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Dysbiosis in inflammatory bowel disease promotes clostridium difficile colonization

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Boston University
DYSBIOSIS IN INFLAMMATORY BOWEL DISEASE PROMOTES CLOSTRIDIUM DIFFICILE COLONIZATION

by

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NICHOLAS HAFFTEN

ABSTRACT

Research into the gut microbiome has revealed the widespread influence that microbial species have on their host. Host genetics and environmental factors influence the abundance and diversity of the bacterial species living within the gastrointestinal tract. When the normal composition of the gut microbiota is altered, a dysbiotic state incurs. Inflammatory bowel disease (IBD) is a chronic/relapsing inflammatory disorder of the intestinal mucosa, which is characterized by a state of dysbiosis. Despite the large amount of information studying the role dysbiosis has in the pathogenesis of IBD, it is not clear how the altered microbial composition of the gut in IBD patients leads to susceptibility to enteric pathogens such as Clostridium difficile. This study aims to highlight the features of the gastrointestinal tract that are modified as a result of dysbiosis in the IBD population, and how these features facilitate colonization by C. difficile and symptom development. Review of the available literature demonstrated that the depletion of Clostridial cluster XIVa in IBD-associated dysbiosis alters bile acid metabolism and butyrate fermentation in the colon, ultimately promoting germination of C. difficile spores and weakening the gut’s immune response against toxin-mediated inflammation. From continued research into the gut microbiota, more will be understood of how these microbial organisms influence human health and disease.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE</td>
<td>i</td>
</tr>
<tr>
<td>COPYRIGHT PAGE</td>
<td>ii</td>
</tr>
<tr>
<td>READER APPROVAL PAGE</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Sequencing Methods</td>
<td>2</td>
</tr>
<tr>
<td>1.2 The Human Microbiome</td>
<td>5</td>
</tr>
<tr>
<td>1.3 The Gut Microbiome</td>
<td>10</td>
</tr>
<tr>
<td>1.4 The Immune System and Gut Microbiota</td>
<td>15</td>
</tr>
<tr>
<td>1.5 Inflammatory Bowel Disease and the Gut Microbiome</td>
<td>20</td>
</tr>
<tr>
<td>1.6 Clostridium Difficile Infection and the Gut Microbiome</td>
<td>24</td>
</tr>
<tr>
<td>SPECIFIC AIMS</td>
<td>27</td>
</tr>
<tr>
<td>PUBLISHED STUDIES</td>
<td>28</td>
</tr>
<tr>
<td>2.0 Dysbiosis in IBD</td>
<td>28</td>
</tr>
</tbody>
</table>
2.1 Clostridial Cluster XIVa Functions in the Colon............................31
2.2 Clostridial cluster XIVa and Loss of Colonization Resistance.............32
2.3 Bile acid Metabolism..............................................................34
2.4 Metabolite Imbalance..............................................................40

DISCUSSION.................................................................................43

CONCLUSION..............................................................................47

REFERENCES..............................................................................49

CURRICULUM VITAE.....................................................................73
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Major antimicrobial peptides of the skin and intestines</td>
<td>18</td>
</tr>
<tr>
<td>2</td>
<td>Risk factors for <em>Clostridium difficile</em> infections</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>Documented microbial alterations in IBD</td>
<td>29</td>
</tr>
<tr>
<td>4</td>
<td>Changes in the gut microbiota by different types of antibiotics identified by 16S rRNA analysis</td>
<td>34</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The Process of 16S rRNA analysis</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>The Process of Whole Genome Shotgun Sequencing</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>HMP sample sites</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Clustering of bacterial composition based on microbial habitat</td>
<td>7</td>
</tr>
<tr>
<td>5</td>
<td>Variation of alpha and beta diversities between niches of the human microbiome</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>Phyla distribution varies across subjects and sample sites while metabolic pathways remain stable</td>
<td>10</td>
</tr>
<tr>
<td>7</td>
<td>Representation of the gut microflora in adults and the elderly</td>
<td>15</td>
</tr>
<tr>
<td>8</td>
<td>Diversified community of gut microbiota expand IgA repertoire in regulatory loop mechanism</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>The intestinal epithelium in inflammatory bowel disease</td>
<td>23</td>
</tr>
<tr>
<td>10</td>
<td>Proposed mechanism of the colonization resistance imparted by the gut microbiota</td>
<td>25</td>
</tr>
<tr>
<td>11</td>
<td>Regulation of gut homeostasis by <em>Clostridial</em> cluster XIVa</td>
<td>32</td>
</tr>
<tr>
<td>12</td>
<td>Bacteria biotransformation of bile salts in the colon</td>
<td>35</td>
</tr>
<tr>
<td>13</td>
<td>Bile acid metabolism and <em>C. difficile</em></td>
<td>37</td>
</tr>
<tr>
<td>14</td>
<td>Bile acid composition in the gall bladder and feces</td>
<td>38</td>
</tr>
<tr>
<td>15</td>
<td>Schematic of the gut microbiota involved in the metabolism of short chain fatty acids</td>
<td>41</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

