopoulos et al (1). Assays used the LightCycler 480 Probes Master reaction mixture (Roche, cat# 04707494001) at 1x concentration and appropriate primer-probe sets to increase the specificity of the signals detected from the sample DNA (100 ng). For detection of the bacterial signal, 100 nmol of each of the forward and reverse primers and the flourescent probe were included in the reaction mixtures. Sequences for the forward primer (5'-TCCTACGGGAGGCAGCAGT-3'), the reverse primer (5'-GGACTACCAGGGTATCTAATCCTGTT-3'), and the probe (5'-[6-carboxyfluorescein]-CGTATTACCGCGGCTGCTGGCAC- [6-carboxytetramethylrhodamine]-3') were based on the work of Nadkarni et al (38).

Signals were detected with a LightCycler 480 instrument (Roche). Detection of the host signal used 200 nmol of the forward (TNFa_mu_se; 5'-GGCTTTCCGAATTCACTGGAG-3') and reverse (TNFa_mu_as; 5'-CCCCGGCCTTCCAAATAAA-3') primers and 100 nmol of the probe (TNFa_mu_probe; 5'-Cy5-ATGTCCATTCCTGAGTTCTGCAAAGGGA-Iowa Black RQ-3') adapted from Nitsche et. al. (40). Relative bacterial loads were compared via the C_T method by normalizing the 16S rRNA gene signal to the host signal (56).

2.2.11 Monitoring *C. difficile* colonization

The colonization status of *C. difficile* infected animals was monitored using a *C. difficile* toxin multiplex qPCR assay of fecal pellets collected at various time points pre and post challenge from mice in each group. For the C. *difficile* Toxin Multiplex qPCR (LightCycler 480) 8 and 10 pmol/µl for *tcdA* and *tcdB* primers, respectively, were prepared from 200 pmol/µl original stocks. Primer and probe sets are as follows: tcdA_F: 5'-GGTAATAATTCAAAAGCGGCT, tcd_R: 5'-AGCATCCGTATTAGCAGGTG, tcdA_probe_FAM: 5'-6FAM-AGCCTAATACAGCTATGGGTGCGAA-BHQ1, tcdB_F: 5'-GAAAGTCCAAGTTTACGCTCAAT, tcdB_R: 5'-GCTGCACCTAAACTTACACCA, tcdB_probe_Hex: 5'-Hex-ACAGATGCAGCCAAAGTTGTTGAATT-BHQ1 (James Versalovic, personal communication, manuscript in preparation). For each 20 µl reaction, 4 µl template, 10 pmol *tcdA* primers, 12.5 pmol *tcdB* primer, 1.6 pmol *tcdA* probe and 2 pmol *tcdB* probe were used. The following cycling conditions were used for the qPCR run: Activation - 95°C for 15min, 95°C for 15sec, Cycling

(X45) - 60°C for 20sec, 72°C for 10sec, Hold - 37°C for 30sec. Values were normalized to mouse TNF alpha gene content and the mean fold change of *tcdA* *tcdB* gene content were calculated using the $2^{-\Delta Ct}$ method (56).

2.2.12 Measurement of RegIIIγ expression

Total nucleic acid was isolated from mouse ileal tissue using the MagNA Pure Compact Nucleic Acid isolation kit (Roche). Samples were deoxyribonuclease (Roche) treated and then reverse transcribed using the RT^2 First Strand kit (SA Biosciences) to yield cDNA for real-time PCR analysis. SYBR Green-based real-time PCR was performed on a LightCycler 480 instrument (Roche) using RegIIIγ-specific primers (forward primer: 5'-TTCCTGTCCTCCATGATCAAAA; reverse primer: 5'-CATCCACCTCTGTTGGGTTCA) from Cash et al. (6). Control experiments were performed to establish that amplicons were derived from cDNA and not from genomic DNA or primer-dimers. Relative levels of RegIIIγ were compared via the C_T method by normalizing to mouse GAPDH (Roche) (56).

2.2.13 *C. difficile* cytotoxin assay

The assay was performed in 96-well flat-bottom microtiter plates (Corning) and was adapted from Corthier et al (10). Green African monkey kidney epithelial cells (Vero) (provided by M. Imperiale, University of Michigan) were grown to confluency in DMEM (GIBCO Laboratories, cat# 11965) containing 10% heat inactivated fetal bovine serum (GIBCO Laboratories, cat# 16140) and 1% penicillin streptomycin solution (GIBCO Laboratories, cat# 15140). The cells were trypsinized using 0.25% trypsin (GIBCO Laboratories, cat# 25200) and

washed with 1 volume of DMEM medium. Cells were diluted in DMEM medium and approximately $1x10^5$ cells were distributed per well and incubated at 37°C with 5% CO_2 for 18-24 hours. Samples of luminal contents or intestinal tissue were weighed and 500 µl of 1x PBS was added. Intestinal tissue was homogenized using a Medimachine (Becton Dickenson). Samples were vortexed then spun at 13,000 rpm for 5 minutes and then the supernatant was filtered through a 0.2 µm membrane. Each sample was titrated in two-fold dilutions within the wells to a maximum dilution of 2^{-12} and each well had a corresponding control to which both antitoxin (TechLabs, cat# T5000) and sample were added. After an overnight incubation at 37°C, plates were fixed with 10% buffered formalin for 2 hours then stained with geimsa (50 µl per well) for 15 minutes followed by a wash with 1x PBS. Wells with approximately 100% round cells were easily recognized under 200x magnification. The cytotoxic titer was defined as the reciprocal of the highest dilution that rounds 100% of Vero cells per gram of sample. Vero cells with purified *C. difficile* toxin and antitoxin (TechLabs, cat# T5000) were used as controls.

2.2.14 Statistical analysis

Statistical analyses were performed using Prism 5 for Mac OS X GraphPad Software. *t* tests were used for treatment group comparisons, except for categorical histology scores, where the nonparametric Krustal Wallis test was used. Statistical significance was set at a *P* value of <0.05.

2.3 Results

2.3.1 Overcoming colonization resistance to *C. difficile*

To compare the ability of different antibiotic regimens to overcome colonization resistance against *C. difficile,* six to eight week old C57BL/6 mice were treated with either an antibiotic cocktail (kanamycin, gentamicin, colistin, metronidazole, and vancomycin), clindamycin or the combination of both prior to challenge with 1 x 10^5 CFU of *C. difficile* (VPI 10463) via oral gavage (Figure 2.1A, 2.1B). Control animals were challenged with *C. difficile* in the absence of any antibiotic pretreatment. Colonization was monitored by culture and *C. difficile*-specific PCR was performed on DNA isolated from stool pellets or from gut tissue harvested at necropsy, which occurred 2 to 4 days post-challenge. Animals were monitored daily for the signs of clinical CDI including diarrhea, weight loss and hunched posture. *C. difficile* was never recovered from animals that were challenged without antibiotic pretreatment. Animals that received the antibiotic cocktail without clindamycin prior to *C. difficile* challenge were also resistant to colonization. Of the 9 animals that received only a single dose of clindamycin prior to *C. difficile* challenge, 4 of them shed low amounts of the organism in their feces for the first 2 days following challenge, but the organism was no longer detectable in stool or tissue when the animals were euthanized four days following challenge. All 12 animals that received both the antibiotic cocktail and clindamycin prior to *C. difficile* challenge shed the organism in their feces throughout the experiment and *C. difficile* was found in tissue at the time of necropsy. These results indicate that the combination of the antibiotic cocktail

and clindamycin is required to completely overcome colonization resistance against *C. difficile*.

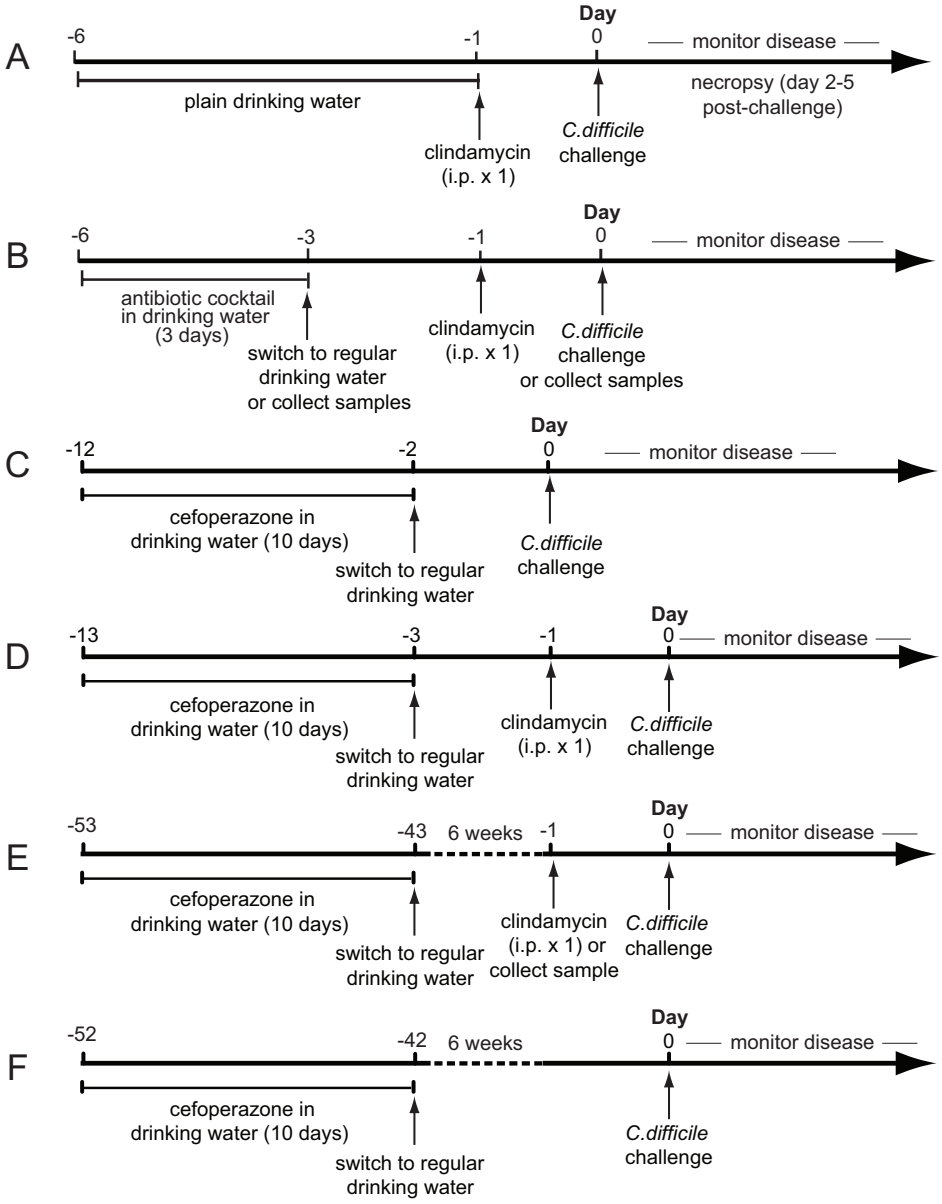

Figure 2.1 Experimental designs for alteration of microbiota and *C. difficile* infection. Wild-type mice were treated with: (A) a single intraperitoneal dose of clindamycin and challenged with 10^5 CFU of *C. difficile (*VPI 10463) (B) a 5 antibiotic cocktail in drinking water for 3 days; a 5 antibiotic cocktail in drinking water for 3 days followed by a 2-day period without the drug and a single dose of clindamycin; or a 5 antibiotic cocktail in drinking water for 3 days followed by a 2-

day period without the drug and a single dose of clindamycin followed by challenge with 10^5 CFU of *C. difficile* one day later (C) 10 days cefoperazone treatment followed by 2 days off drug with or without *C. difficile* challenge (D) 10 days cefoperazone treatment followed by 2 days off drug, a single dose of clindamycin followed by one day recovery with or without *C. difficile* challenge (E) 10 days cefoperazone treatment followed by 6 weeks off drug, a single dose of clindamycin followed by one day recovery prior to *C. difficile* challenge (F) 10 days cefoperazone treatment followed by 6 weeks off drug then *C. difficile* challenge.

2.3.2 Clinical disease in *C. difficile* infected mice

We monitored the development of disease in mice that received both the antibiotic cocktail and clindamycin prior to *C. difficile* challenge. Five of 12 animals that received both the antibiotic cocktail and clindamycin did not show overt clinical signs of CDI despite remaining colonized with *C. difficile*. The remaining seven mice exhibited signs of disease including diarrhea, hunched posture and significant (>20% from baseline) weight loss (Figure 2.2). One animal was found dead at 2 days post challenge while six animals were moribund and euthanized 2 to 4 days post challenge.

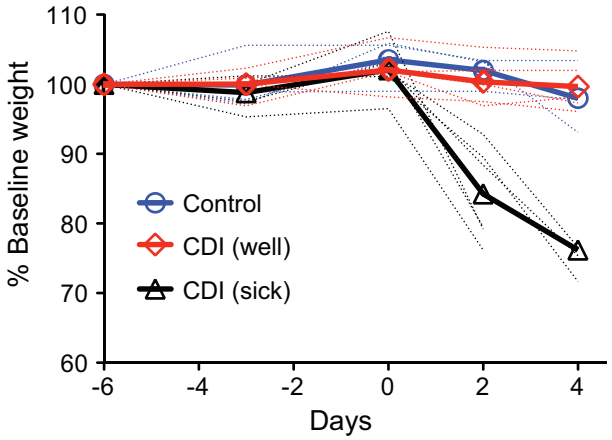

Figure 2.2 Weight loss in *C. difficile* infected mice. Weight loss curves for untreated (control) animals (n=5), and animals treated with the 5 antibiotic

cocktail for 3 days (days -6 to -3) then given a single dose of clindamycin after 2 days off antibiotics followed by challenged with *C. difficile* one day later (n=11). 6 of 11 mice lost >20% body weight (sick) while one animal died 1-2 days post challenge (not shown). The remaining 5 animals did not lose significant body weight (well) when compared to control animals. Weight loss percentage is based on the starting weight at day -6 with thick lines showing the average for animals in each clinical group and dotted lines showing the data for each individual. CDI- *C. difficile* infection

A central feature of the pathogenicity of *C. difficile* is the production of two large glucosyltransferase toxins encoded by *tcdA* and *tcdB* that modify and inactivate the small GTPases Rho, Rac, and Cdc42 (63). We measured the activity levels of *C. difficile* toxin in the gut using a tissue culture cytotoxin assay. High levels of *C. difficile* cytotoxin were detected in samples obtained from animals with severe clinical disease (Figure 2.3A). Additionally, quantitative PCR analysis indicates that animals with severe clinical disease had significantly higher numbers of *C. difficile* in their gut at the time of necropsy compared to those animals that were clinically well (Figure 2.3B). These results suggest that the animals that did not develop severe CDI in the multi-antibiotic treatment protocol had the ability to control the population size of colonizing *C. difficile* and to limit the production of toxin.

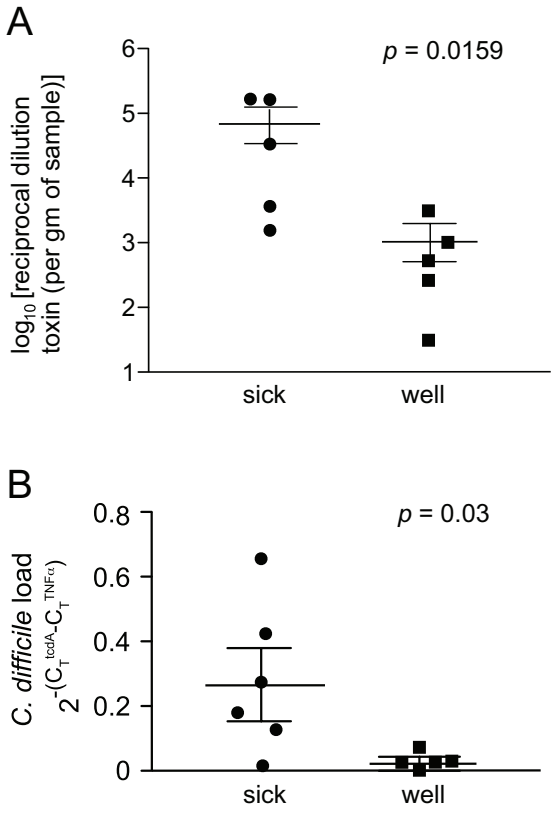

Figure 2.3 Increased cytotoxin activity and *C. difficile* load are associated with increased CDI severity. (A) Vero cell tissue culture was used to determine the log10 reciprocal cytotoxin dilution per gram of sample. Each point represents individual animals that were either sick (n=5) or well (n=5) after treatment with antibiotics/clindamycin and exposure to *C. difficile.* Sick animals had increased levels of cytotoxin production compared to animals with less clinical disease. (B) Quantitative PCR was performed on cecal DNA from animals treated with antibiotics/clindamycin and exposed to *C. difficile.* Values represent the relative abundance of the *tcdA* gene normalized to the single copy mouse tumor necrosis factor α (TNFα) gene. Sick animals (n=6) had increased levels *C. difficile* compared to well animals (n=5) with less clinical disease.

Histopathologic changes were seen in both the cecal and colonic tissue of all animals that received the antibiotic cocktail, clindamycin and *C. difficile.* Pathologic changes consisted of neutrophilic inflammation in the mucosa and submucosa with varying degrees of submucosal edema (Figure 2.4). Of note, animals that were infected and clinically ill had significantly more severe colonic

inflammation and, particularly, submucosal edema than animals that were infected but remained clinically well (Figure 2.4E, 2.4F). In the most severely affected animals, there were areas of erosion and in rare cases, ulceration. Occasionally, severely affected animals had luminal exudates comprised of degenerate neutrophils, hemorrhage, and sloughed epithelium embedded in a fibrinous matrix suggestive of pseudomembranes (Figure 2.4D). Untreated animals or those that received antibiotics without *C. difficile* challenge had no histological alterations.

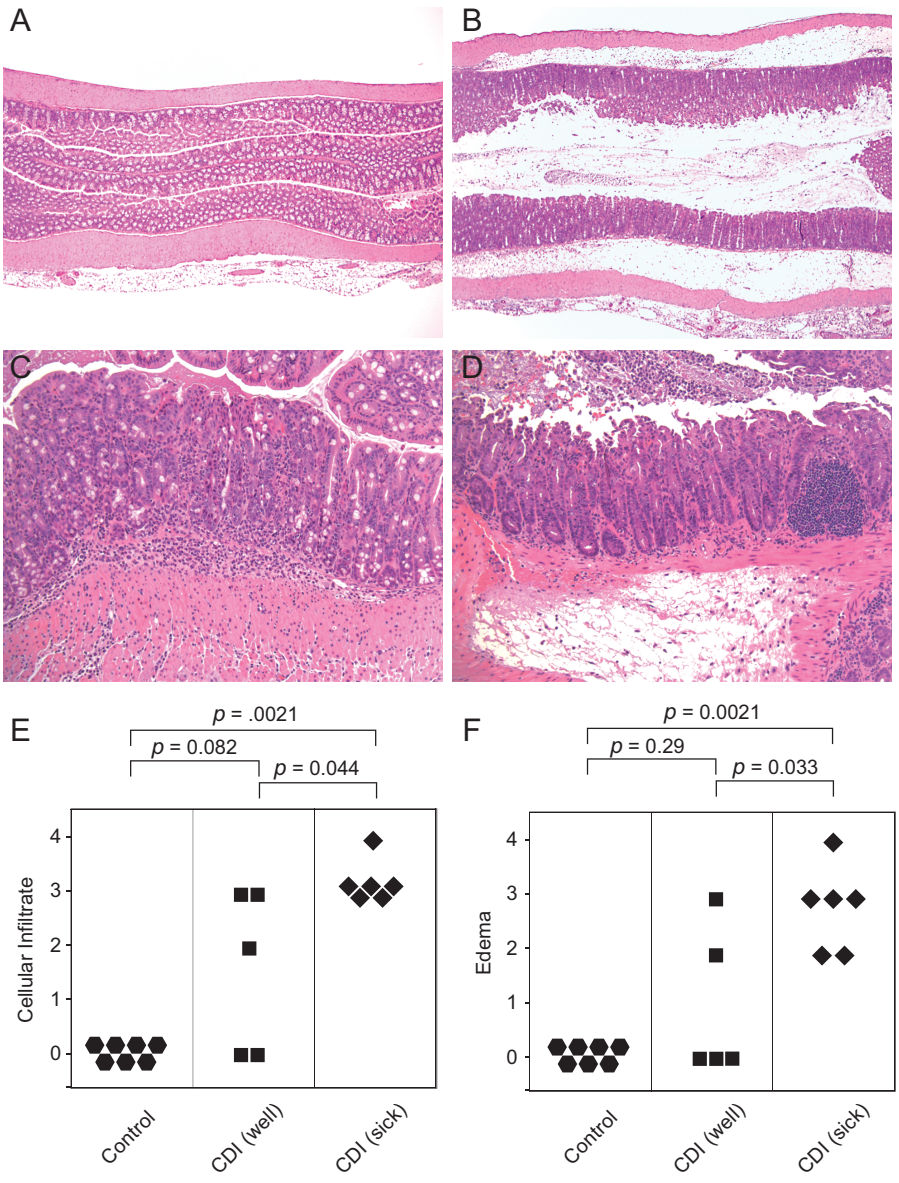

Figure 2.4 Animals with clinically severe CDI have increased colonic histopathology. Proximal colon of an untreated (control) mouse. HE. Original magnification x40. (B) Colon of a sick antibiotic treated mouse infected with *C. difficile* showing severe submucosal edema. HE. Original magnification x40. (C)

Increased magnification of colon from a well antibiotic treated mouse infected with *C. difficile* showing moderate neutrophilic mucosal and submucosal inflammation but lacking significant submucosal edema. HE. Original magnification x200. (D) Sick *C. difficile* infected mouse showing marked submucosal edema in addition to neutrophilic inflammation. There is also a pseudomembrane on the luminal surface consisting of degenerate neutrophils, sloughed epithelial cells, and hemorrhage within a fibrinous matrix. HE. Original magnification x200. (E) Categorical cellular infiltration scores of untreated (control) animals, *C. difficile* infected well animals, and *C. difficile* infected sick animals. (F) Categorical edema scores of untreated (control) animals, *C. difficile* infected well animals, and *C. difficile* infected sick animals. Comparisons between groups were performed using the nonparametric Krustal Wallis test.

2.3.3 *C. difficile* colonization resistance is independent of RegIIIγ expression levels

RegIIIγ is a secreted C-type lectin with potent bactericidal activity against Gram-positive bacteria (6). It binds the surface-exposed peptidoglycan layer of Gram-positive organism with high affinity in a calcium-independent manner (33). Expression of RegIIIγ appears to be driven by the indigenous gut microbiota through host sensing of microbial-associated molecular patterns, primarily from Gram-negative organisms. RegIIIγ has been shown to be important in mediating colonization resistance to vancomycin resistant *Enterococcus* (VRE) (5). Treatment with antibiotics is associated with decreased expression of RegIIIγ and susceptibility to colonization with VRE. We used quantitative PCR to examine the role of RegIIIγ in mediating colonization resistance in the murine model of CDI. RegIIIγ expression was decreased 50 fold in animals treated with the antibiotic cocktail compared to the untreated group (Figure 2.5). Clindamycin treatment alone did not alter RegIIIγ expression, but when preceded by treatment with the antibiotic cocktail, clindamycin treatment was associated with significantly

decreased expression levels. Interestingly, animals that developed severe clinical disease after treatment with the antibiotic cocktail and clindamycin prior to challenge with *C. difficile* had a significant (6-fold) increase in RegIIIγ compared to the untreated controls while this increase was not seen in animals that were clinically well.

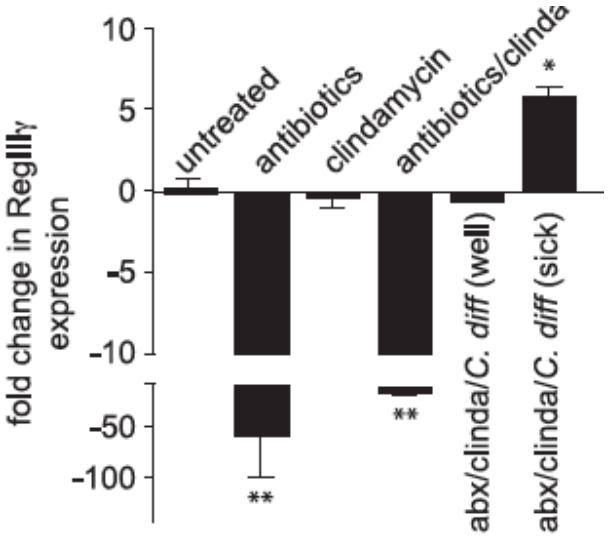

Figure 2.5 *C. difficile* colonization is not correlated with RegIIIγ expression

RegIIIγ mRNA expression levels in ileal tissue from animals in the untreated (control) (n=5), antibiotics (n=3), clindamycin (n=5), antibiotics/clindamycin (n=4), antibiotics/clindamycin/*C. difficile* (well) (n=5), and antibiotics/ clindamycin/ *C. difficile* (sick) (n=6) treatment groups. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and the mean fold expression of RegIIIγ relative to the untreated group ± standard deviation is plotted. The antibiotic cocktail as well as antibiotics/clindamycin are associated with decreased RegIIIγ expression but not clindamycin only. Sick animals pretreated with antibiotics/clindamycin and exposed to *C. difficile* had a significant increase in RegIIIγ expression compared to untreated animals.

2.3.4 Shifts in microbial ecology associated with antibiotic treatment and *C. difficile* infection

We have previously shown that antibiotic administration can decrease the overall mass of bacteria within the gut (1). The administration of the five antibiotic cocktail significantly decreased the overall bacterial population by 20-fold when measured immediately after the treatment period (Figure 2.6A). However, a single administration of clindamycin did not change the total microbial population size when measured 24 hours after the dose was given (Figure 2.6A). Furthermore, following administration of both the antibiotic cocktail and clindamycin, at the time corresponding to *C. difficile* challenge, the overall bacterial population size was similar to untreated animals (Figure 2.6A). Therefore, the loss of colonization resistance against *C. difficile* following antibiotic administration was not directly related to changes in the overall density of the gut microbiota.

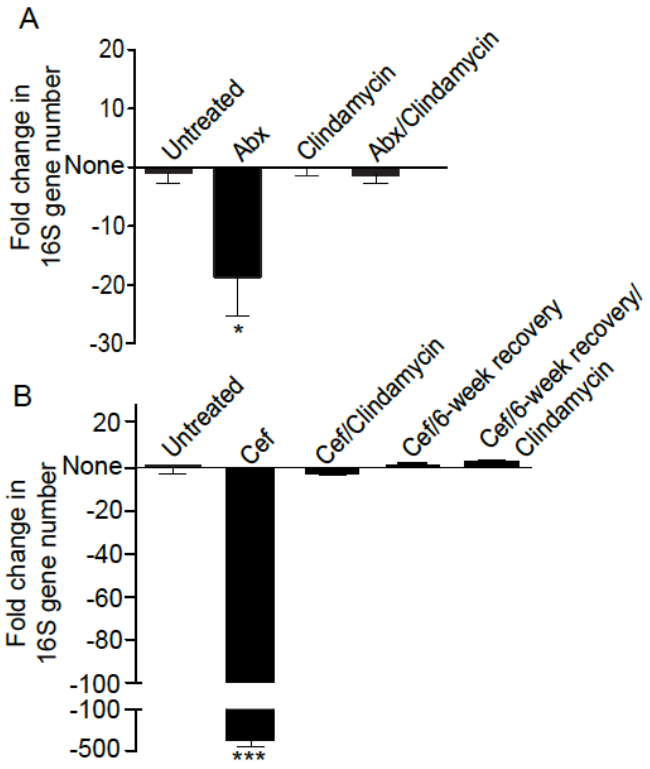

Figure 2.6 *C. difficile* colonization is not correlated with overall levels of the indigenous microbiota. (A and B) Total 16S rRNA-encoding gene quantitative PCR was performed on cecal DNA from animals treated as (A) untreated (n=6), 5-antibiotics (n=7), clindamycin (n=5), and 5-antibiotics/clindamycin (n=4). Bacterial load was decreased after treatment with the antibiotic cocktail but remained unchanged after treatment with clindamycin alone or treatment with both the antibiotic cocktail and clindamycin when compared to controls. (B) untreated (n=3), cefoperazone (n=4), cefoperazone and clindamycin (n=4), cefoperazone with 6 weeks recovery (n=4) and cefoperazone with 6 weeks recovery followed by a dose of clindamycin (n=4). Bacterial load was severely decreased following cefoperazone treatment with a 2-day recovery period but recovered after clindamycin was administered a day later and remained unchanged after a 6-week recovery period. Values were normalized to host DNA content as described in the methods and the mean fold change of 16S rRNA gene expression relative to the controls ± standard deviation is plotted. Group comparisons were performed using Krustal Wallis. (* P= <0.05, *** P= <0.0001) cef – cefoperazone, Abx- 5-antibiotics.

Since the loss of colonization resistance against *C. difficile* was not associated with an overall decrease in bacterial density, we analyzed 16S rRNA gene sequences retrieved from gut tissue at the time of necropsy to examine the specific changes in the community structure of the gut microbiota that resulted from antibiotic treatment and *C. difficile* infection. In control mice that never received antibiotics, the gut microbial community was dominated by members of the phylum Firmicutes with lower numbers of Bacteroidetes. The administration of either the antibiotic cocktail, clindamycin or both resulted in a significant change in the structure of the gut microbial community (Figure 2.7A, 2.7B). The administration of the antibiotic cocktail resulted in a shift in the community structure to one dominated by bacteria from the family Lactobacillaceae. Clindamycin treatment alone shifted the community composition to a dominance of Proteobacteria belonging to the family Enterobacteriaceae (Figure 2.7A). When clindamycin was administered following treatment with the antibiotic cocktail, there was again a predominance of Proteobacteria in the gut community (Figure 2.7B).

Interestingly, subsequent changes in the gut microbial community structure following *C. difficile* challenge followed two distinct courses. Animals that developed severe clinical disease harbored a gut microbial community at the time of necropsy that remained dominated by Proteobacteria (Figure 2.7B). Animals that remained clinically well and had significantly less severe histologic colitis at the time of necropsy possessed a gut microbiota that appeared to be returning towards the baseline state. Members of the Firmicutes again became

significant members of the community and Proteobacteria were no longer dominant. 16S rRNA gene sequences corresponding to *C. difficile* were detected in the gut communities of both clinically well and sick animals but in agreement with the quantitative PCR results, *C. difficile* sequences composed 8.5% (± 7.7) of those recovered in clinically ill mice, but only 1.1% (± 1.5) of the sequences in well mice. Thus, mice that were clinically well harbored an indigenous microbial community that was more similar to the baseline state seen in untreated controls than those with severe clinical CDI.

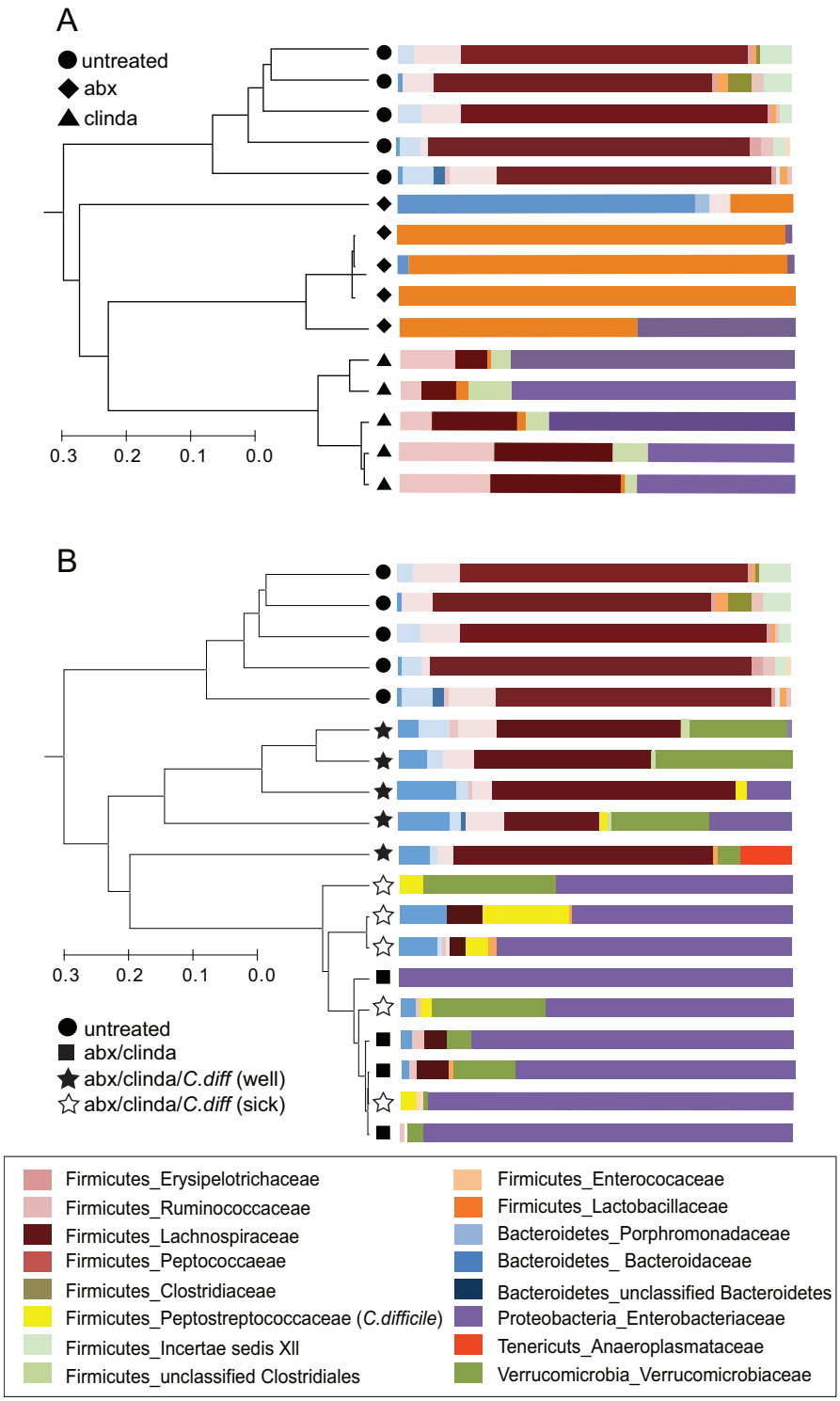

Figure 2.7 Shifts in microbial community structure and composition associated with antibiotic administration and *C. difficile* infection. The community structure of the gut microbiota was determined by 16S rRNA gene clone library construction. (A) The microbial communities in animals that were treated with the antibiotic

cocktail (abx) or with clindamycin alone (clinda) were altered from that seen in untreated controls. The antibiotic cocktail alone resulted in the appearance of significant numbers of lactobacilli, whereas clindamycin administration was associated with an increase in Proteobacteria. (B) The microbial communities in animals that received the combination of the antibiotic cocktail and clindamycin were also dominated by Proteobacteria. Animals that were challenged with *C. difficile* after antibiotic treatment harbored gut communities that were distinguished by the clinical disease that developed. Proteobacteria dominated sick antibiotic treated animals exposed to *C. difficile* while the communities of animals that remained well appeared to resemble controls with a predominance of Firmicutes. Dendrograms were constructed using Morisita-Horn community similarities based on >97% sequence similarity while taxonomic assignments were made using the Ribosomal Database Project Classifier. antibiotic cocktail- abx, clindamycin- clinda, *C. difficile- C.diff*

2.3.5 Cefoperazone treatment renders mice susceptible to colonization and colitis following *C. difficile* challenge

We previously demonstrated that administration of the beta-lactam antibiotic cefoperazone had significant and long-lasting effects on the indigenous gut microbiota (1). Even after a six-week recovery period following a 10-day course of cefoperazone, the gut microbiota exhibited altered community structure and diminished diversity. The 5 antibiotic cocktail did not have such long-lasting effects as gut microbial composition returned to baseline within four weeks of discontinuing the drug (data not shown).

To determine if the greater disturbance of the gut microbiota associated with cefoperazone administration differentially altered the course of experimental *C. difficile* infection, six to eight week old C57BL/6 mice were treated with cefoperazone prior to oral challenge with *C. difficile*. One group of mice received cefoperazone in drinking water for 10 days followed by a 2-day period on plain

water (Figure 2.1C). Another group received the same cefoperazone treatment followed by a single dose of clindamycin after 2 days (Figure 2.1D). Two final groups received 10 days of cefoperazone followed by a 6-week period without the drug with or without a single dose of clindamycin (Figure 2.1E, F). All four groups of animals then received a challenge of 1 x 10^5 CFU of *C. difficile* via oral gavage. All animals in the 2 groups of mice that were challenged 2-3 days after cefoperazone treatment (with or without clindamycin) were moribund by 2 days post challenge while one animal died between 1-2 days post challenge. High levels of *C. difficile* were present in these animals (Figure 2.8). Similarly, all animals treated with cefoperazone followed by a 6-week recovery period that received a single dose of clindamycin and *C. difficile* challenge also exhibited signs of CDI and were moribund by 4 days post challenge. However, animals that were challenged with *C. difficile* after the 6-week recovery period without a dose of clindamycin were not colonized (Figure 2.8).

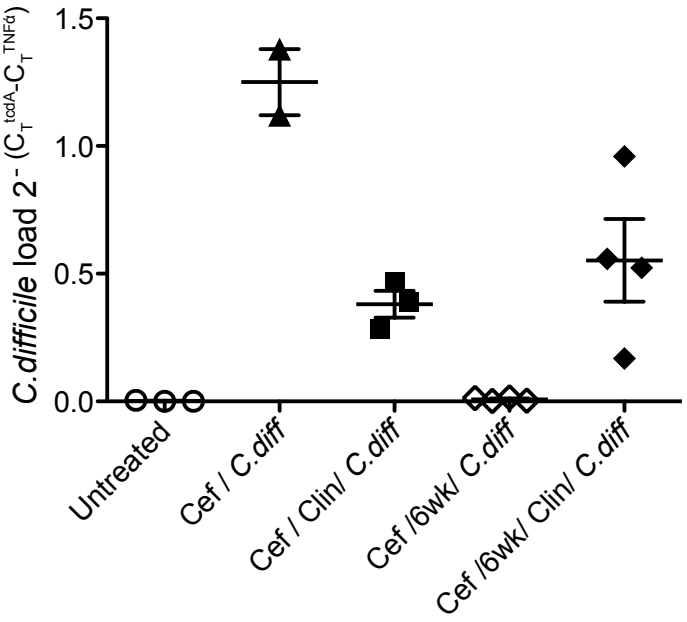

Figure 2.8 Increased *C. difficile* load after cefoperazone treatment. Quantitative PCR was performed on cecal DNA from animals treated with cefoperazone with or without clindamycin then exposed to *C. difficile.* Values represent the abundance of *tcdA* gene normalized to the single copy mouse tumor necrosis factorα (TNF α) gene. Animals treated with cefoperazone then infected with *C. difficile* 2 days later had very high levels of *C. difficile* (n=4). However *C. difficile* colonization following a 6-week recovery period only occurred after clindamycin treatment (n=4). All animals with detectable *C. difficile* levels succumbed to CDI. Cef- cefoperazone, Clin- clindamycin, *C.diff- C. difficile,* 6wk- 6-week recovery off cefoperazone.

16S rRNA-encoding gene analysis on tissue collected from cefoperazone-treated animals indicated that a 10-day treatment with this antibiotic resulted in a bacterial gut community dominated by bacteria in the family Pseudomonadaceae (Figure 2.9A). Following a 2-day period without cefoperazone and a single dose of clindamycin, the gut microbiota became dominated by members of the Lactobacillaceae (Figure 2.9A). After infection with *C. difficile* the communities of all animals pretreated with cefoperazone with or without clindamycin became

dominated by *C. difficile* (Figure 2.9B) with between 48-92% (69 ± 15%) of the 16S rRNA-encoding gene sequences retrieved corresponding to this organism. *C. difficile* 16S rRNA-encoding gene sequences were detected in 3 of the 4 animals infected 6 weeks after cefoperazone treatment followed by a dose of clindamycin and *C. difficile* challenge (12.8 ± 10%).