AMP ................................................................. Antimicrobial Peptides
CD ................................................................. Crohn’s Disease
CDI ...................................................... Clostridium difficile Infection
FMT .................................................. Fecal Microbiota Transplantation
FISH ............................................. Fluorescent In Situ Hybridization
HMP ...................................................... Human Microbiome Project
IBD ...................................................... Inflammatory Bowel Disease
LPS ....................................................... Lipopolysaccharide
NIH ............................................................... National Institutes of Health
NF-κβ ........................................................ Nuclear Factor κβ
SCFA ...................................................... Short Chain Fatty Acids
UC ................................................................. Ulcerative Colitis
WGS ........................................................ Whole Genome Shotgun
INTRODUCTION

The human microbiome is characterized by the microorganisms that live in or on the human body. It is estimated that 100 trillion microbes reside in the human body, correlating to 10 times the number of human cells, and 100 times the number of gene products (Ley et al. 2006). Historically, science has focused on identifying pathological bacterial species and their involvement in disease. However, with the advent of high throughput technology such as 16S rRNA and whole genome shotgun (WGS) sequencing, there has been a vast increase in studies attempting to identify the commensal and symbiotic bacterial species living within the human body (NIH HMP Working Group et al. 2009).

The growing interest in the human microbiome spurred the initiation of the Human Microbiome Project (HMP). The overall goal of this NIH sponsored, 5-year, $157 million dollar project is to identify the residential bacterial species of the human body and determine their role in human health and disease (NIH HMP Working Group et al. 2009). In its jumpstart phase, initiated in 2007 and completed in 2012, the HMP aimed to generate reference databases representative of the human microbiome and to develop the necessary computational tools to analyze such data (National Institutes of Health, 2015). A total of 4,788 samples were collected across 15-18 body sites (15 for male and 18 for female including vaginal sites) from “healthy” participants ranging in ages 18-40 years old (Human Microbiome Project Consortium 2012b). “Health” was determined through clinical screening criteria set forth by the consortium. Each participant gave 1-3
samples, which were collected by swabs with the exception of stool, which was collected by the participant using a collection kit. As of early 2015, the HMP has sequenced over 1,300 genomes related to the human microbiome (NIH HMP Working Group et al. 2009; Human Microbiome Consortium 2012a; Human Microbiome Project - DACC HMP Reference Genomes 2015).

The HMP is currently in its second phase and has 3,024 reference genome and 1,265 metagenomics projects in progress (Human Microbiome Project - DACC HMP Data Catalog 2015; National Institute of Health 2015). With plans to complete this phase in late 2015, the HMP hopes to have compiled databases that will facilitate the understanding of what constitutes the “core microbiome”, a term used to describe the bacteria normal to the human body (NIH HMP Working Group et al. 2009; National Institute of Health, 2015). Ultimately, by knowing what constitutes a “healthy” microbiome, physicians and scientists will be able to identify an “unhealthy” microbiome, and hopefully develop methods to manipulate its status to improve human health.