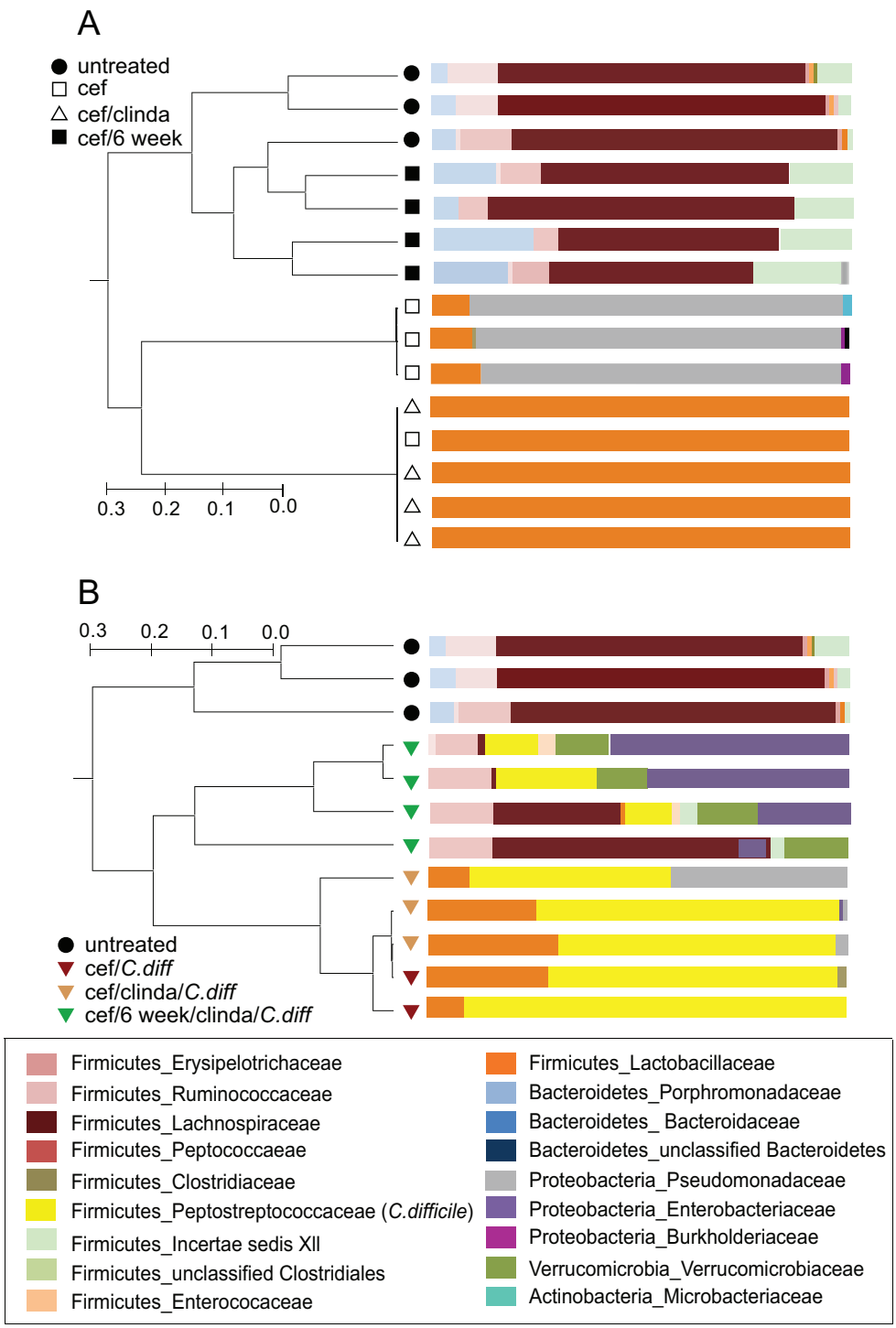

Figure 2.9 Shifts in microbial community structure and composition associated with cefoperazone administration and *C. difficile* infection. The composition of the gut microbiota was determined by 16S rRNA clone library construction. (A) The microbial communities in animals that were treated with cefoperazone with or without clindamycin were altered from that seen in untreated controls. Six weeks

after cefoperazone treatment, the communities returned to a community structure that resembled untreated controls. (B) Animals that were challenged with *C. difficile* within three days after stopping cefoperazone treatment (with or without clindamycin treatment) had very high relative levels of *C. difficile* in their microbial communities. Animals that were challenged with *C. difficile* 6 weeks after antibiotic treatment was stopped followed by a single dose of clindamycin had less *C. difficile* present. The relative abundance of *C. difficile* appeared to directly correlate with the amount of Proteobacteria and inversely with the abundance of Lachnospiraceae. Dendrograms were constructed using Morisita-Horn similarities based on >97% sequence similarity while taxonomic assignments were made using the Ribosomal Database Project Classifier. cefoperazone- cef, clindamycin- clinda, *C. difficile- C.diff*

Given the apparent increased susceptibility to *C. difficile* infection after cefoperazone treatment, we determined if decreasing the challenge dose would alter the severity of the resultant CDI. Groups of 9 to10 animals were treated with a 10-day course of cefoperazone followed by a 2-day recovery period without the drug, and were then challenged with varying doses of *C. difficile* ranging from 2 x 10^2 CFU to 2 x 10^5 CFU. The animals were monitored for weight loss and clinical signs of severe disease, and euthanized when the appropriate clinical endpoints were reached. There was a strict dose dependence on the rate at which clinical endpoints were reached (Figure 2.10). The majority of animals receiving the highest dose of *C. difficile* became moribund within 2 days post challenge while all of the animals receiving the lowest dose of 10^2 CFU remained clinically well until 5 days post challenge at which time all became ill and required euthanasia.

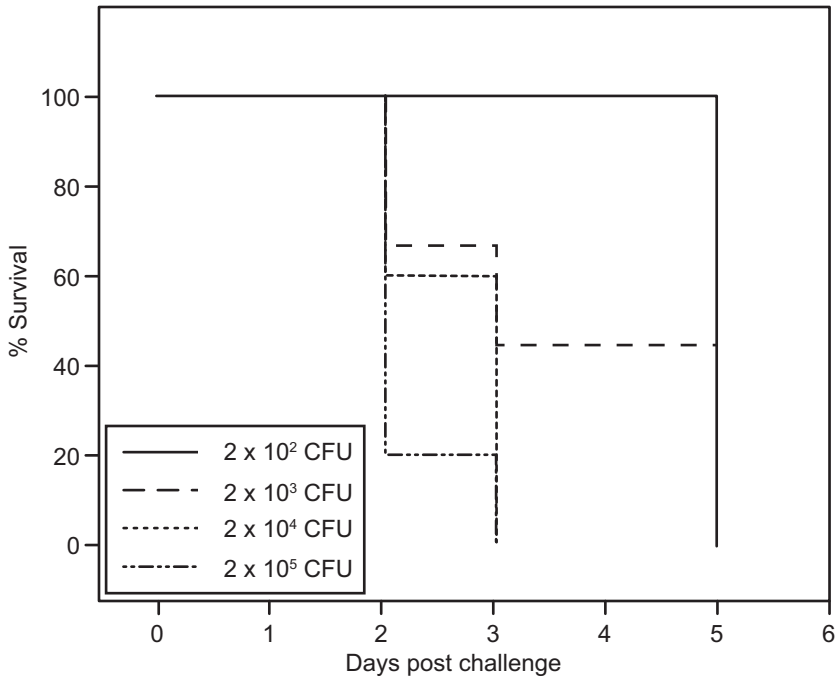

Figure 2.10 Dose dependence of disease in infected cefoperazone-treated mice infected with *C. difficile*. Kaplan-Meier survival plot for mice infected with different doses of *C. difficile* VPI 10463 (2 x 10^2, 2 x 10^3, 2 x 10^4, 2 x 10^5 CFU) after 10 days of cefoperazone pretreatment and 2-day recovery. (n=9 for 10^2, 10^3 groups and n=10 for 10^4, 10^5 groups). Survival curves are significantly different (p=0.0123 by the Log-rank (Mantel-Cox) Test) and there is a significant trend (p=0.001).

2.4 Discussion

The term “colonization resistance” was coined to refer to the ability of an established gut microbial community to resist invasion by additional microbes (15, 19, 62). Although this initially applied to pathogenic microbes, the concept was derived from concepts of community robustness derived from studies of classical ecologic systems (for example grasslands and lakes) and thus could be applied to any invading microbe (52). Current hypotheses suggest that the normal indigenous microbiota is not permissive for the establishment of colonization by *C. difficile* (64). In rare cases where normal individuals are

colonized by *C. difficile* without overt clinical disease, it is further hypothesized that the normal indigenous microbiota can at least limit the production of toxin, perhaps by directly interfering with toxin production or limiting the population size of *C. difficile* and preventing significant amounts of toxin from accumulating in the gut (50, 64). Accordingly, disruption of the indigenous microbiota by antibiotics leads to a loss of colonization resistance, making the gut vulnerable to colonization by exogenous *C. difficile* spores or, in previously colonized patients, expansion and toxin production. In support of this concept, Wilson and colleagues provided evidence for the ability of the normal gut microbiota to inhibit *C. difficile* by demonstrating that administration of normal cecal homogenates would decrease the number of viable *C. difficile* and prevent colitis in antibiotic-challenged hamsters (64, 66).

Using a murine model of *C. difficile* infection involving pretreatment of mice with antibiotics to overcome colonization resistance, we found that administration of 10^5 CFU of *C. difficile* to animals treated with a cocktail of five antibiotics and clindamycin results in uniform colonization and a mortality rate of about 60%. The initial description of this model noted that by increasing the challenge dose of *C. difficile*, mortality would increase in direct relationship to the dose of organism (8). In the current study, we also found that a more significant disruption of the microbiota, using the antibiotic cefoperazone could also result in uniform mortality in animals that were challenged with a dose of *C. difficile* that was lethal to only about half the animals that were treated with the five antibiotic cocktail and clindamycin.

Although the combination of the antibiotic cocktail and clindamycin was able to overcome colonization resistance, it is important that neither alone had the same effect. Therefore, loss of colonization resistance is not simply associated with creating an overall “depauperate” community but is dependent on the specific changes to the community structure as well. Administration of the antibiotic cocktail alone significantly decreased the overall biomass of the community, but this decrease in bacterial community size alone did not lead to a loss of colonization resistance. Furthermore, after administration of the antibiotic cocktail and clindamycin, at the time of successful challenge with *C. difficile*, the overall bacterial population had recovered, but the community structure was markedly altered from baseline. This further supports the idea that the specific changes brought on by antibiotic administration determine susceptibility to *C. difficile* colonization. This is consistent with the clinical observation that the risk of subsequent CDI differs with different antibiotics (4) and in vitro and animal studies that also differentiate antibiotics on the basis of their ability to overcome colonization resistance against *C. difficile* (25, 39, 50).

Taken together these results suggest that this murine infection model accurately represents competition between two opposing processes that are thought to be at the center of the pathogenesis of CDI (Figure 2.11). On one hand there is the expansion of the population of *C. difficile* once it has colonized an altered/susceptible microbial community and the subsequent production of toxin. On the other hand, there is the tendency of stable microbial communities to return towards their baseline state following a perturbation, in this case, antibiotic

administration (1, 11, 14, 52). According to this model, the observation of 50% mortality in animals treated with the antibiotic cocktail and clindamycin and then challenged with 10^5 CFU of *C. difficile* reflects a point at which the two processes are in close balance. In this case, if the pathogen can grow and produce toxin more rapidly than the recovery of the indigenous microbiota clinically severe disease would result. Alternately, if the microbiota recovers prior to sufficient expansion of *C. difficile* there could be control of the infection. This balance can be shifted in favor of *C. difficile* colonization and severe disease either by administering a larger challenge dose of *C. difficile* or causing a greater perturbation in the microbial community structure by administering cefoperazone. Alternately, administration of a smaller inoculum of *C. difficile* results in less disease. In terms of the antibiotic cocktail with clindamycin, decreasing the inoculum prevented the development of clinical disease, but in the setting of cefoperazone, this merely delayed the onset of disease. This further supports the idea that cefoperazone administration results in a greater disturbance of the indigenous gut microbiota.

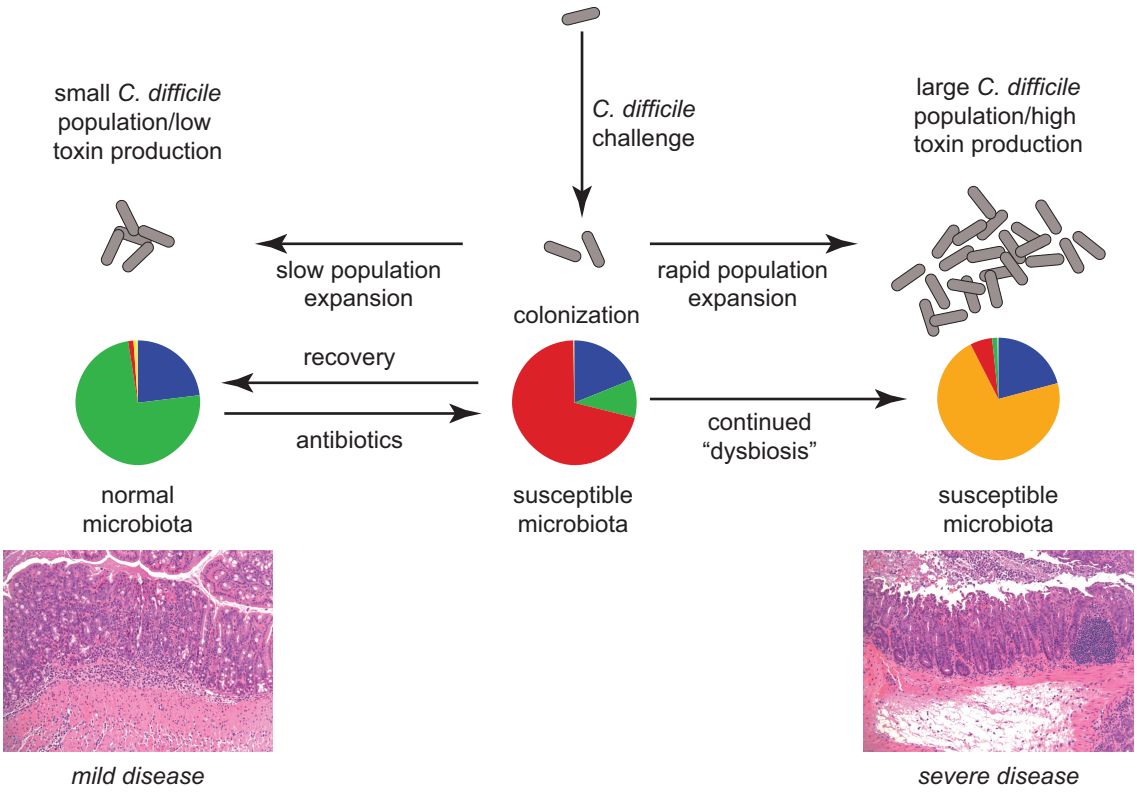

Figure 2.11 Model of the interaction between dynamics of the gut microbiota and *C. difficile* in antibiotic-treated mice. Antibiotic administration alters the community structure of the indigenous gut microbiota to a state that is susceptible to colonization with *C. difficile*. Subsequent clinical outcome is determined by the balance between the recovery of the gut microbiota following withdrawal of antibiotics and the expansion of the population of *C. difficile* and toxin production.

Additionally, the overall gut bacterial load remained reduced two-days following the end of cefoperazone treatment. This community was able to rebound to baseline levels 24-hours later following a single administration of clindamycin. Regardless of the overall bacterial mass, all animals were moribund two days after *C. difficile* infection. This suggested that loss of colonization resistance against *C. difficile* was independent of the absolute load of the gut microbiota but dependent on specific alterations within the gut microbial community structure. After a six-week period off cefoperazone the gut microbiota

seemed to resemble that of untreated control animals which suggested that the gut community had recovered. Direct infection with *C. difficile* at this point resulted in no *C. difficile* colonization or disease development suggesting that the gut microbiota had recovered to the point where colonization resistance was restored. However, administration of a single dose of clindamycin following a six-week recovery period off cefoperazone rendered all mice susceptible *C. difficile* colonization and disease. These mice all succumbed to CDI by seven days post *C. difficile* challenge and their microbial community once again shifted to a predominance of Proteobacteria with an overall decrease in the Firmicutes, specifically members of the Lachnospiraceae family. This result suggested that although the community seemed to recover enough to prevent *C. difficile* colonization after six weeks off cefoperazone, it did not recover to its initial baseline community.

Initial examination of the taxonomic community composition at the family level suggested that the gut community of mice that had recovered from cefoperazone for six weeks was similar to untreated controls. However, further examination of the operational taxonomic units (OTUs) on the specie level (or at 97% similarity) indicated that the recovered community only shared approximately 50% of the OTUs that were in the starting baseline community (untreated controls) (Figure 2.12), a community which was able to resist *C. difficile* colonization and disease even after pre-treatment with a single dose of clindamycin. Antonopoulos and colleagues observed that mice treated with cefoperazone had long term alterations in the gut community (1). Similarly our

results confirmed this finding. Therefore, cefoperazone treatment was associated with long-term alterations in the gut community which resulted in increased susceptibility to *C. difficile* colonization and disease following clindamycin treatment.

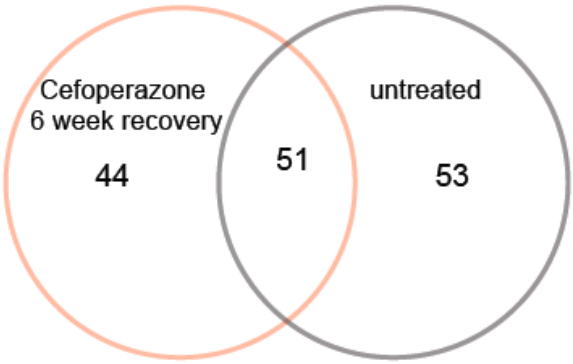

Figure 2.12 Long-term alteration in gut community after cefoperazone treatment**.** Venn diagram showing the number of shared OTUs between untreated mice (n=4) and mice treated with 10 days of cefoperazone followed by a 6-week recovery period (n=4). The total number of OTUs in the untreated community was 104 and 95 in the cefoperazone recovery community. Only 51 OTUs were shared between the two groups suggesting that the microbial community of cefoperazone treated mice had long-term alterations. 16S rRNA gene sequences from each group were placed into OTUs at 97% similarity using mothur (54). OTU- operational taxonomic unit.

The specific microbiota harbored by an individual plays a key role in mediating colonization resistance. In our hands, wild-type C57BL/6 mice from our breeding colony could not be colonized by *C. difficile* without antibiotic pre-treatment. However, Lawley and colleagues have reported that low-level *C. difficile* colonization of C57BL/6 mice could be achieved without antibiotic pre-treatment (31). These investigators also demonstrated that administration of clindamycin to *C. difficile*-colonized mice would result in a transient increase in

the shedding of the organisms which again indicates that disturbances in the microbial community structure of the microbiota alters the ecology of *C. difficile* in the GI tract. These results suggest that clinical outcomes following antibiotic pre-treatment and *C. difficile* infection may depend on the level of recovery of the altered microbial community toward the baseline state.

The mechanisms by which the indigenous microbiota can resist colonization and limit disease are not clear. Although direct competition between organisms within the GI tract is possible (59), it has been recently demonstrated that changes in the gut microbial community can indirectly affect colonization resistance via differential host responses. For example, decreasing the overall bacterial community through the administration of antibiotics can result in decreased host production of the antimicrobial peptide RegIIIγ (6). RegIIIγ binds the surface-exposed peptidoglycan layer of Gram-positive organisms with high affinity in a calcium-independent manner (33). Expression of RegIIIγ appears to be driven by the indigenous gut microbiota through host sensing of microbial-associated molecular patterns, primarily from Gram-negative organisms. RegIIIγ has been shown to be important in mediating colonization resistance to vancomycin resistant *Enterococcus* (5). However, in our animals we did not find a direct relationship between changes in RegIIIγ expression following antibiotic treatment and colonization resistance to *C. difficile*.

The development of specific host immune responses against *C. difficile* appears to have an important role in determining the severity of CDI, including the development of recurrent disease (29). This observation underscores the

exploration of *C. difficile* vaccines as a novel treatment/ prevention modality (16, 32, 46, 48). Our current model is characterized by the acute development of disease and employs naïve mice, and thus *C. difficile*-specific adaptive responses are not thought to play a role. However, since this model can be manipulated such that the disease is not uniformly fatal (unlike the hamster model of disease), it remains to be determined if this model will be useful for studying adaptive immunity in CDI.

Recently, Theriot and colleagues (60) demonstrated that the cefoperazone model can be manipulated by utilizing less severe strains of *C. difficile* to produce a non-lethal murine CDI model. This model could provide a useful tool to study the adaptive immune response in mice infected with *C. difficile*. There are other reports that the five antibiotic and clindamycin model is useful for studying the role of innate immune responses in CDI (18, 24). It is important to note that the role of immune responses in CDI is likely not independent of that of the indigenous microbes. It is clear that the gut microbiota has a key role in modulating the development of mucosal immune responses (21, 22). Therefore, changes in the gut microbiota, can influence the response to pathogens by altering the nature of host immunity (51, 67).

Another mechanism by which altered gut microbial communities could affect a pathogen is by changing the overall chemical environment of the gut. Changes in the community structure of the gut microbiota can dramatically alter the concentrations of microbial metabolites (53, 69). When comparing the gut microbial communities found in our animals that were clinically well versus those

that were succumbing to CDI, the most obvious differences were the dominance of Proteobacteria in the ill animals and the return of Firmicutes, specifically members of the family Lachnospiraceae in well animals. These latter organisms are notable in that many are able to ferment complex carbohydrates to short-chain fatty acids (SCFA), which have an important role in maintaining intestinal homeostasis (2, 49, 68). With regards to *C. difficile*, SCFA are able to inhibit the growth of the organism and decrease toxin production in vitro (36).

In summary, our results demonstrate that the community structure (not the absolute level) of the indigenous gut microbiota plays a crucial role in shaping the outcome of *C. difficile* infection. The use of tractable murine models of disease should provide insight into the role that the indigenous gut microbiota plays in defense against pathogenic microbes. It remains to be seen which of the possible interactions between the host, indigenous microbiota and pathogen are important in determining the clinical outcome of infection. However, further study could lead to novel methods for the treatment and prevention of this increasing clinically important infection.

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Chapter 3

Suppression of *Clostridium difficile* in the Gastrointestinal Tract of Germ-free Mice Inoculated with a Murine Lachnospiraceae Isolate

3.1 Introduction

Clostridium difficile is a Gram-positive toxin-producing bacterium first described as a commensal organism in the fecal microbiota of healthy newborn infants (18). Currently, *C. difficile* is the most common cause of health care-associated diarrhea and colitis. *C. difficile* infection (CDI) is responsible for significant morbidity, mortality and increased economic burden in hospitalized patients (9, 23). Risk for the development of CDI is associated with the use of broad-spectrum antibiotic therapy as well as increasing patient age and hospitalization (2).

The human gastrointestinal (GI) microbiota protects the host against colonization by exogenous pathogenic organisms, a function referred to as colonization resistance (17, 40, 42). Administration of broad-spectrum antibiotics is theorized to destroy this protective function of the indigenous microbiota, allowing *C. difficile* to proliferate and colonize the GI tract (32, 35, 41). In support of this hypothesis, mice or hamsters challenged with *C. difficile* are not readily susceptible to *C. difficile* colonization or disease (CDI) (5, 45, 47) while antibiotic administration will render animals susceptible to infection (3, 5, 12, 20, 30).

In previous studies, it was demonstrated that wild-type mice treated with a cocktail of five antibiotics and clindamycin prior to *C. difficile* challenge would follow one of two clinical courses. At the appropriate challenge dose mice would either develop rapidly lethal CDI or were stably colonized with the development of only mild disease (5, 30). We reported that members of the bacterial family Lachnospiraceae dominated the gut communities of animals with mild disease. Members of the Lachnospiraceae were also the primary component of the GI community in untreated mice from our colony. On the other hand the GI community of moribund animals had a predominance of *Escherichia coli* (30). Based on this observation, we hypothesize that members of the Lachnospiraceae family (but not *E. coli*) were responsible for at least a portion of the natural colonization resistance against *C. difficile* in the murine GI tract.

In order to directly examine this hypothesis I isolated Lachnospiraceae and *E. coli* from the cecum of mice and tested their ability to suppress *C. difficile* colonization, toxin production and disease in germ-free mice. Our results indicate that Lachnospiracaeae can play an important role in limiting *C. difficile* colonization. Further study could lead to new modes of *C. difficile* suppression and greater insight to the function of these organisms in health and disease.

3.2 Materials and Methods

3.2.1 Animals and housing

Wild-type C57BL/6 mice from a breeding colony established using animals purchased from Jackson Laboratories were housed with autoclaved food,

bedding and water under specific pathogen free conditions. Cage changes were performed in a laminar flow hood. Infection studies were performed with 6-8 week old germ-free Swiss Webster mice from a breeding colony established at the University of Michigan germ-free core facility. Mice were housed in sterile soft-sided plastic isolators with autoclaved food, bedding and water for the duration of the experiments. Each experimental group was housed in a separate isolator. All animal protocols used during the conduct of these experiments were reviewed and approved by the University Committee on Use and Care of Animals of the University of Michigan, Ann Arbor. The protocol was reviewed following guidelines for the care and use of laboratory animals set by the Office of Laboratory Animal Welfare, United States Department of Health and Human Services.

3.2.2 Development of Lachnospiraceae 16S rRNA-encoding gene primers

Partial 16S rRNA gene sequences for Lachnospiraceae from our previous study (30) were used to generate CLUSTALW multiple sequence alignments. Regions of conserved homology from the most common Lachnospiraceae operational taxonomic units (OTUs) were identified and used for PCR primer design. These conserved regions were compared against 16S rRNA gene sequences of other non-Lachnospiraceae bacteria to ensure primer specificity. Primer specificity was confirmed by performing PCR amplification on representative 16S rRNA sequences of non-Lachnospiraceae clones including *E. coli*, *Pseudomonas*, Porphyrmonodaceae, Bacteroides, Verrucomicrobia, *Lactobacillus*, Clostridiaceae, *Staphylococcus,* Ruminococcaceae and Peptococcaceae strains.

The final Lachnospiraceae-specific forward primer (LachnoF 5'- ACC GCA TAA GCG CAC AGC-3') was used with the broad-range reverse bacterial primer 1492R (1492R 5'-GGT TAC CTT GTT ACG ACT T-3') for PCR with the following cycling conditions; initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 45 sec and extension at 72°C for 90 sec. A final extension at 72°C for 10 min was performed. PCR was performed with 20 pmol of each primer (LachnoF, 1492R), 8 mM dNTP master mix (Promega-U1511), 1 unit GoTaq DNA polymerase (Promega- M3005), PCR buffer (Promega- M3005) and water in a total of 25 µl per reaction.

3.2.3 Bacterial isolation from specific-pathogen free mice

The plate wash PCR technique (36) was adapted for the isolation of murine Lachnospiraceae strains. Ceca from wild-type C57BL/6 mice were removed in a sterile manner and immediately transferred to an anaerobic chamber (Coy Industries). Anaerobically equilibrated 1x phosphate-buffered saline (PBS) was added to the cecum and the organ was opened with a sterile scalpel to release the cecal contents. Serial dilutions of this cecal suspension were plated in triplicate on various types of culture media including trypticase peptone (BD, cat# 211043) with 5% blood (blood agar), chocolate agar (consisting of 5% lysed blood) with trypticase peptone base, reinforced clostridial agar (BD cat# 218081), modified peptone yeast glucose agar (ATCC medium #1237), routine growth media (RGM) (8) and brain heart infusion agar (BD, cat# 211065) with 0.1% cysteine (Sigma-Aldrich, cat# C7352) added (BHIS) in combination with aztreonam (Sigma-Aldrich, cat# A6848) and gentamicin (Sigma-Aldrich, cat#

G1914) to determine which media would provide the greatest enrichment of Lachnospiraceae. The surface of one agar plate was scraped to remove all bacterial colonies and bacterial DNA was extracted using an automated system (Roche MagNA Pure, cat# 03730964001). Enrichment for Lachnospiraceae was determined by performance of the Lachnospiraceae specific 16S rRNA gene PCR and the amplification of the expected 1320 base pair band. The greatest enrichment for Lachnospiraceae was obtained using BHIS plates supplemented with 2 mg/L gentamicin and 1 mg/L aztreonam (BHIS gen/az). Subsequently, individual colonies from the remaining BHIS gen/az plates in the anaerobic chamber were inoculated into 250 µl of BHIS gen/az broth in a sterile 96-well plate then incubated anaerobically at 37°C and growth was monitored. PCR using 1 µl of bacterial culture was used to identify putative Lachnospiraceae. Genomic bacterial DNA was extracted using an automated system (Roche MagNA Pure) from cultures of putative Lachnospiraceae isolates. Sequencing of full-length 16S rRNA gene sequences was obtained using the following primers: 8F (5'-AGA GTT TGA TCC TGG CTC AG-3'), 515F (5'- GTG CCA GCM GCC GCG GTA-3'), E939R (5'- CTT GTG CGG GCC CCC GTC AAT TC-3') and 1492R (5'-GGT TAC CTT GTT ACG ACT T-3'). DNA sequencing was performed by the DNA Sequencing Core facilities at the University of Michigan. Isolation of *Escherichia coli* was performed by selectively plating murine cecal contents on MacConkey agar anaerobically at 37°C. The identity of putative *E. coli* isolates was confirmed by sequencing the 16S rRNA-encoding gene amplicon. 16S rRNA

gene sequence analysis and taxonomic classification was performed using the RDP Bayesian classifier implemented in mothur (33).

3.2.4 *Clostridium difficile* strains and growth conditions

C. difficile spores were prepared from strains VPI 10463 (ATCC 43255) and 630 (ATCC BAA-1382) as follows. *C. difficile* was cultured overnight anaerobically at 37ºC in brain heart infusion broth with 0.1% cysteine (BHIS). On the following day, 100 µl of these cultures were spread onto BHIS plates (four plates per strain) and the plates incubated for seven days anaerobically at 37ºC. The plates were removed from the anaerobic chamber and exposed to ambient oxygen for 24 hr at room temperature to kill vegetative cells. Plates were flooded with 15 ml cold water and bacteria were removed by scraping with a sterile loop. Bacterial suspensions were centrifuged and washed in cold water at least three times. Spore stocks were stored at 4ºC in sterile water. The presence of spores was confirmed using phase contrast microscopy and stocks were enumerated by plating for viable colony forming units on TCCFA (Taurocholate cycloserine cefoxitin fructose agar). *C. difficile* spores were heat treated for 20 min at 65°C to ensure that all spores were viable prior to gavaging animals with 100 µl of a specific dose of the spore suspension (39). Culture plates were incubated in an anaerobic chamber (Coy Industries) at 37°C for 24 hr.

3.2.5 Germ-free mouse infection studies

Six to 8 week-old germ-free Swiss Webster mice were divided into groups of 3 - 9 animals. Each treatment group was housed in separate sterile isolators.

Initially, mice were challenged via oral gavage with varying spore doses of two *C. difficile* strains VPI 10463 or strain 630 to determine the appropriate dose of spores to use for the remaining experiments. For *C. difficile* VPI 10463, mice were challenged with 3.8×10^1, 3×10^2, 3.3×10^3 and 1×10^5 spores while for *C. difficile* strain 630 mice were challenged with 1×10^1, 1×10^2, 1×10^3 and 1×10^4 spores (Figure 3.1) with each group consisting of 3 to 5 mice. Based on these experiments 100 spores were used as the challenge dose for all experiments. Mice were pre-colonized via oral gavage with 1×10^8 CFU of either *Escherichia coli* or Lachnospiraceae D4 for four days. As a control, mice were also orally gavaged with single dose of cecal content homogenate obtained from a wild-type mouse for four days. Each treatment group consisted of 5 to 9 animals. Mice were then challenged by oral gavage with 100 spores of *C. difficile* (VPI 10463 or strain 630) and monitored daily for signs of disease such as diarrhea, hunched posture and weight loss. Control groups consisted of animals colonized with only *C. difficile* (n=11 and n=15 for strain 630 and VPI 10463 respectively), *E. coli* (n=4), Lachnospiraceae D4 (n=4), cecal content homogenate from wild-type mice (n=3) or no bacteria (n=5) (Figure 3.1). Bacterial colonization was monitored daily by anaerobically culturing fecal pellets and cecal contents at necropsy at 37°C for 24 hr. *E. coli* colonization was monitored by culture on MacConkey agar while the levels of Lachnospiraceae D4 were monitored by culture on BHIS gen/az. The colonization status of *C. difficile* challenged animals was monitored by anaerobic culture on TCCFA.

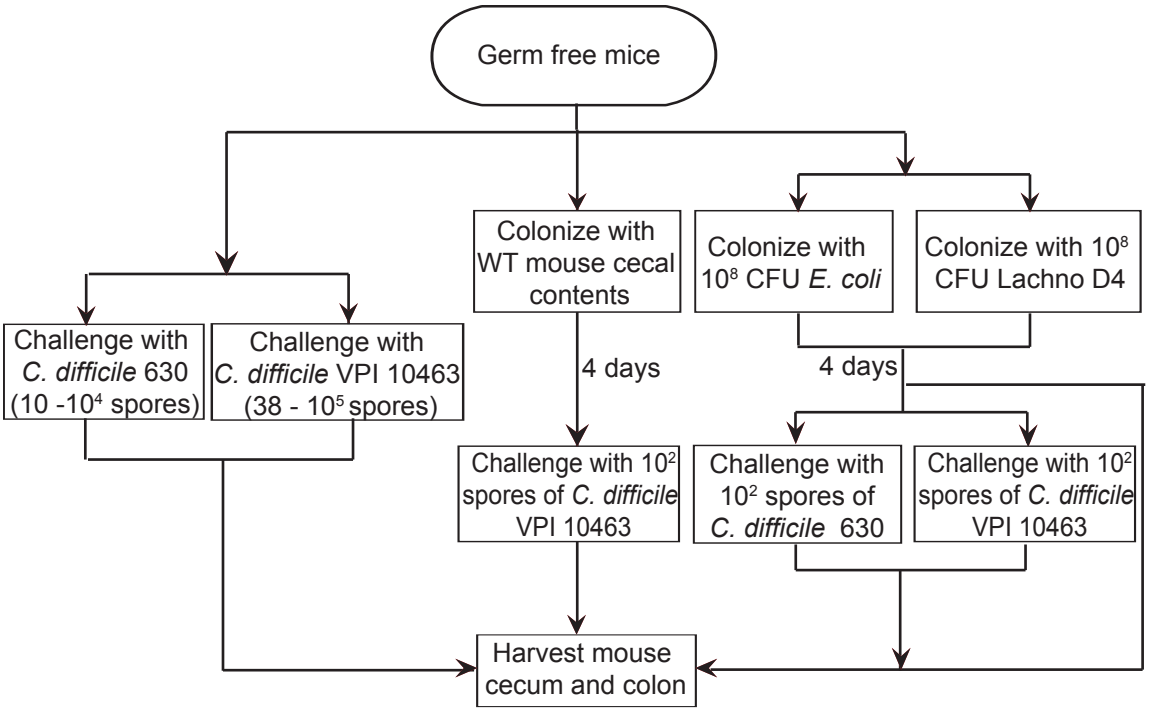

Figure 3.1 Schematic for examining the effect of Lachnospiraceae D4 and *E. coli* on *C. difficile* colonization in germ-free mice. Mice were infected with titrating doses of *C. difficile* 630 (10, 10^2, 10^3, 10^4) spores (n=3 per dose) or *C. difficile* VPI 10463 (38, $3x10^2$, $3.3x10^3$, $1x10^5$) spores (n=4 per dose) and monitored for colonization. Animals were colonized with cecal contents from a wild-type mouse for 4 days prior to *C. difficile* VPI 10463 challenge (n=5). Additionally, mice were either pre-colonized with *E. coli* then challenged with *C. difficile* 630 (n=9) or *C. difficile* VPI 10463 (n=7) spores or pre-colonized with Lachnospiraceae D4 then challenged with *C. difficile* 630 (n=8) or *C. difficile* VPI 10463 (n=14) spores. Mice mono-colonized with *C. difficile* 630 (n=11) or *C. difficile* VPI 10463 (n=15) were done as controls. Other groups of mice were colonized with either *E. coli* (n=4) or Lachnospiraceae D4 (n=4) for 4 days and harvested. The cecum and colon from each animal was harvested for bacterial and cytotoxin quantification.

3.2.6 Necropsy and histological procedures

Mice were euthanized by CO_2 asphyxiation. Approximately 5 mm of the proximal colon, terminal ileum tissue, and luminal contents were collected for each animal, snap frozen in liquid nitrogen and stored at -80°C. The remaining cecum and

colon tissue were placed intact into histology tissue cassettes and stored in 10% buffered formalin for 24 hr then transferred to 70% ethyl alcohol. Tissue cassettes were then processed, paraffin embedded and 5 μm sections prepared. Haematoxylin and eosin stained slides were prepared for histologic examination (McClinchey Histology Lab Inc. Stockbridge, MI.)

3.2.7 Histopathologic examination

Histological changes were coded, randomized, and scored in a blinded manner by a board-certified veterinary pathologist. A scoring system was adapted from a previously published method (5, 22). Edema, neutrophilic inflammation and epithelial damage in colon and cecum were scored from 0-4 according to the following defined criteria: Edema scores – 0, no edema; 1, mild edema with minimal (<2x) multifocal submucosal expansion; 2, moderate edema with moderate (2-3x) multifocal sub-mucosal expansion; 3, severe edema with severe (>3x) multifocal sub-mucosal expansion; and 4, same as score 3 with diffuse sub-mucosal expansion. Neutrophilic inflammation scores were graded as follows: 0, no inflammation; 1, minimal multifocal neutrophilic inflammation (marginating or perivascular neutrophils in submucosa, minimal intraepithelial and proprial neutrophils); 2, moderate multifocal neutrophilic inflammation (perivascular and interstitial neutrophils in submucosa, mild to moderate intraepithelial and proprial neutrophils); 3, severe multifocal to coalescing neutrophilic inflammation (perivascular and increased interstitial neutrophils in submucosa +/- extension to muscular wall, moderate intraepithelial neutrophils); and 4 same as score 3 with abscesses. Epithelial damage was scored as follows:

0, no epithelial changes; 1, minimal multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); 2, moderate multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); 3, severe multifocal epithelial damage (same as above) +/- pseudomembrane (intraluminal neutrophils, sloughed epithelium in a fibrinous matrix); and 4, same as score 3 with significant pseudomembrane or epithelial ulceration (focal complete loss of epithelium).

3.2.8 *C. difficile* cytotoxin assay

Quantification of *C. difficile* toxin present in luminal contents was performed using a method adapted from Corthier et al. (7). Green African monkey kidney epithelial cells (Vero) (provided by M. Imperiale, University of Michigan) were grown to confluency in DMEM (GIBCO Laboratories, cat# 11965) containing 10% heat inactivated fetal bovine serum (GIBCO Laboratories, cat# 16140) and 1% Penicillin streptomycin solution (GIBCO Laboratories, cat# 15140). The cells were trypsinized using 0.25% trypsin (GIBCO Laboratories, cat# 25200) and washed with 1 volume of DMEM medium. Cells were diluted in DMEM medium and approximately $1x10^5$ cells were distributed per well in a 96-well flat bottom microtiter plate (Corning) and incubated at 37°C for 18-24 hours.100 mg of luminal contents were weighed and 500 µl of 1x PBS was added to make a suspension. Samples were mixed and then particulate material removed by centrifugation at 9000 x *g* for 5 min and then the supernatant was filtered through a 0.2 µm membrane. Each sample was titrated in ten-fold dilutions within the wells to a maximum dilution of 10^{-12} and each well had a corresponding control to

which both neutralizing *C. sordelli* antitoxin (TechLabs, cat# T5000) and sample were added. After an overnight incubation at 37°C, plates were fixed with 10% buffered formalin for 2 hours then stained with geimsa (50 µl per well) for 15 min followed by a wash with 1x PBS. Wells with approximately 100% round cells were easily recognized under 200x magnification. The cytotoxic titer was defined as the reciprocal of the highest dilution that rounds 100% of Vero cells per gram of sample.

3.2.9 Short-chain fatty acid (SCFA) measurement

In vivo SCFA measurement was performed as follows: groups of four 6-8 week old Swiss Webster germ-free mice were colonized with either 10^8 CFU of *E. coli* or Lachnospiraceae D4 for four days. Additionally, a third group remained germ-free as a control. All mice were necropsied, cecal contents were removed, weighed, flash frozen and stored at -80°C. Samples were sent to the Michigan Metabolomics and Obesity Center at the University of Michigan for SCFAs measurement by gas chromatography-mass spectrometry (GC-MS). GC (Agilent 6890) separation was performed using a ZB-Wax plus column (0.25 µm x 0.25 mm x 30 m) and a quadruple mass spectrometer (Agilent, 5973 inert MSD) was used to identify and quantitate SCFAs using Agilent Chemstation software.

3.2.10 Fluorescent in situ hybridization (FISH)

Representative 5 µm paraffin embedded cecal and colon sections from mice colonized with *C. difficile* only, *E. coli* and *C. difficile* or Lachnospiraceae D4 and *C. difficile* were prepared on slides. Each tissue section was de-paraffinized by

performing two 5-minute washes in xylene, followed by two 5-minute washes in 100% ethanol then each section was rinsed in sterile water. Hybridization buffer (2 ml per slide) was made using the following: 360 µl 5M NaCl, 40 µl 1M Tris-HCl pH 7.2, 700 µl formamide, 2 µl 10% SDS and 900 µl sterile water. Fluorescently labeled probes CY3-Eub338 (general bacteria probe) and CY5-Cd198 (*C. difficile* specific probe) each at 5 ng/ µl was added to 100 µl hybridization buffer then applied to each section. The slide was then placed in a hybridization chamber with the remaining buffer for 2 hours at 46ºC. A wash buffer pre- warmed to 48ºC was made as follows: 700 µl 5M NaCl, 1 ml 1M Tris-HCl pH 7.2, 500 µl 0.5M EDTA pH 8, 50 µl 10% SDS and water added to make 50 ml total. Each slide was removed from the hybridization chamber and placed in 25 ml of pre-warmed wash buffer and incubated at 48ºC for 40 minutes. After wash, each slide was mounted using VectaShield mounting medium (Vector Laboratories Inc., cat# H-1000) to protect from photobleaching and then images were captured using a fluorescent microscope. Images labeled with CY3-Eub338 were false colored red while images labeled with CY5-Cd198 were false colored green. Background fluorescence for each image was captured and false colored magenta. Images were merged using Adobe Photoshop CS5 version 12.0.

3.2.11 Statistical analysis

Statistical analyses were performed using Prism 5 for Mac OS X GraphPad Software. The nonparametric Krustal Wallis test was used to determine significance for all treatment groups while Student's t test was used for individual comparisons. Statistical significance was set at a *P* value of <0.05.