1.1 Sequencing Methods

16S rRNA analysis and other sequencing methods, such as whole genome shotgun (WGS) sequencing, have facilitated the large growth in classification of unculturable microorganisms. Culture-based experiments were heavily relied upon before the development of molecular techniques, which greatly limited the study of microbial communities, as approximately 80% of bacterial are unculturable (Hugenholtz, 2002; Connon and Giovannoni, 2002). The development of 16S rRNA analysis resolved this
issue as this provided a way to analyze the genomes of these unculturable bacteria (Woese and Fox 1977; Woese 1982). The analysis of 16S rRNA is utilized since these sequences are sufficiently conserved in bacteria and yet varied enough to distinguish between bacterial species and other microbes such as Archaea (Weng et al. 2006). 16S rRNA analysis proceeds by extracting bulk DNA from the environment and then amplifying the 16S rRNA sequences using highly conserved PCR primers (Hugenholtz 2002) (Figure 1). The amplified sequences (typically 1,500 base pairs in length) are then cloned using vectors, clustered based on similarity, and compared to genomic libraries for phylogenetic classification (Weng et al. 2006). Once identified, these sequences give evidence to the microbial composition of the sample, both in terms of relative abundance and membership. By identifying the relative abundances of the bacteria present, the impact that particular species may have on the environment can be discerned.

**Figure 1. The Process of 16S rRNA analysis**
The extracted bulk DNA is amplified using site-specific primers for the 16S rRNA genomes. The products are then cloned and compared to genomic databases allowing classification of the species. Adapted from Weng et al. 2006.
While the 16S rRNA analysis is beneficial for the identification of species, it does not describe the functions of these microorganisms. To determine the functions of the bacteria in an environment, WGS sequencing is used (Tyson et al. 2004; Venter et al. 2004). WGS assesses the “metagenome” or collective genomic content of the microbial community within an environment (Handelsman et al. 1998). Instead of amplifying the genomic sequences as in 16S rRNA analysis, the DNA extracted from the environment is randomly cut, which creates many fragmented pieces of DNA (Figure 2) (Riesenfeld et al. 2004). These fragments are then cloned into vectors and subjected to functional-driven analysis and/or sequence-driven analysis (Handelsman, 2004). Functional-driven analysis entails transforming the clones into a host bacterium in order to determine the function of the cloned sequences. This process is helpful in identifying the functions of the metagenome, however, it is difficult to know from which bacteria the expressed gene product originated (Handelsman, 2004). Sequence-driven analysis provides insight into the types of bacteria contributing genomic content to the metagenome by looking for phylogenetic markers, or “anchors”, in the DNA flanking the cloned sequence (Handelsman, 2004). Researchers are then able to link gene products to the bacteria of origin (Handelsman, 2004). The utilization of sequencing methods such as 16S rRNA analysis and WGS has provided tools to studying the bacteria that live within or on the human body.
Figure 2. The Process of Whole Genome Shotgun Sequencing
Schematic representation of the construction of metagenomic libraries obtained from WGS. After creation of a metagenomic library, these sequences are subjected to either functional-driven or sequence-driven analysis in order to determine the function or species of origin, respectively. Adapted from Handelsman, 2004.

1.2 The Human Microbiome

The human microbiome is a complex collection of microbial ecosystems. Based on the areas sampled in the HMP, the human microbiome is divided into 5 main microbial habitats: oral cavity, nasal cavity, urogenital tract, skin, and GI tract (Figure 3) (Human Microbiome Project Consortium 2012b). The microbial composition of each area is assessed by the diversity of the genomic data obtained at each site. The variation in diversity exhibited by the different body habitats demonstrate that each site differs in its microbial composition in comparison to other sites (Figure 4) (Human Microbiome Project Consortium 2012b).
Additionally, the diversity of species observed varies at each site. For example, the oral cavity and GI tract exhibit a much more diverse bacterial composition, whereas the vagina harbors a relatively low diversity of bacteria (Costello et al. 2009; Human Microbiome Project Consortium 2012b).

**Figure 3. HMP sample sites**
Multiple sites were sampled from the oral cavity, skin, and vagina. Only stool and anterior nares samples were obtained from gut and respiratory tract, respectively. Appended from “Human Microbiome Project DACC - Microbiome Analyses,” 2015.
Figure 4. Clustering of bacterial composition based on microbial habitat
Principal coordinate plot demonstrates distinct variation patterns between body areas. Gastrointestinal, oral, and urogenital samples are distinguished from all areas. Skin and nasal samples overlap and are separate. Adapted from Human Microbiome Project Consortium 2012b.