3.3 Results

3.3.1 Isolation and characterization of murine Lachnospiraceae and *E. coli*

A strain of *E. coli* was isolated from the murine gut by selectively plating murine cecal contents on MacConkey agar. The identification of potential *E. coli* cultivars was confirmed by 16S rRNA gene sequence analysis. To isolate Lachnospiraceae, 16S-targeted rRNA-encoding gene primers were designed based on the partial 16S rRNA gene sequences from our previous study (30), to guide cultivation efforts via the plate wash PCR technique (36). The greatest enrichment for Lachnospiraceae was achieved by selectively plating murine cecal contents on brain heart infusion agar supplemented with 0.1% cysteine, 2 mg/L gentamicin and 1 mg/L aztreonam (BHIS gen/az) under anaerobic conditions. A total of 14 Lachnospiraceae isolates based on 16S rRNA-encoding gene sequence were confirmed (Figure 3.2). We determined the phylogenetic relationship of these isolates to the most abundant phylotypes (clostridial clusters) of the low molecular % G+C Gram-positive bacteria in the Fimicutes phylum (Figure 3.2). All 14 Lachnospiraceae isolates were members of the clostridial cluster XIVa (10).

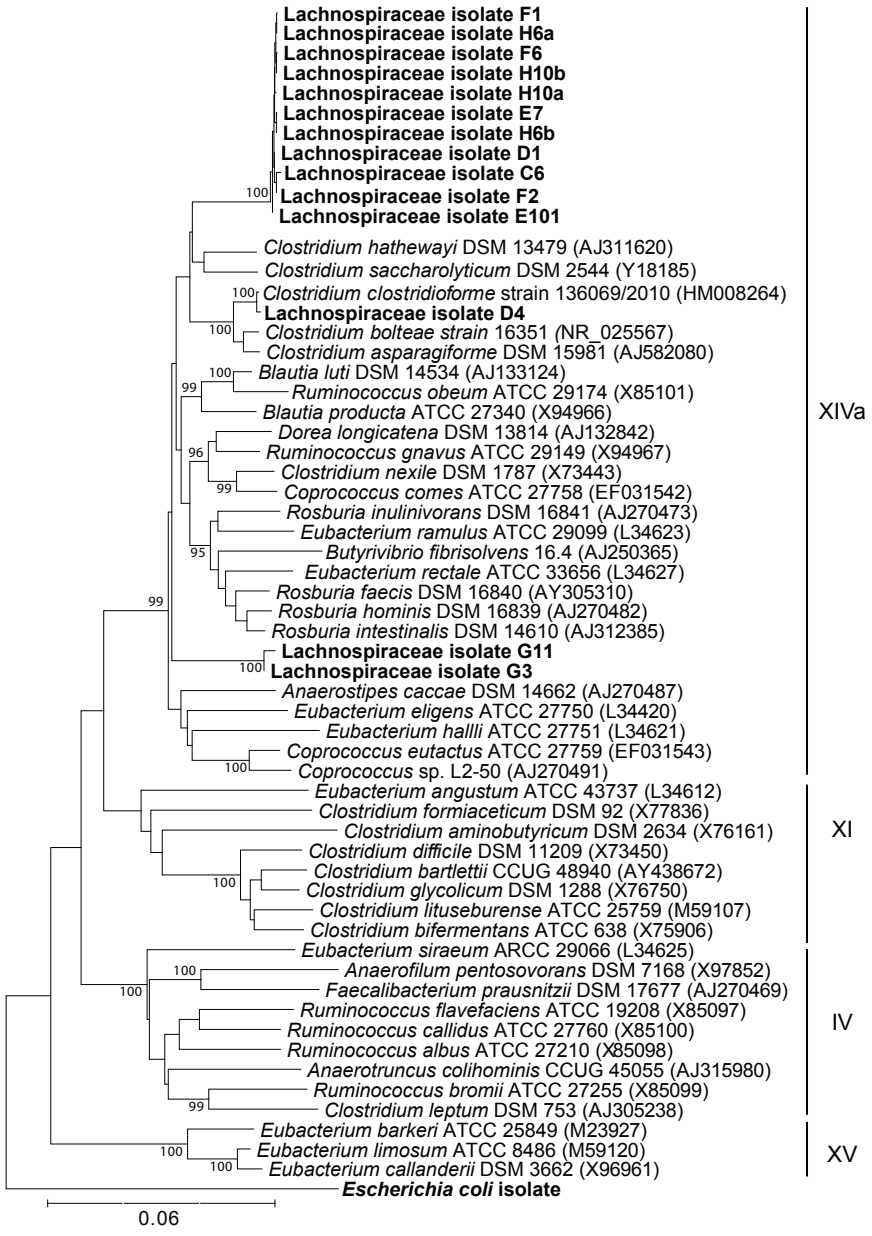

Figure 3.2 Phylogenetic tree showing clostridial clusters of low molecular % G+C Gram-positive bacteria based on 16S rRNA sequence. The tree was constructed using the neighbor joining method with the murine *E. coli* isolate used as the out-group. Newly isolated murine Lachnospiraceae strains are shown in bold face. Accession numbers for sequences are given in brackets. Boot-strap values greater than 95 (per 500 replicates) are shown at branch points. The scale bar represents genetic distance and clostridial clusters are indicated by Roman numerals.

These Lachnospiraceae isolates were further characterized based on growth on BHIS gen/az plates and BHIS gen/az broth. Of the 14 Lachnospiraceae isolates, 13 took 3-4 days to grow on solid media and grew poorly in broth culture. The remaining isolate (referred to as D4) was most closely related to *Clostridium clostridioforme* based on 16S rRNA gene sequence (Figure 3.2). Lachnospiraceae isolate D4 grew very well on solid media and in broth culture which allowed us to test its ability to inhibit *C. difficile* growth and toxin production in germ-free mice.

3.3.2 Colonization of germ-free mice with Lachnospiraceae D4 interferes with subsequent *C. difficile* colonization

We recently demonstrated that cefoperazone-treated mice were readily colonized with *C. difficile* strain 630 but did not exhibit signs of clinically severe CDI such as weight loss, diarrhea or hunched posture (39). As such, infection of cefoperazone-treated mice with *C. difficile* 630 permits the examination of the effect of Lachnospiraceae D4 and *E. coli* on *C. difficile* colonization and cytotoxin production in a non-lethal infection model. We initially challenged germ-free mice with varying doses of *C. difficile* 630 spores ranging from 10^1 to 10^4 (Figure 3.1) to determine if the lack of lethality observed in antibiotic-treated animals would extend to mono-colonization with this strain. *C. difficile* colonization levels were monitored daily by culture of fecal pellets and culture of cecal contents at necropsy.

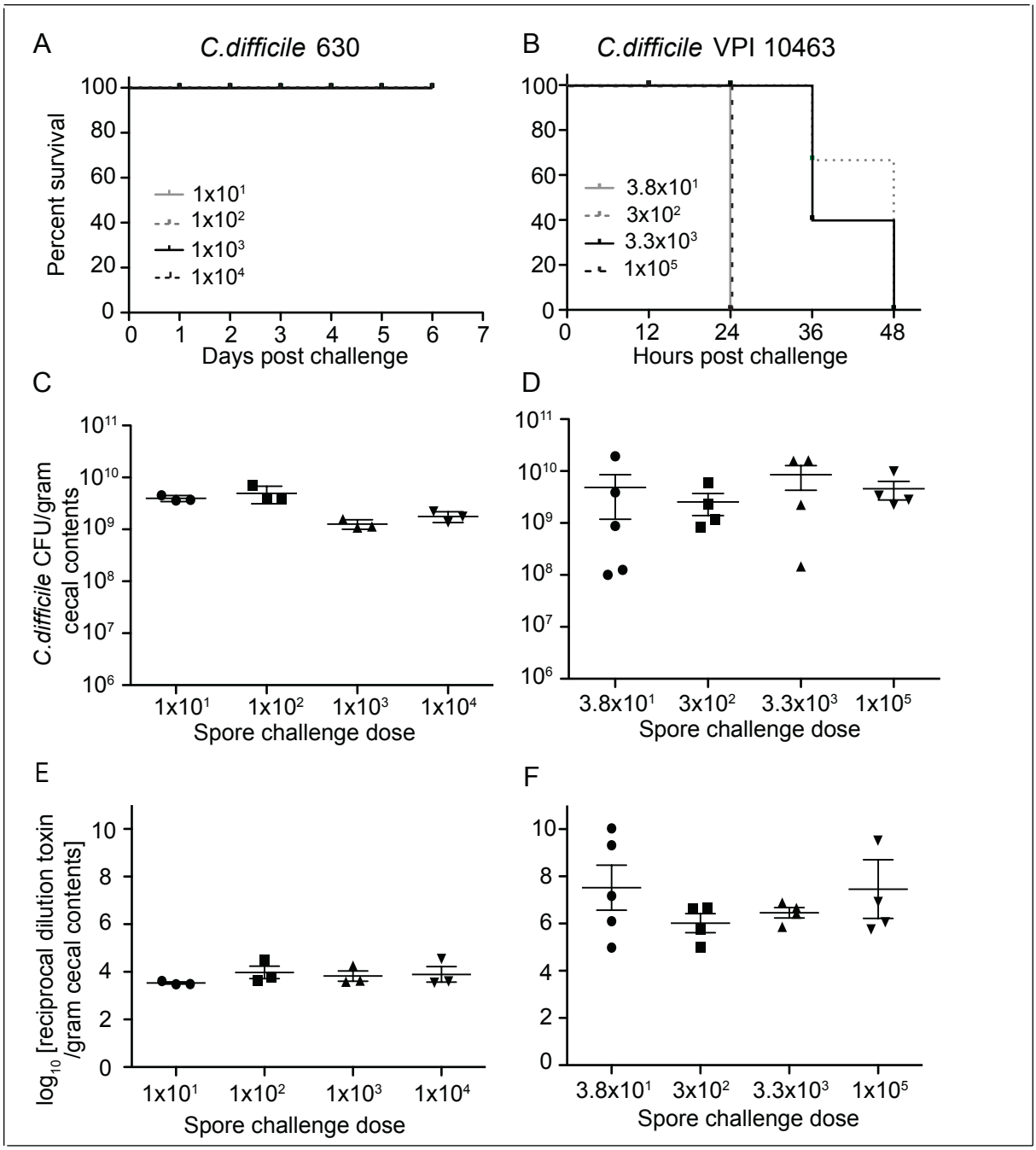

Figure 3.3 *C. difficile* infection in germ-free mice. Kaplan-Meier survival plot for mice infected with titrating doses of (A) *C. difficile* 630 spores (1 x 10^1, 1 x 10^2, 1 x 10^3, 1 x 10^4) and (B) *C. difficile* VPI 10463 spores (3.8 x 10^1, 3 x 10^2, 3.3 x 10^3, 1 x 10^5). Quantification of *C. difficile* was determined by culturing cecal contents at the time of necropsy: (C) at day 6 from mice infected with *C. difficile* 630 for each challenge dose (n=3) or (D) at days 1 or 2 for mice infected with *C. difficile* VPI 10463 for each challenge dose (n=4). Vero cell tissue culture was used to determine the $\log_{10}$ reciprocal cytotoxin dilution per gram of cecal contents from

mice in (C and D) infected with (E) *C. difficile* 630 or (F) *C. difficile* VPI 10463 for each challenge dose. Points on each graph represent individual animals. Error bars represent standard deviation.

Levels of *C. difficile* cytotoxin were measured using a Vero cell assay. As with conventional mice, germ-free mice challenged with *C. difficile* 630 did not exhibit clinical signs of CDI and survived the infection (Figure 3.3A). Mono-colonized animals had high levels of *C. difficile* colonization (>10^9 CFU/ gram) (Figure 3.3C). Similar results were seen regardless of the infectious dose administered. Subsequently, we employed an infectious dose of 100 *C. difficile* 630 spores for the following experiments.

Germ-free mice received 10^8 CFU of either Lachnospiraceae D4 or *E. coli* via oral gavage. Mice challenged with either bacteria were readily colonized but the levels of Lachnospiraceae D4 in the feces of colonized animals was approximately 2 logs lower than that reached by *E. coli* in mono-colonized mice (Figure 3.4). Four days after pre-colonization with either Lachnospiraceae D4 or *E. coli* animals were challenged with 100 spores of *C. difficile* strain 630. All mice challenged with *C. difficile* were successfully colonized and this colonization did not alter the level of fecal Lachnospiraceae or *E. coli* colonization (Figure 3.4). No clinical disease was apparent in any of the experimental groups.

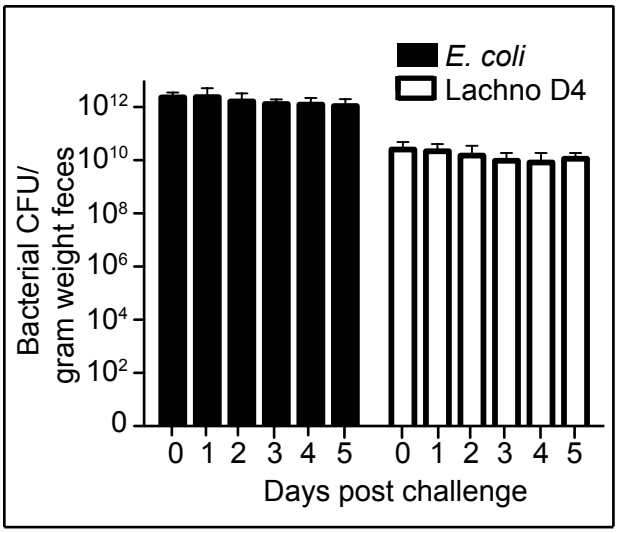

Figure 3.4 Intestinal cecal colonization levels by *E. coli* and Lachnospiraceae D4 after *C. difficile* challenge. Mice were pre-colonized with either *E. coli* or Lachnospiraceae D4 for 4 days then infected with *C. difficile* 630. Both *E. coli* and Lachnospiraceae D4 levels were measured daily in feces by culture. For each day of the experiment colonization levels for *E. coli* reached 10^{12} CFU while Lachnospiraceae D4 reached 10^{9}-10^{10} CFU. *P* value was calculated using student's t test and was significant (p = <0.0001) between the two groups. Error bars represent standard deviation. CFU- colony forming units. Lachno D4- Lachnospiraceae D4

In mice pre-colonized with *E. coli* prior to *C. difficile* challenge, there was no difference in the cecal levels of colonization by *C. difficile* compared to mice monocolonized with *C. difficile* strain 630 (Figure 3.5A). On the other hand, mice pre-colonized with Lachnospiraceae D4 prior to *C. difficile* challenge had a significant decrease in the levels *C. difficile* colonization (>1.5 log) (Figure 3.5A) and a corresponding decrease in the amount of *C. difficile* cytotoxin in the cecal contents when compared to animals monocolonized with *C. difficile* (Figure 3.5B).

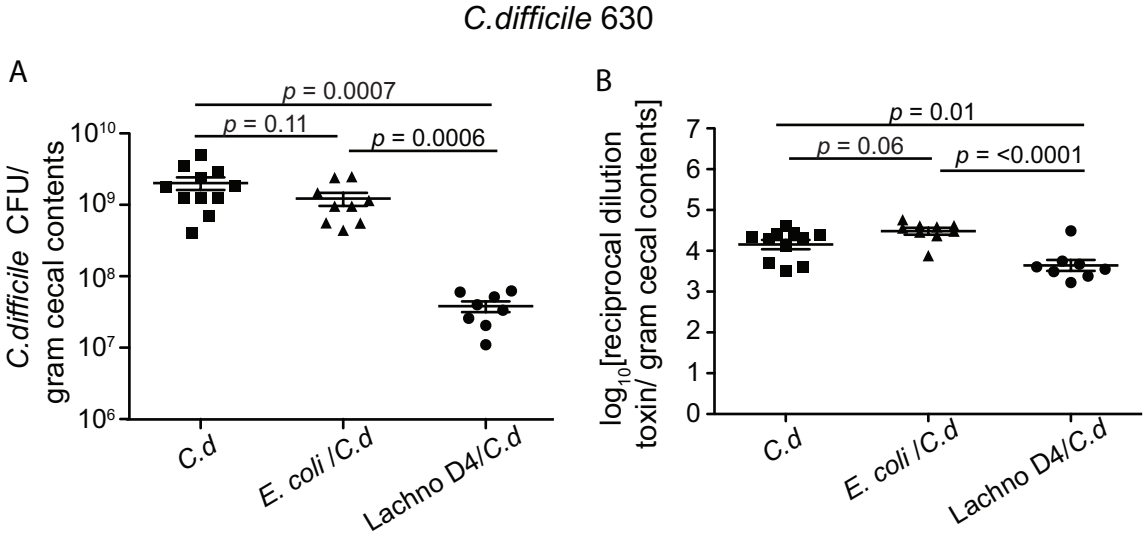

Figure 3.5 Decreased levels of *C. difficile* 630 and cytotoxin in Lachnospiraceae D4 pre-colonized mice. (A) Quantification of *C. difficile* was determined by culturing cecal contents at the time of necropsy (day 6) from mice infected with *C. difficile* only (n=11) or pre-colonized with either *E. coli* (n=9) or Lachnospiraceae D4 (n=8) then infected with *C. difficile.* Each point represents the *C. difficile* level from an individual animal. Mice pre-colonized with Lachnospiraceae D4 had significantly decreased levels of *C. difficile* compared to *C. difficile* controls or *E. coli* pre-colonized mice. Error bars represent standard deviation. Comparisons between groups were performed using the non-parametric Krustal Wallis test. (B) Vero cell tissue culture was used to determine the $\log_{10}$ reciprocal cytotoxin dilution per gram of cecal contents from mice in (A) infected with *C. difficile* only or pre-colonized with either *E. coli* or Lachnospiraceae D4 then infected with *C. difficile.* Error bars represent standard deviation. Comparisons between groups were performed using the non-parametric Krustal Wallis test. *C.d* - *C. difficile*, Lachno D4- Lachnospiraceae D4. CFU- colony forming units

3.3.3 Colonization of germ-free mice with Lachnospiraceae isolate D4 decreases subsequent disease severity after challenge with *C. difficile* strain VPI 10463

C. difficile VPI 10463 has been shown to cause acute, severe and often lethal form of CDI in antibiotic-treated wild-type and germ-free mice (21, 30, 39). Germ-free mice challenged with various doses of *C. difficile* VPI 10463 spores

(Figure 3.3) all developed clinically severe CDI with diarrhea, hunched posture and significant (>20% from baseline) weight loss. All mice regardless of challenge dose were either dead or moribund by 1-2 days post challenge (Figure 3.3B). High levels of *C. difficile* (>10^9 CFU/ gram) (Figure 3.3D) and cytotoxin were detected in cecal contents at the time of necropsy when compared to animals monocolonized with *C. difficile* 630 (Figure 3.3E, 3.3F). Similar to animals monocolonized with *C. difficile* VPI 10463, mice pre-colonized with *E. coli* prior to *C. difficile* challenge lost > 20% of baseline body weight by 2 days post infection (Figure 3.6) and had high levels of *C. difficile* colonization and cytotoxin production at necropsy (Figure 3.7).

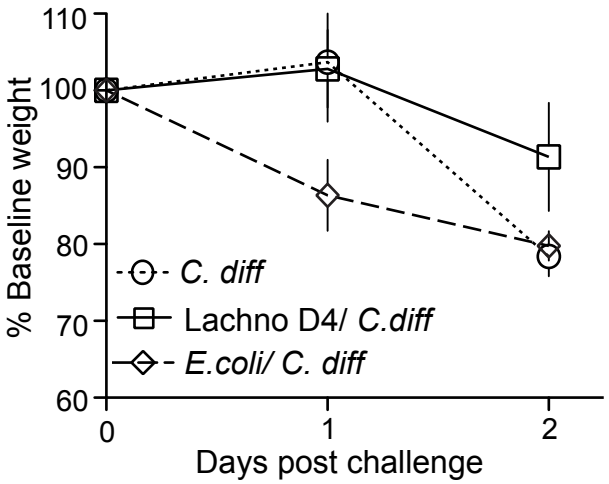

Figure 3.6 Weight loss in *C. difficile* infected mice. Weight loss curves for *C. difficile* infected mice (n=15), mice pre-colonized with Lachnospiraceae D4 and infected with *C. difficile* (n=14) and mice pre-colonized with *E. coli* and infected with *C. difficile* (n=7). Lachnospiraceae D4 pre-colonized mice lost less weight than *C. difficile* or *E. coli* pre-colonized mice. Weight loss percentage is based on the starting weight on day 0. Error bars represent the standard deviation of the weights for animals within each group. Lachno D4- Lachnospiraceae D4, *C. diff*- *C. difficile*

Conversely, mice pre-colonized with Lachnospiraceae D4 demonstrated significantly less clinically severe disease following challenge with *C. difficile* VPI 10463. Of a total of 14 mice, 3 were moribund and lost significant weight while the remaining 11 had minimal weight loss and were clinical well 2 days post infection (Figure 3.6). The 11 surviving mice also had lower levels of *C. difficile* colonization and measureable cytotoxin (Figure 3.7) compared to the moribund mice and mice challenged *C. difficile* alone or following prior colonization with *E. coli*.

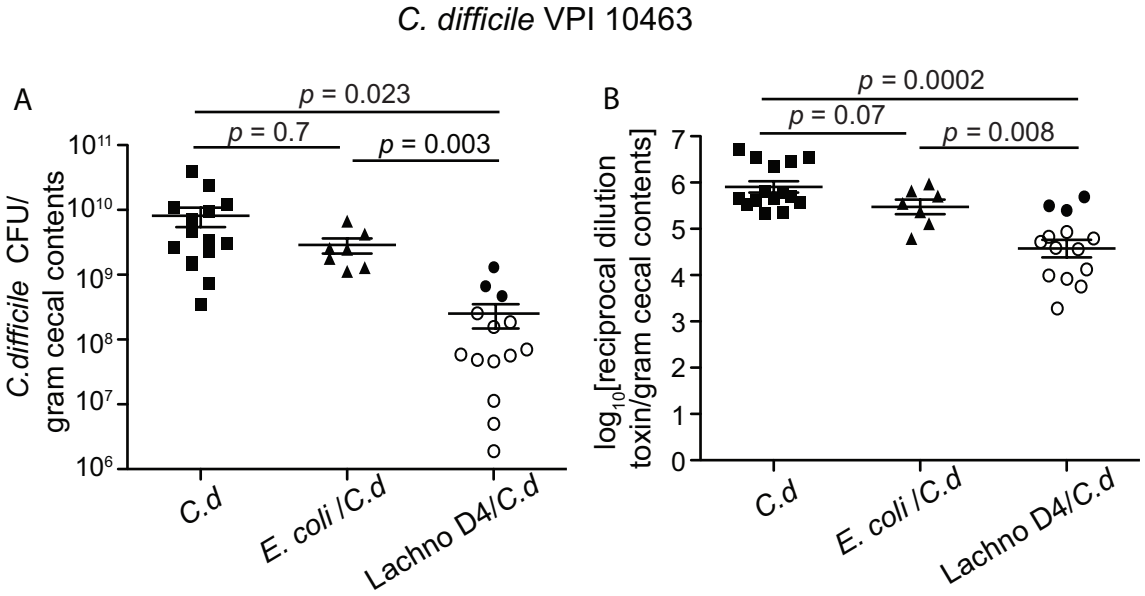

Figure 3.7 Decreased *C. difficile* VPI 10643 colonization and cytotoxin levels in Lachnospiraceae D4 pre-colonized mice. (A) Quantification of *C. difficile* was determined by culturing cecal contents at the time of necropsy (day 2) from mice infected with *C. difficile* only (n=15) or pre-colonized with *E. coli* (n=7) or Lachnospiraceae D4 (n=14) then infected with *C. difficile*. The levels of *C. difficile* colonization was decreased in Lachnospiraceae D4 pre-colonized compared to *C. difficile* controls or *E. coli* pre-colonized mice. Each point represents the *C. difficile* level from an individual animal. The open points represent animals that had improved CDI signs and did not lose significant weight while the closed points (in black) represent animals that were moribund or dead at the time of

necropsy. (B) Vero cell tissue culture was used to determine the $\log_{10}$ reciprocal cytotoxin dilution per gram of cecal contents from mice in (A) infected with *C. difficile* only or pre-colonized with *E. coli* or Lachnospiraceae D4 then infected with *C. difficile.* Error bars represent standard deviation. Comparisons between groups were performed using the non-parametric Krustal Wallis test. *C.d* - *C. difficile,* Lachno D4- Lachnospiraceae D4.

Germ-free mice pre-colonized with Lachnospiraceae D4 prior to challenge with *C. difficile* VPI 10463 had significantly less colonic inflammation and submucosal edema than either *C. difficile* infected controls or animals pre-colonized with *E. coli* before *C. difficile* challenge (Figure 3.8E, 3.8F). Mice that were maintained germ-free and mice mono-associated with either Lachnospiraceae D4 or *E. coli* had no histologic alterations (data not shown). In addition to the differences in the colon, there was also significantly less mucosal epithelial damage in the cecum of mice pre-colonized with Lachnospiraceae D4 compared to *E. coli* pre-colonized and *C. difficile* control mice (data not shown). Representative histological alterations within the *C. difficile* VPI 10463 challenged groups are shown in Figure 3.8. Alterations consisted predominantly of edema within the submucosa and the mucosal lamina propria. There was also neutrophilic inflammation perivascularly and interstitially within the submucosa and multifocally within the mucosa (Figure 3.8). Epithelial damage was not prominent but consisted of vacuolar degeneration and increased loss of apical tip enterocytes.

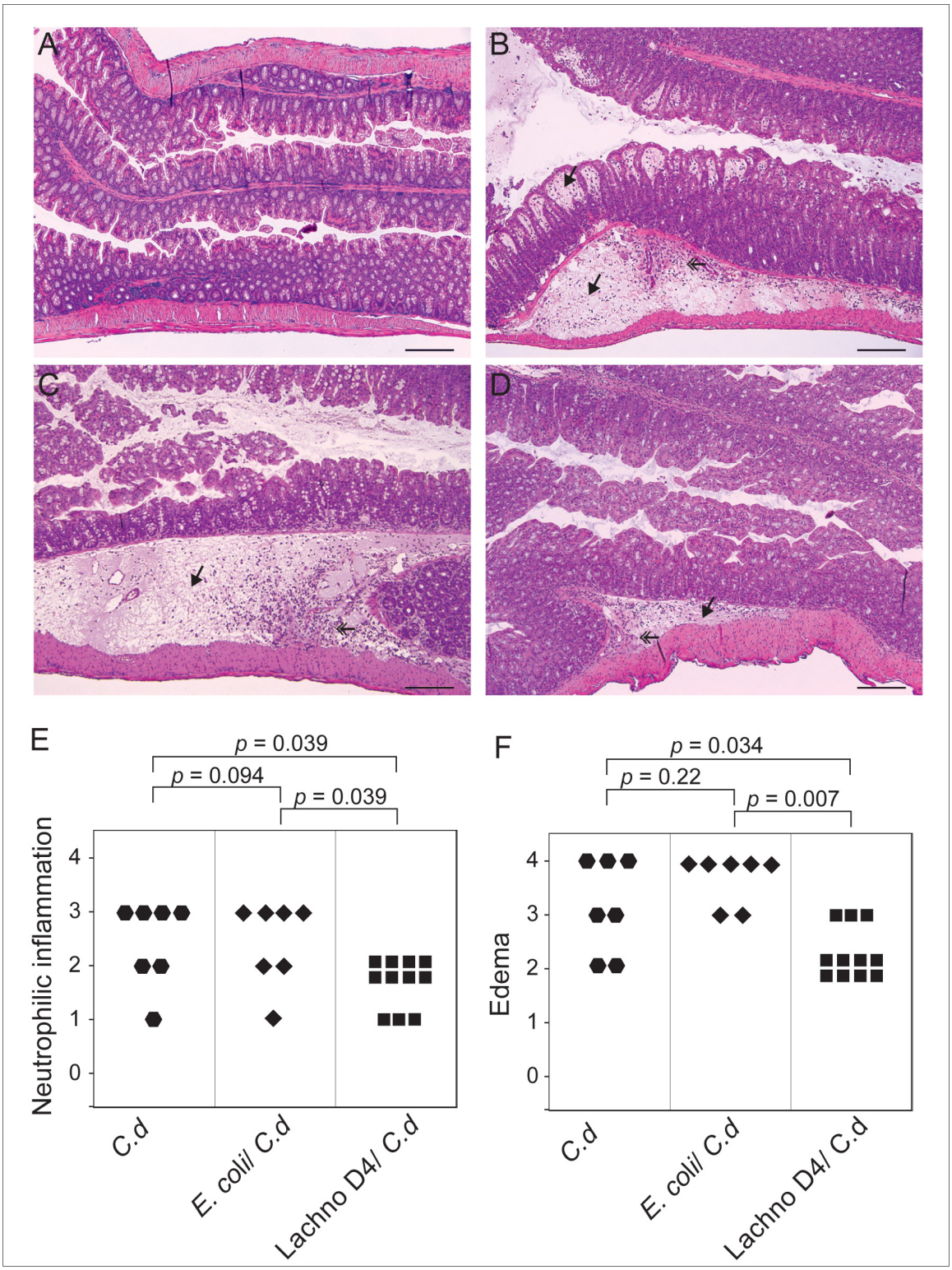

Figure 3.8 Germ-free animals pre-colonized with Lachnospiraceae D4 have improved colonic histopathology after *C. difficile* challenge. (A) Colon of a germ-free mouse. HE. Original magnification x100. (B) Colon of a *C. difficile* infected

mouse showing severe edema in the submucosa and mucosal lamina propria (arrow) accompanied by neutrophilic inflammation (arrowheads). HE. Original magnification x100. (C) Colon from a mouse pre-colonized with *E. coli* and infected with *C. difficile* showing submucosal edema (arrow) and neutrophilic inflammation (arrowheads) similar to a *C. difficile* infected mouse. HE. Original magnification x100. (D) Colon of a mouse pre-colonized with Lachnospiraceae D4 and infected with *C. difficile* showing moderate neutrophilic mucosal inflammation (arrowheads) but decreased submucosal edema (arrow) in comparison to *C. difficile* only and *E. coli* pre-colonized *C. difficile* infected mice. HE. Original magnification x100. Bar represent 100 μm. (E) Categorical scores of neutrophilic inflammation and (F) edema in *C. difficile* infected controls, *C. difficile* infected *E. coli* pre-colonized mice and *C. difficile* infected Lachnospiraceae D4 pre-colonized mice. Comparisons between groups were performed using the non-parametric Krustal Wallis test. *C.d- C. difficile,* Lachno D4- Lachnospiraceae D4

3.3.4 In vivo association of *C. difficile*, *E. coli* and Lachnospiraceae D4 in the colon

Thus far, I have demonstrated that Lachnospiraceae D4 decreases the level of *C. difficile* colonization in mice. Next I wanted to determine whether Lachnospiraceae D4 occupied a specific physical niche within the colon that affected *C. difficile* colonization. Therefore, I performed FISH on colonic tissue from mice colonized with both *C. difficile* and *E. coli* or Lachnospiraceae D4 to visualize the location of *C. difficile, E. coli* and Lachnospiraceae D4 and to determine whether any interactions with the gut epithelium were occurring between these organisms. *C. difficile* control mice demonstrated the presence of *C. difficile* primarily in the lumen of the colon (Fig. 3.9A, 3.9B). Mice pre-colonized with *E. coli* and infected with *C. difficile* demonstrated the presence of both *E. coli* and *C. difficile* localized to the colonic lumen. Numerous *E. coli* and *C. difficile* organisms were also observed visually in the colonic section examined

further confirming the inability of *E. coli* to limit *C. difficile* colonization (Fig. 3.9C, 3.9D). Mice colonized with both Lachnospiraceae D4 and *C. difficile* also demonstrated Lachnospiraceae D4 localization to the lumen. However, only few *C. difficile* cells were observed in the sample (Fig. 3.9E, 3.9F) further confirming the ability of Lachnospiraceae D4 to decrease the levels of *C. difficile* colonization. Based on these results no specific interaction with the gut epithelium or between each organism could be ascertained.

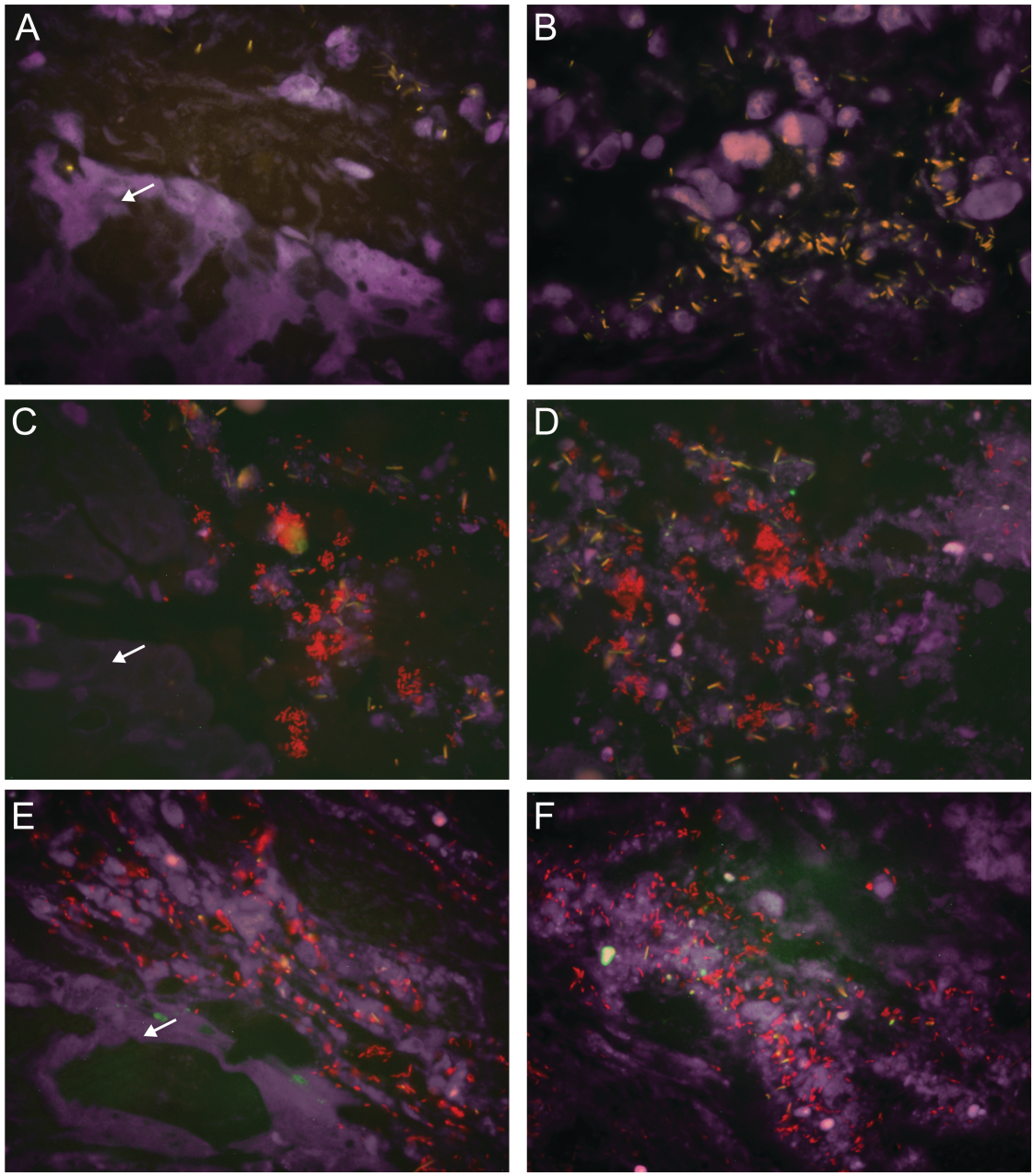

Figure 3.9 Examination of *C. difficile* infected colonic tissue sections by fluorescence in situ hybridization. Tissue sections were dual labeled with two fluorescence-labeled oligonucleotide probes, one specific for *C. difficile* and the other general for all bacteria. *C. difficile* appears orange/ yellow while *E. coli* and Lachnospiraceae D4 are red in color. Colon sections of: (A) *C. difficile* infected mice demonstrating the presence of *C. difficile* primary in the lumen and (B) central lumen, (C) mice pre-colonized with *E. coli* and infected with *C. difficile* demonstrated the presence of both organisms primarily in the lumen with high numbers of *C. difficile* present and (D) central lumen. (E and F) Colonic section

from a mouse pre-colonized with Lachnospiraceae D4 and infected with *C. difficile.* Lachnospiraceae D4 was predominant in the lumen with only few *C. difficile* present and (F) central lumen. Arrows represent position of intestinal epithelial mucosa. Original magnifications of all images are x1000.

3.3.5 Prevention of *C. difficile* colonization the GI tract of germ-free mice inoculated with cecal contents from wild-type mice

Previous results indicate that Lachnospiraceae strain D4 partially restores colonization resistance against *C. difficile* in germ free mice. As a control, I tested whether the full complement of cecal microbiota from wild-type mice could completely restore colonization resistance in germ free mice. Germ-free mice received cecal contents obtained from a wild-type mouse via oral gavage. Four days later, these mice were challenged with 100 *C. difficile* VPI 10463 spores (Figure 3.1). Unlike *C. difficile* mono-associated control mice, mice that were colonized with the cecal content homogenate prior to *C. difficile* challenge did not exhibit signs of clinical CDI such as weight loss, diarrhea and hunched posture. No detectable *C. difficile* was present in feces at one-day post challenge or in the cecal contents at two days post challenge or at the time of necropsy (data not shown).

3.4 Discussion

The indigenous GI microbiota plays a fundamental role in colonization resistance against *C. difficile* (43). However, the specific components of the gut microbiota that are important in mediating colonization resistance are not well defined. In this study we demonstrate that a single component of the murine gut

microbiota, a member of the family Lachnospiraceae, is able to partially restore colonization resistance against *C. difficile* in germ-free mice.

Until now no study has examined the ability of Lachnospiraceae organisms to contribute to colonization resistance against *C. difficile* or other pathogens. We recently reported that antibiotic treated mice with clinically severe CDI were predominated with *E. coli* while mice with mild CDI were predominated with Lachnospiraceae. Likewise, other studies have associated the microbial gut community of patients with inflammatory bowel disease (IBD) to have an increased prevalence of *E. coli* and decreased prevalence of Lachnospiraceae (15, 24, 25). Furthermore, patients with IBD are at a higher risk for developing CDI (14, 28). These findings prompted us to isolate and investigate the relative roles of Lachnospiraceae and *E. coli* in mediating colonization resistance against *C. difficile*

The Lachnospiraceae family is made up of many different genera including *Butyrivibrio, Lachnospira, Rosburia, Coprococcus, Blautia, Robinsoniella* only to name a few. Members within this family are usually Gram-positive, though some may appear Gram-negative due to the presence of a very thin peptidoglycan later in their bacterial cell wall (19). These organisms are strict obligate anaerobes and the nutritional requirement for growth of many of these organisms remains largely unknown. As a result only few members within this family have been cultured and studied. The Lachnospiraceae are phylogenetically placed within the clostridial cluster XIVa of the *Clostridium* subphylum based on 16S rRNA gene sequence (6, 8). Clostridial cluster XIVa is

one of the three main clostridial clusters of the low molecular % G+C Gram-positive bacteria that make up approximately 25% of the total bacterial species found in the human colon (10). Since many gut bacteria are adapted to an environment of low partial oxygen pressures they may lack electron transport chains usually present in facultative anaerobic bacteria to regenerate $NADH_2$ and therefore may not gain energy by electron transport level phosphorylation (10). As a result, the formation of acidic fermentation products due to the regeneration of NAD^+ occurs resulting in many Lachnospiraceae members being capable of producing large amounts of SCFAs such as acetate, propionate and butyrate. These SCFAs serve important roles in maintaining the overall health of the gut epithelium and aids in preventing mucosal inflammation (8, 24, 29, 48).

Our murine Lachnospiraceae isolates were members of the clostridial cluster XIVa (3.1). Lachnospiraceae organisms found in both humans and mice can have highly similar 16S rRNA gene sequences. In our study the murine Lachnospiraceae isolate D4 was phylogenetically similar to *Clostridium clostridioforme*, a Lachnospiraceae organism found in humans (Figure 3.1) (13). This organism is present as part of the indigenous gut microbiota in humans and has also been associated with opportunistic infections (13). However, Lachnospiraceae D4 did not produce disease in our monocolonized mice.

Germ-free mice have proven to be a useful tool for studying host-microbe and microbe-microbe interactions within the GI tract (11). Germ-free mice have been used to examine how individual bacteria or bacterial communities influence colonization resistance against *C. difficile* (7, 21, 37, 44, 46). In many cases, the

bacteria employed in an attempt to interfere with *C. difficile* were previously described as “probiotic” organisms or undifferentiated groups of bacteria derived from healthy animals. In the current study, we examined bacteria that were previously observed to be associated with normal or diminished colonization resistance to *C. difficile* (30). In this way, we used the results of culture-independent study of gut microbial ecology to inform and guide subsequent hypothesis-testing studies utilizing cultured bacterial isolates. We feel that this coupling of sequence-based microbial ecology studies with more traditional methods such as experimental animal infection represents a powerful way study bacterial pathogenesis.

Multiple mechanisms have been proposed that explain how the indigenous microbiota can mediate colonization resistance (reviewed (4)). It is likely that several factors are involved in mediating colonization resistance, but the production of bacterial products that directly inhibit pathogens has received significant experimental attention. Several investigators have examined the ability of bacterial fermentation products including SCFAs to inhibit *C. difficile* growth. Some studies have shown that butyrate is capable of inhibiting *C. difficile* in vitro (26, 31) although contradicting reports exist (37). Lachnospiraceae organisms are notable in that many are capable of fermenting complex carbohydrates to SCFAs, which have an important role in maintaining intestinal homeostasis (1, 8, 29, 48). We investigated whether SCFAs were associated with less *C. difficile* colonization in Lachnospiraceae pre-colonized mice and found that SCFA production did not correlate to lower *C. difficile* colonization levels (data not

shown). Therefore the decrease in *C. difficile* colonization levels due to Lachnospiraceae colonization is most likely attributed to the production of other metabolites or through other mechanisms.

It has been proposed that rather than specific inhibition by the production of metabolites such as SCFA or antimicrobial compounds including bacteriocins, the indigenous microbiota could simply be competing for limiting nutrients, the so-called nutrient niche hypothesis. Stated in brief, this hypothesis maintains that an organism can outcompete another if it utilizes a limiting nutrient more efficiently. It is possible that the diminished levels of *C. difficile* in the presence of Lachnospiraceae is due to less effective utilization of specific nutrients by the former (16). It should be noted however, that our experiments don't suggest that there is a simple mass effect with regards to nutrient utilization. When the levels of Lachnospiraceae D4 and *E. coli* colonization were measured, Lachnospiraceae D4 reached colonization levels 100-fold less than *E. coli* suggesting that simply occupying more "space" in gut, and presumably consuming proportionately more of the available resources does not necessarily contribute to colonization resistance.

Corthier and colleagues demonstrated that a neonatal *E. coli* strain significantly inhibited *C. difficile* cytotoxin (7). In our studies an *E. coli* strain indigenous to wild-type mice had no such effect on *C. difficile* cytotoxin or colonization levels in germ-free mice. Naaber and colleagues examined the effect of over 50 *Lactobacillus* strains on *C. difficile* growth inhibition and found only five strains that had antagonistic activity toward *C. difficile* (27). These

studies suggest that although strains may belong to the same genus/species, variation in their individual genetic content results in functional differences. Similarly, it would be important to test the ability of other Lachnospiraceae isolates for their ability to inhibit *C. difficile* in vivo. It is possible that this is an ability that is shared by the Lachnospiraceae as a group or is associated with a function that is more restricted to certain members.

We have demonstrated that a single Lachnospiraceae organism (D4) was able to decrease the level of *C. difficile* colonization in vivo and improve clinical outcome. Therefore, we wanted to test the hypothesis that colonizing mice with a community of Lachnospiraceae organisms could further restore colonization resistance against *C. difficile*. Germ-free mice were inoculated with a combination of the remaining 13 slow growing Lachnospiraceae isolates and monitored for colonization. However, these isolates were unable to colonize the germ-free mouse gut. There are a number of reasons why this may have occurred. After examining the growth of each of these Lachnospiraceae isolates on enrichment agar, the average time for visible growth was between 3 and 4 days. Additionally, growth in liquid culture was much less efficient. This slow growth may have hindered colonization since an organism must proliferate at an adequate rate if it is to maintain colonization. Additionally, many bacteria respond to co-colonization in species and sequence dependent manners. For example, in infants it has been shown that facultative anaerobes colonize the GI tract first, followed by obligate anaerobic organisms (34). A study performed by Syed and colleagues found that some strictly anaerobic bacteria could only colonize germ-

free mice after mice were pre-colonized with *E. coli* (38). These studies all suggest that our 13 Lachnospiraceae isolates possible relied on other bacterial sources to provide metabolites or a gut environment conducive for colonization and proliferation in germ-free mice.

Although monocolonization with Lachnospiraceae isolate D4 only partially restored *C. difficile* colonization resistance, there was complete restoration following the transfer of cecal contents from a wild-type mouse to germ-free mice. This implies that there are likely additive effects of specific microbiota in determining colonization resistance. Each member of the microbiota may partially contribute, but the entire community (or a specific subset) is required for complete colonization resistance. Others have observed only partial restoration of colonization resistance against *C. difficile* (7, 21). For instance, Itoh and colleagues colonized germ-free mice with multiple strains of Bacteroides and Lactobacilli and observed little antagonism toward *C. difficile*. Only when feces containing clostridia were administered to mice was *C. difficile* eliminated (21).

In summary, our results show that a single component of the gut microbiota, a murine Lachnospiraceae isolate, was able to partially restore colonization resistance against *C. difficile* and improve clinical CDI outcome. Further investigation of the members within the Lachnospiraceae family potentially in combination with other taxonomically distinct members of the indigenous microbiota could lead to a greater understanding of mechanisms of *C. difficile* suppression and the role that these organisms play in protection against a variety of other pathogens and disease states.

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Chapter 4

Discussion

4.1 Overview

The work described in this thesis examine the role of the microbiota in colonization resistance against *C. difficile*. The major findings are: 1) antibiotic administration resulted in shifts in the GI microbiota and these shifts were associated with the loss of colonization resistance to *C. difficile;* 2) specific microbial communities were associated with conferring resistance or susceptibility to *C. difficile* colonization; 3) a single component of the indigenous gut microbiota, a murine Lachnospiraceae isolate, was able to confer partial colonization resistance against *C. difficile*. The findings presented in previous chapters lay the foundation for many other studies that will further our knowledge on the role of the GI microbiota in preventing *C. difficile* colonization and pathogenesis. Future investigations developed from these studies will contribute to the field by attempting to unravel the mechanism(s) by which the indigenous GI microbiota contribute to colonization resistance against *C. difficile* and possibly other pathogens, thereby forging novel avenues towards treatment or prevention. This chapter aims to discuss the important findings in this thesis and elucidate possible mechanisms by which a specific subset of microbes, the Lachnospiraceae, contributes to colonization resistance against *C. difficile*.

4.2 Mechanisms by which Lachnospiraceae D4 contributed to partial restoration of colonization resistance against *C. difficile* in germ-free mice.

In the past, many theories have been put forward on the various mechanisms by which the GI microbiota suppress invading pathogens. Several investigators have shown that the indigenous GI microbiota present a natural barrier that interferes with the establishment of pathogens (1, 21). Common mechanisms that have been proposed include: competitive exclusion which includes occupation of attachment sites (2, 62), consumption of nutrient sources (13, 22, 68), changes in oxidation-reduction potential and pH (21), and production of antimicrobial substances such as bacteriocins, SCFAs and hydrogen sulphide (13, 21, 50). Additionally, the gut microbiota may also stimulate the host to produce various antimicrobial compounds such as defensins (55). We will discuss which of these potential mechanisms Lachnospiraceae D4 possibly utilized to contribute to partial restoration of colonization resistance against *C. difficile* in germ-free mice.

4.2.1 Competitive exclusion

A proposed mechanism by which the indigenous gut microbiota inhibit pathogen colonization is competition for specific physical niches within the intestine. These include either competition for attachment sites or physical space thereby competitively excluding the pathogen from the gut (12, 14, 23, 46, 66). Many researchers assume that adhesion of pathogenic bacteria to mucosal surfaces is the initial step of colonization and speculate that adhesion can be inhibited by physically blocking attachment receptors or by providing some form

of steric hindrance against the pathogen. Therefore, indigenous bacteria capable of attaching to the mucosal surface could prevent attachment and colonization by invading pathogens, a mechanism termed competitive exclusion (31).

Many studies have examined the ability of *C. difficile* to attach to intestinal epithelial cells and mucus and concluded that *C. difficile* could attach to epithelial cells. However, these studies were conducted in vitro (59, 62, 63) which may not reflect the in vivo state. Borriello and colleagues attempted to elucidate the GI mucosal association of *C. difficile* in hamsters and concluded that *C. difficile* was able to adhere to mucosal sites of the GI tract (2). However, there is one caveat pertaining to how this experiment was conducted. The adherence of *C. difficile* was measured by removing luminal contents from mice and then *C. difficile* was cultured by plating gut tissue (2). This is not an ideal method to conclusively determine if, and more importantly, where *C. difficile* attaches in the gut. Direct visualization of *C. difficile* attachment to the gut epithelium using microscopic techniques such as scanning or electron microscopy would be conclusive. Therefore, it is still remains unclear whether *C. difficile* attaches directly to the gut epithelium or where it colonizes in mice (4).

The epithelial layer of the colonic mucosa produces mucus, antimicrobial peptides and other proteins capable of forming electrostatic interactions with the lumen of the intestines (24, 37). Lachnospiraceae organisms have been shown to be closely associated with mucus on the colonic epithelial mucosa in both humans and conventional mice (11, 36, 37). However, there is a fundamental difference in the thickness of the mucus layers associated with conventional mice

compared to germ-free mice. Petersson and colleagues have shown that in germ-free mice the thickness of the mucus layer associated with the colonic epithelial mucosa is approximately four times thinner than that of conventional mice (45). These observations suggest that the germ-free state might not be ideal for examining specifically where certain bacteria might be localizing. However, for our purposes, I thought that determining the in vivo localization of *C. difficile*, *E. coli* and Lachnospiraceae D4 would provide some insight as to how these organisms were interacting in germ-free mice. Therefore, I examined colonic sections from germ-free mice pre-colonized with either Lachnospiraceae D4 or *E. coli* and infected with *C. difficile* VPI 10463 using fluorescent in situ hybridization (FISH) analysis. Results from this experiment indicated that *C. difficile, E. coli* and Lachnospiraceae D4 were primarily localized to the lumen of the colon. No attachment to the intestinal epithelium was observed by any of the organisms. However, comparatively fewer *C. difficile* were present in the lumen of mice pre-colonized with Lachnospiraceae D4 as compared with *C. difficile* or *E. coli* pre-colonized mice. This provided further confirmation of the decrease in *C. difficile* colonization observed in Lachnospiraceae D4 pre-colonized mice (Chapter 3, Figure 3.9). However, it was difficult to determine whether *E. coli* and Lachnospiraceae D4 had attached to mucus that was associated with the epithelial mucosa because of: (i) the presence of a loose and thin mucus layer that has been documented in germ-free mice (45); (ii) the fact that a fluorescently labeled probe specific for mucus identification was not used; and (iii) the FISH technique only provided visual data and could not provide direct evidence of

specific bacterial interaction. Therefore the fact that (i) *E. coli* and Lachnospiraceae D4 were present visually in comparatively similar numbers using FISH analysis and, (ii) *E. coli* colonized mice more than 100 times higher than Lachnospiraceae D4, suggests that physical competition for space was not associated with the decrease in *C. difficile* colonization by Lachnospiraceae D4.

4.2.2 Changes in the chemical environment of the gastrointestinal tract

One mechanism by which the GI microbiota could affect a pathogen is by changing the overall chemical environment of the gut. Changes in the community structure of the GI microbiota can dramatically alter the concentrations of various microbial metabolites (70). Many Lachnospiraceae organisms are capable of fermenting complex carbohydrates to SCFAs (7, 10). SCFAs, specifically acetate, propionate and butyrate are an important energy source for colonic enterocytes and provide other beneficial effects to the host. These include decreasing oxidative stress, inhibiting inflammation and even preventing carcinogenesis (20, 54, 60). Many studies have examined the ability of SCFAs to inhibit *C. difficile* growth. Rolfe and colleagues have shown that butyrate is capable of inhibiting *C. difficile* growth and causes loss of viability in vitro (50). In contrast, Su and colleagues repeated many of Rolfe's experiments and found contradictory results. Su and colleagues did not observe any *C. difficile* growth inhibition when acetic, propionic or butyric acids were tested at physiological concentrations in vitro (57). To further confirm this result, these researchers administered a SCFA solution to *C. difficile* mono-associated germ-free mice by daily oral gavage but found no change in the levels of *C. difficile* colonization

since SCFAs did not accumulate in the GI tract (57). Finally to assume a more natural state of SCFA production, germ-free mice were di-associated with *Clostridium butyricum*, (an organism capable of producing high levels of butyrate in vivo) and *C. difficile* but the level of *C. difficile* colonization remained unchanged compared to mono-associated *C. difficile* controls (57).

Other studies have shown that the concentration of SCFAs in mice and hamsters with an intact indigenous GI microbiota are high. These animals are only susceptible to *C. difficile* colonization following antibiotic treatment (50, 51, 61). Therefore, it is possible that SCFAs could be a contributing factor to colonization resistance against *C. difficile* (50, 51, 61). The cecal microbial communities of our conventional C57BL/6 mice are predominated by Lachnospiraceae (49). Previous studies conducted in our lab demonstrate the presence of high levels of SCFAs in wild-type mice (data not shown). We have also shown that following antibiotic treatment, the levels of Lachnospiraceae are decreased (Chapter 2, Figure 2.7). Additionally, measurement of SCFAs following antibiotic treatment are reduced dramatically when compared to control animals. This led us to hypothesize that the decrease in *C. difficile* colonization levels observed by pre-colonization of germ-free mice with Lachnospiraceae D4 might be associated with the production of SCFAs. Therefore, the level of SCFAs produced by Lachnospiraceae D4 was measured in vitro in rich broth (brain heart infusion) by gas chromatography mass spectrometry (GC-MS). Both Lachnospiraceae D4 and *E. coli* produced only acetate. However, Lachnospiraceae D4 produced 40% more acetate than *E. coli* (data not shown).

Furthermore, we also measured the concentration of SCFAs present in the cecal contents of germ-free mice mono-associated with either *E. coli* or Lachnospiraceae D4 and found no difference in the SCFA concentration as compared to germ-free mice controls. This result suggested that SCFA production by Lachnospiraceae D4 was not associated with the decrease in *C. difficile* colonization levels observed in germ-free mice. The decrease in *C. difficile* colonization levels by the Lachnospiraceae D4 could be due to the production of other metabolites that changed the chemical environment of the gut making it unfavorable for efficient *C. difficile* colonization. On the other hand, the Lachnospiraceae family is very diverse and consists of numerous genera that are associated with the production of high concentrations of SCFAs. Hence, the fact that SCFAs were not associated with lowered *C. difficile* colonization in our germ-free studies does not rule out the fact that these substances may play a role in contributing to colonization resistance against *C. difficile* in wild-type mice with a fully intact microbiota.

4.2.3 Production of antimicrobial substances

Another possible mechanism by which the indigenous GI microbiota directly inhibits growth and proliferation of pathogenic bacteria is through the production of antimicrobial substances (8). This antimicrobial activity can be multi-factoral. It can include the production of bacteriocins, SCFAs (as discussed earlier) which lower gut pH, nitric oxide, hydrogen sulphide, and hydrogen peroxide (27, 35, 43, 48). It is estimated that many bacterial species in the gut can produce antimicrobial substances. For instance, *Lactococcus lactis* has been

shown to secrete a cationic peptide that has antimicrobial activity against several *C. difficile* strains in vitro (48). Additionally, O'Shea and colleagues performed a culture-based screening of over 40,000 lactic acid bacterial colonies to assess antimicrobial activity from a variety of intestinal sources. These researchers demonstrated that only 23 strains were capable of producing a bacteriocin-like substance (40). The Lachnospiraceae family consists of many genera that have not been widely studied. Hence little information is available on their ability to produce bacteriocins or inhibitory compounds. One Lachnospiraceae member that has been well studied, *Butyrivibrio fibrisolvens,* has been shown to produce compounds (Butyrivibriocins) that inhibit the growth of a number of different bacterial species (7, 25). However the ability of Lachnospiraceae to inhibit *C. difficile* growth through the production of bacteriocins have not been demonstrated.

In this thesis I initially performed a simple in vitro test to determine whether Lachnospiraceae D4 was capable of producing and secreting an antimicrobial substance against *C. difficile.* In this experiment, *C. difficile* was streaked onto BHIS plates and then cross-streaked with Lachnospiraceae D4. Here, we would predict that if Lachnospiraceae D4 produced a bacteriocin or antimicrobial substance that inhibited *C. difficile* growth it would be secreted into the media surrounding the area of Lachnospiraceae D4 growth. Ideally this would result in a clear zone which would indicate that *C. difficile* growth was inhibited. However, no *C. difficile* inhibition was observed using this technique. In another in vitro experiment, Lachnospiraceae D4 and *C. difficile* were co-cultured in BHIS

broth and a growth curve of both organisms was performed over a 12-hr period followed by selective plating at 3-hour intervals. The expectation for this experiment was if Lachnospiraceae D4 produced an inhibitory substance, little to no *C. difficile* growth would be observed. Our results revealed that *C. difficile* growth was not significantly decreased compared to a *C. difficile* only control. These data do not provide evidence that production of bacteriocins or antimicrobial compounds by Lachnospiraceae D4 was responsible for the relative decrease of *C. difficile* colonization in germ-free mice.

4.2.4 Competition for Nutrients

It is also theorized that the indigenous GI microbiota utilize an appreciable portion of available nutrients or substrates in the gut thereby preventing the availability of nutrient reservoirs to invading pathogens (13, 15, 19). Disruption of the normal indigenous microbiota allows this nutrient reservoir to become available. This was demonstrated by Guiot and colleagues where *E. coli* growth was suppressed when directly injected into the cecum of a live rat but upon antibiotic administration, the *E. coli* population grew efficiently (19). Previous studies have investigated nutrient competition in the suppression of *C. difficile* using an in vitro model of continuous-flow (CF) culture, which closely reproduces the GI ecosystem found in a mouse cecum (16, 67, 68). Wilson and colleagues have investigated the role of nutrient competition by the colonic microbiota in *C. difficile* suppression (69). These researchers observed that *C. difficile* proliferated efficiently in CF culture in the presence of carbohydrate sources such as glucose, N-acetylglucosamine and N-acetylneuraminic acid but not when galactose,

mannose, xylose, arabinose and fucose was added. These results suggested that the latter carbohydrates were not *C. difficile's* primary substrate source (69). It has been demonstrated that bacteria from murine luminal contents inoculated into CF culture media consume between 75-99.5% of the total carbohydrates present. On the other hand, amino acids are not extensively used for metabolism by *C. difficile* or other cecal bacteria in CF culture (68).

The nutrient–niche hypothesis was initially presented by Freter and colleagues. In general, this concept states that species can coexist in the gut because each organism is capable of growing faster than all others on one or a few limiting nutrients. The rate of growth of each organism during colonization must be at least equal to it wash out rate from the gut (12, 14(29). In other words, two strains cannot grow equivalently in the gut as the metabolically efficient strain will out compete the other for similar nutrients unless the less efficient organism attaches to the gut wall or mucus layer associated with the gut epithelium. Based on my results I could not confirm that Lachnospiraceae D4 or *C. difficile* adheres to the mucus associated with the gut wall in germ-free mice. Therefore a possible mechanism by which Lachnospiraceae D4 contributed to colonization resistance against *C. difficile* was through direct competition for similar nutrients or substrates (not utilized by *E. coli*) within the gut. Later in this chapter I will discuss experiments that could be used to provide insight on whether Lachnospiraceae D4 and *C. difficile* competed for similar nutrient sources.

4.2.5 Immuno-modulatory effects

There are indirect mechanisms by which the indigenous gut microbiota inhibit enteric pathogen growth (4). Gut bacteria modulate the innate and adaptive immune systems by stimulating toll like receptors (TLRs) and by up-regulating cytokine expression in dendritic cells and peripheral white blood cells (18, 47). One way this is done is through the stimulation of host antimicrobial defense pathways, for example, the production of antimicrobial peptides such as RegIIIγ and defensins.

Changes in the gut microbial community can indirectly affect colonization resistance. For example, decreasing the overall bacterial community through the administration of antibiotics can result in decreased host production of the antimicrobial peptide RegIIIγ (6). RegIIIγ is one of several antimicrobial peptides produced by Paneth cells that specifically target Gram-positive bacteria because it binds to their surface peptidoglycan layer. The alteration of host immunity can lead to colonization with vancomycin-resistant Enterococcus (VRE) (3). In the murine model of CDI, the use of the five antibiotic cocktail resulted in marked decreases in the expression of RegIIIγ. However, unlike the case with VRE, this decrease in antimicrobial peptide expression was not associated with decreased colonization resistance to *C. difficile*. In fact, administration of clindamycin alone resulted in a temporary decrease in colonization resistance without any changes in RegIIIγ expression. These results suggested that RegIIIγ was not associated

with inhibiting *C. difficile* colonization. Since RegIIIγ targets Gram-positive bacteria it is highly unlikely that Lachnospiraceae D4 would stimulate its release.

Defensins are a family of proteins that can be secreted by immune cells such as neutrophils, intestinal Paneth cells and epithelial cells, which have bactericidal properties against enteric pathogens (32, 52). Gut bacteria may stimulate defensin activity by stimulating defensin expression, thereby strengthening intestinal defenses (4, 34). Additionally, many defensins are synthesized in an inactive form and must be activated by matrilysin (proteolyic) cleavage (52). One study has shown that mice defective in matrilysin production were more susceptible to severe *Salmonella* infection. This study also demonstrated that germ-free mice do not produce matrilysin which suggests that bacteria must be present in mice to stimulate matrilysin production in the intestine (30). Therefore, an alternative way in which gut bacteria could stimulate defensin activity is by stimulating the production of matrilysin, resulting in increased levels of activated defensins in the GI tract. Currently, defensin production has not been implicated as a mechanism of colonization resistance against *C. difficile* in vitro or in vivo. Germ-free mice colonized with Lachnospiraceae D4 prior to *C. difficile* exhibited a decrease in the level of *C. difficile* colonization. It is not known whether Lachnospiraceae organisms are capable of stimulating defensin expression in the host or are capable of producing matrilysin that could cleave and activate defensin molecules. Therefore, I will propose experiments to examine whether the production of

defensins stimulated by Lachnospiraceae D4 colonization, contributed to the suppression of *C. difficile* in germ-free mice.

Bifidobacterium and *Lactobacillus* have been shown to stimulate the host innate immune response and lamina propria dendritic cells by providing an anti-inflammatory effect (9, 38, 41). The host immune response has an important influence on *C. difficile* colonization and disease severity. For instance, it has been demonstrated that increased titers of antibodies to toxin A were associated with a decrease in CDI severity and *C. difficile* asymptomatic carriage (26, 28). Clinical evidence has linked a reduction in the level of colonization of members of the Lachnospiraceae family to chronic inflammatory disorders such as inflammatory bowel disease (56, 65). However, many of these studies have theorized that the inflammatory effect was due to differences in host immune response and metabolite production in the gut. For instance, Lachnospiraceae are capable of fermenting complex carbohydrates to SCFAs (7, 10). Specifically, butyrate is essential for maintaining intestinal homeostasis and has anti-inflammatory properties (20). However, due to limited knowledge and availability of cultivated members from the Lachnospiraceae family, work on the regulation of the host immune response in relation to *C. difficile* colonization have not been previously performed.

Many of the experiments performed in this thesis include the use of germ-free animals. Germ-free mice contain abnormal numbers of several immune cell types and cell products and possess deficiencies in local and systemic lymphoid structures. As a result, there may be reduced levels of secreted immunoglobulins

(IgA, IgG) and irregular cytokine levels/profiles (32, 39). However, bacterial colonization reverses this observed phenotype (33). Therefore, further examination of the level of pro-inflammatory (IL-1, IL-6, TNF alpha) or anti-inflammatory (IL10, IL-12) cytokines produced in germ-free mice pre-colonized with Lachnospiraceae D4 prior to and after *C. difficile* challenge could provide a more complete analysis on the engagement of the murine immune system in response to *C. difficile.*

4.3 Summary model by which Lachnospiraceae D4 partially suppressed *C. difficile* colonization in germ-free mice

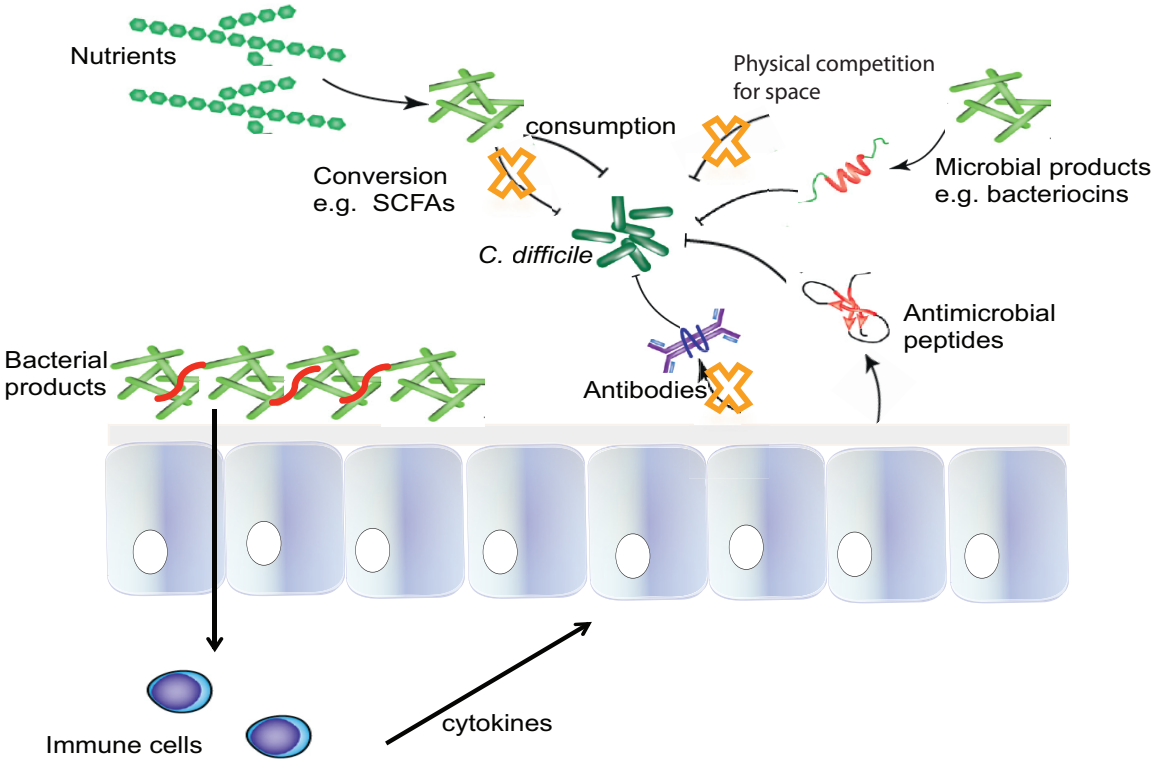

Figure 4.1 Model summarizing potential mechanisms by which Lachnospiraceae D4 partially restored colonization resistance against *C. difficile*. The gut microbiota could inhibit *C. difficile* colonization by direct competition for similar nutrients, production of SCFAs, competition for physical space and production of microbial products such as bacteriocins. It can also stimulate the immune system indirectly which can influence the adaptive immune response resulting in the production of antibodies, or it can stimulate an innate immune response causing the release of antimicrobial peptides. However, for Lachnospiraceae D4, some of these potential mechanisms can be eliminated as designated by the orange colored X. Stimulation of the adaptive immune response, physical competition for space and SCFA production can be eliminated. Figure adapted from (5)

Many mechanisms have been implicated in the suppression of *C. difficile* colonization by the indigenous gut microbiota. These include competition for nutrients and space, SCFA production, production of bacteriocins, and

stimulation of the adaptive and innate immune response (5). I have demonstrated that Lachnospiraceae D4 partially restores colonization resistance against *C. difficile.* Although the exact mechanism by which this occurred is undefined, my data suggest that SCFA production by Lachnospiraceae D4 was not associated with the decrease in *C. difficile* colonization and toxin production in germ-free mice. Additionally, competition for physical space was also not associated for the decrease in *C. difficile* colonization levels. My results demonstrated that even though the level of colonization by Lachnospiraceae D4 was approximately 100 times lower than *E. coli, E. coli* was not associated with colonization resistance against *C. difficile.* Finally, stimulation of the adaptive immune response can be eliminated as a potential mechanism by which Lachnospiraceae D4 suppressed *C. difficile* colonization. The adaptive immune response takes weeks to produce antibodies against pathogens. In Lachnospiraceae D4 colonized mice challenged with *C. difficile* VPI 10463, CDI was acute. As a result, this time period was not a long enough for the development of antibodies against *C. difficile*.

Therefore, I am postulating two mechanisms by which Lachnospiraceae D4 potentially contributed to colonization resistance against *C. difficile.* These are: (i) both Lachnospiraceae D4 and *C. difficile,* but not *E. coli,* competed for similar nutrient resources. As a result of this competition, Lachnospiraceae D4 was able to keep the *C. difficile* population under control; (ii) Lachnospiraceae D4 had a positive effect on the host immune response by inducing increased production of defensins (or matrilysin which activate defensins) that targeted *C. difficile*. Because of the reduction in the level of *C. difficile* colonization, there was

a corresponding decrease in toxin production. In turn, this resulted in only moderate disease and improved histopathology in germ-free mice pre-colonized with Lachnospiraceae D4 and infected with *C. difficile.*

4.4 Future directions

The goals of the research presented in this thesis were two-fold. First, I wanted to determine the effect of antibiotic administration in conferring susceptibility or resistance to *C. difficile* infection in murine models. Our findings suggested that specific microbial communities, the Lachnospiraceae family, may play a role in conferring resistance to *C. difficile* colonization and disease severity. Lastly, I wanted to determine whether a single component of the indigenous gut microbiota, a Lachnospiraceae isolate, was involved in mediating colonization resistance against *C. difficile.* My findings demonstrated that a single Lachnospiraceae isolate (D4) restored partial colonization resistance against *C. difficile.* However, the mechanism by which Lachnospiraceae D4 contributed to colonization resistance against *C. difficile* is undefined. To test the model described previously, I would propose the following specific aims and experiments to unravel the mechanism by which Lachnospiraceae D4 mediated colonization resistance against *C. difficile.*

4.4.1 Aim 1: To investigate whether partial suppression of *C. difficile* colonization in Lachnospiraceae D4 pre-colonized germ-free mice is associated with defensin production

Rationale

Defensins are a family of proteins that are secreted by immune cells and have bactericidal properties against enteric pathogens (32, 52). GI bacteria may stimulate defensin activity by stimulating the synthesis of defensin expression or proteases such as matrilysin that activate defensins (4, 34). Matrilysin, a matrix metalloproteinase (MMP7), is predominantly expressed by mucosal epithelial cells in normal tissues and in the ileal tissue in mice. A study performed by Lopez-Boudo and colleagues suggest that bacterial exposure is a potent and physiologically relevant signal capable of regulating matrilysin expression in epithelial cells (30). The only previous report exploring whether defensins protect against *C. difficile* was performed in vitro using a human epithelial cell line. These investigators reported that human alpha defensins inhibited *C. difficile* TcdB in vitro (17). However, the effects of these molecules have not been previously reported in vivo. I also reported RegIIIγ, another antimicrobial peptide produced by the host in response to certain bacteria, was measured in mice presenting with mild and severe CDI. Mice with severe CDI had a six-fold increase in the level of RegIIIγ when compared to mice with mild CDI. This result suggested that a direct relationship between changes in RegIIIγ expression and colonization resistance against *C. difficile* could not be made. However, the fact that RegIIIγ was not associated in preventing *C. difficile* colonization does not necessarily suggest that other antimicrobial peptides such as defensins function in the same way. In mice, there is evidence for the expression of 19 or more (highly similar) defensin genes in the small intestine (42). In order to determine whether Lachnospiraceae D4 stimulated host defensin expression, I will measure the

level of Paneth cell defensin effector expression in intestinal tissue of germ-free mice monocolonized with Lachnospiraceae D4 and di-associated with *C. difficile*.

Experimental approach

Comparison of Paneth cell defensin effector expression will be measured in mice monocolonized with Lachnospiraceae D4, *E. coli* and *C. difficile*. Germ-free mice monocolonized with *C. difficile* and *E. coli* will function as controls to determine whether the defensin levels are increased or decreased when compared to Lachnospiraceae D4 monocolonized mice. Additionally, germ-free mice will also be di-associated with Lachnospiraceae D4 or *E. coli* and *C. difficile* similar to that described in Chapter 3, section 3.2.5. Specifically, RNA will be isolated and quantified from ileal tissue as described by Wehkamp (64) then reverse transcribed to cDNA. Gene specific real time PCR using cDNA as a template will be done with specific oligonucleotide primer pairs for the following murine alpha defensin effectors: cryptidin 1, cryptidin 2, cryptidin 4, cryptidin 5, as previously described by Salzman and colleagues (52). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) will be used as a housekeeping control gene for data normalization.

Expected results and alternative approaches

I expect that there will be differential expression of specific defensins across all experimental groups of mice. Ideally, I expect that defensins present or up regulated in response to both *E. coli* and *C. difficile* colonization will not be associated with contributing to *C. difficile* colonization resistance. On the other

hand, defensins up regulated in response to Lachnospiraceae D4 and *C. difficile* co-colonization, but not in response to *E. coli* and *C. difficile* co-colonization may be important for inhibiting *C. difficile* growth. One potential pitfall to this approach is that there are approximately 19 different murine cryptidins currently identified (42). Therefore, additional oligonucleotide primer pairs for the remaining murine cryptidins will need to be designed based on sequences provided by Ouellette and colleagues (42). However, examining which cryptidins are up or down regulated across the various experimental groups of mice may be challenging. Therefore, an alternate approach that can be used to examine whether defensins play a role in *C. difficile* inhibition is to first examine which murine cryptidin inhibits *C. difficile* growth. An in vitro based bactericidal assay can be performed by growing *C. difficile* to log phase and inoculating 5 ml aliquots of *C. difficile* culture with purified murine cryptidin. At 30-minute intervals, I could sample each *C. difficile* aliquot over a 2-hour period and perform dilution plating on TCCFA to measure the level of *C. difficile* inhibition. This experiment will provide information on specific cryptidins that may be important for inhibiting *C. difficile* growth. As a result, this would be a targeted approach to measure the levels of specific cryptidins across the groups of germ-free mice.

One possible experimental outcome is that no change in defensin level will be observed across all experimental groups of mice used. In this case I will measure the levels of matrilysin (MMP7) in all experimental groups of mice. MMP7 can be measured in serum using a fluorescent based immunoassay method as described in Sarkissian et. al. (53). This will determine whether

Lachnospiraceae D4 promoted the activation of defensins, which in turn partially suppressed *C. difficile* colonization. If no association can be made from these experiments then I will rule out the possibility that defensins contributed to the decrease in *C. difficile* colonization in Lachnospiraceae D4 pre-colonized mice.

4.4.2 Aim 2: To determine whether competition for similar nutrients by Lachnospiraceae D4 and *C. difficile* is associated with decreased *C. difficile* colonization

Rationale

Many Lachnospiraceae members are capable of fermenting a plethora of soluble carbohydrate sources such as glucose, maltose, sucrose, fructose, cellobiose, xylose and arabinose (7, 10). Many of these organisms can also utilize amino acids and complex nitrogen sources such as casein, peptone and trypticase (7). Currently, I have not directly tested the carbon sources that Lachnospiraceae D4 utilizes. Both Lachnospiraceae D4 and *C. difficile* can grow on various types of culture media such as modified peptone yeast glucose (MPYG), BHIS and TCCFA (after 72 hours incubation at 37°C). Based on these data, I hypothesize that both of these organisms can utilize fructose, glucose and dextrose as carbohydrate sources and various nitrogen sources. Additionally, *E. coli* can grow on MPYG, BHIS and other enrichment media suggesting it too can utilize similar substrate sources. Thus, understanding the specific amino acids, carbohydrates or other carbon sources utilized by both Lachnospiraceae D4 and *C. difficile,* but not *E. coli* may provide a mechanism as to the exact mode by which Lachnospiraceae D4 partially suppressed *C. difficile* colonization.

Experimental approach

In order to determine substrate sources that are utilized by both Lachnospiraceae D4 and *C. difficile* but not *E. coli,* an in vitro approach can be utilized. Here I will grow *C. difficile, E. coli* and Lachnospiraceae D4 separately, and in co-culture (Lachnospiraceae D4 and *C. difficile; E. coli and C. difficile*) in complex media consisting of many different carbon, nitrogen and amino acid sources. Cultures that consist of individual bacteria will be grown to mid-log phase as determined by optical density (OD) measurement. Samples will be collected and centrifuged to obtain cell free supernatants. Co-cultures will be inoculated with $1x10^3$ CFU of each organism and then grown for 12 hours after which samples will be collected and centrifuged to obtain cell free supernatants. To measure the concentrations of carbohydrates present in each sample, a method described by Perini and colleague using fluoro-metric analysis of acid hydrolyzed samples and liquid chromatography will be done (44). Amino acid analysis will be performed using mass spectrometry (58). These data will provide the concentrations of each substrate utilized by each organism as compared to fresh media. Comparative analysis can be used to determine substrates that are utilized by both *C. difficile* and Lachnospiraceae D4 but not *E. coli*.

Expected results and alternate approaches

Examining the growth of each organism in complex media will provide information on the types of nutrients utilized. I expect that Lachnospiraceae D4, *E. coli* and *C. difficile* when grown in monoculture, will utilize many carbohydrates

and amino acids. This data will be important to compare the levels of carbohydrates and amino acids that are utilized when the organisms are grown in co-culture. For example, if both *C. difficile* and Lachnospiraceae D4 but not *E. coli* utilized mannose in mono-culture, then Lachnospiraceae D4 and *C. difficile* will be competing for the same sugar when grown in co-culture. In this case, I will expect that the level of mannose will be completely exhausted in the supernatant. On the other hand, I would expect that the level of mannose measured when *E. coli* and *C. difficile* are grown in co-culture to be higher. One caveat to this experiment is that the generation time for *E. coli* is faster than *C. difficile*. As a result of the static nature of the culture, *C. difficile* may be out competed by *E. coli* since it will utilize other resources in the media which may adversely affect *C. difficile* growth.

Although in vitro approaches can be useful, it does not represent what occurs in vivo. An alternate approach to test nutrient competition is to use germ-free mice. Germ-free mice will be mono-colonized with *E. coli*, Lachnospiraceae D4, *C. difficile* (630 strain) or di-associated with either *E. coli* or Lachnospiraceae D4 and *C. difficile.* Luminal contents will be collected along with contents from untreated germ-free mice as controls. Similar methods for carbohydrate (44) and amino acid analysis (58) will be used to measure the concentrations of various substrates differentially present in the luminal contents of each mouse from each group. Extrapolation of these data will allow us to determine whether competition for similar nutrients contributed to the decrease in *C. difficile* colonization in Lachnospiraceae D4 pre-colonized mice.

Summary and significance

Investigating potential mechanism(s) by which Lachnospiraceae D4 partially suppressed *C. difficile* colonization in germ-free mice could lead to the identification of general mechanism(s) used by other Lachnospiraceae organisms to mediate colonization resistance against *C. difficile*.

4.5 Overall summary and conclusions

A number of important conclusions can be made based on work presented in this thesis. I have demonstrated that antibiotic-treated mice can be utilized to study the effect of altering the indigenous gut microbiota as it relates to loss of colonization resistance against *C. difficile*. I have demonstrated that both the five-antibiotic cocktail and clindamycin were required to overcome colonization resistance to *C. difficile*. A key feature of this model was the ability to modulate disease severity by altering the challenge dose of *C. difficile.* When mice were challenged with 10^5 *C. difficile* VPI 10463 organisms, approximately 60% were moribund with high *C. difficile* load and cytotoxin levels. The remaining mice had less severe disease with significantly less *C. difficile* load and cytotoxin. This clinical phenotype allowed us to investigate differences in microbial ecology following antibiotic treatment and *C. difficile* infection. Shifts in microbial ecology following antibiotic treatment were associated with susceptibility to *C. difficile* infection. For instance, the gut community of mice pre-treated with the five-antibiotic cocktail and clindamycin shifted from a predominance of Firmicutes

(Lachnospiraceae) to Proteobacteria (*E. coli*). The gut communities of mice with severe CDI were similar to the gut community of antibiotic treated mice. On the other hand, the gut community of mice with clinically mild disease seemed to be recovering to the baseline community with a predominance of Lachnospiraceae.

I have also demonstrated that treatment of mice with cefoperazone results in severe disruption of the gut microbiota. These mice were also highly susceptible to *C. difficile* colonization and severe disease regardless of *C. difficile* VPI 10463 challenge dose. After a six-week recovery period, mice were susceptible to CDI following a single dose of clindamycin which suggested that cefoperazone was associated with prolonged alteration of the gut community. These results suggest that clinical outcomes following antibiotic pre-treatment and *C. difficile* infection may depend on the level of recovery of the altered microbial community toward the baseline state. For instance, if the altered microbial community recovers slowly then *C. difficile* may proliferate and produce toxin readily. This in turn may overwhelm the host and result in severe CDI. However, if the altered community is able to reach a level of recovery that is faster than *C. difficile* proliferation, then the indigenous (still altered) microbial community may be able to control the level of *C. difficile* expansion.

Based on the observed differences of GI communities in mice with mild and severe CDI, I tested the hypothesis that Lachnospiraceae was less permissive to *C. difficile* colonization than *E. coli*. Murine Lachnospiraceae and *E. coli* isolates were isolated from wild-type mice and tested in germ-free mice. This investigation revealed that Lachnospiraceae organisms play an important role in

limiting *C. difficile* colonization. Specifically, colonization of germ-free mice with a single Lachnospiraceae isolate D4 significantly decreased the level of *C. difficile* colonization and toxin by two different *C. difficile* strains and improved clinical CDI outcome when compared to *E. coli* pre-colonized and *C. difficile* control mice. Additionally, higher levels of bacterial colonization were not associated with decreased *C. difficile* colonization which suggests that *C. difficile* colonization was independent of bacterial load. I also investigated whether the production of SCFAs was a potential mechanism by which Lachnospiraceae D4 partially restored colonization resistance against *C. difficile* but found that it was not associated with the decrease in *C. difficile* colonization levels. Additionally, I also determined that competition for physical space was not associated with the partial suppression of *C. difficile* colonization. Although the specific mechanism(s) by which Lachnospiraceae D4 partially restored colonization resistance against *C. difficile* remains undefined, I postulate that nutrient competition and the production of immune-modulatory factors such as defensins may be involved.

Overall, the findings in this thesis demonstrate that: (1) the microbial community structure of the indigenous gut microbiota plays an important role in colonization resistance against *C. difficile* and disease severity, (2) the use of various tractable murine models are useful in studying the indigenous gut microbiota and (3) Lachnospiraceae plays an important role in contributing to colonization resistance against *C. difficile*. These results lay the groundwork for future study of other Lachnospiraceae and members of the GI community and

their role in contributing to colonization resistance against *C. difficile* and other pathogens.

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