The variations in microbial composition differ depending on both the body habitat and the individual (Human Microbiome Project Consortium 2012b). The diversity observed in the different locations of one subject is defined as the alpha diversity and the diversity observed at one location, but between subjects is termed the beta diversity. Thus, the alpha diversity would describe species variety of the skin microbiota of Person A, while beta diversity would compare the skin microbiota between Person A and Person B. The alpha and beta diversities of the human microbiome both vary in every major body area (Figure 5) (Human Microbiome Project Consortium 2012b). For example, the oral cavity demonstrates high alpha diversity and low beta diversity. This means that although the oral cavity was species rich, a majority of these species were conserved...
across individuals. On the other hand, samples taken from the vagina of healthy, non-pregnant women demonstrated both low alpha and beta diversities, correlating to both low species richness and low inter-individual variability.

Figure 5. Variation of alpha and beta diversities between niches of the human microbiome
a. Alpha diversity within subjects, grouped by individual niche, and measured using the relative inverse Simpson index of genus-level phylotypes (cyan), 16S rRNA gene OTUs (blue), shotgun metagenomic reads matched to reference genomes (orange), functional modules (dark orange), and enzyme families (yellow). The mouth and vagina demonstrate high and low alpha diversity, respectively. The other body niches generally display intermediate alpha diversity. Individual variation was often greater than variation between body habitats. b. Bray-Curtis beta diversity among subjects, grouped by niche, and measured in similar colors as (a). The skin demonstrates the greatest beta diversity. The mouth and vagina are relatively stable. The alpha and beta diversity are not directly comparable, though the variation observed in (a) exhibited a greater range than the variation observed in (b). Adapted from Human Microbiome Project Consortium 2012b.

Each body habitat of the human microbiome is dominated by one or several phyla (Figure 6a) (Human Microbiome Project Consortium 2012b). The abundance of the dominant clade varies depending on the individual, representing on average 17-84% of the total genera. However, in some individuals the typical dominant clade may represent 0% or 100% of the genera present. The less abundant taxa are highly diverse depending on the habitat and individual. For example, in the oral cavity the dominant genus was Streptococcus, but the next most abundant genus varies depending on the location within the oral cavity (buccal mucosa, supragingival plaque, and tongue dorsum) (Segata et al. 2012).

The variation in the microbial composition observed across individuals contrasts with the relative stability of the “core” metabolic functions across habitats (Figure 6b) (Human Microbiome Project Consortium 2012b). Turnbaugh et al. 2009 demonstrated that the assemblages of microbial species vary widely from person to person, yet the primary metabolic and biochemical profiles are conserved. This suggests that these “core” functions are not specific to particular clades, but are instead shared across a multitude of species. It is not clear how core functionality is determined given the highly
variable structure of the gut microbiota (Turnbaugh et al. 2009). Interestingly, low abundance metabolic pathways are highly conserved (Human Microbiome Project Consortium 2012b). For example, spermidine biosynthesis, methionine degradation, and hydrogen sulfide production are all active at low levels in the GI tract, but are present in >92% of subjects (Human Microbiome Project Consortium 2012b). It is speculated that many of these lowly expressed, highly conserved pathways are yet to be discovered and harbor the uncharacterized functions observed within the different body habitats.

Figure 6. Phyla distribution varies across subjects and sample sites while metabolic pathways remain stable
Vertical bars represent microbiome samples obtained from the seven niches with both shotgun and 16S rRNA data; bars indicate relative abundances colored by microbial phyla (a) and metabolic modules (b). One or several phyla typically dominate the niche. In contrast, metabolic pathways are stable across subjects and niches. Adapted from Human Microbiome Project Consortium 2012b.

1.3 The Gut Microbiome

The GI tract has received the most attention from the HMP, making up 25.5% (772/3,024) and 25% (253/1,265) of the ongoing reference genome and metagenomic projects, respectively (Human Microbiome Project - DACC HMP Data Catalog 2015).
is estimated that approximately $10^{13}$-$10^{14}$ microbial cells reside in the human gut, the most of any human body habitat (Human Microbiome Project Consortium 2012a). The microbial composition of the human gut varies widely between individuals on account of multiple factors such as race, diet, and medications (Human Microbiome Project Consortium 2012b). The relationship the body has with its microbiota ranges from gaining physiological benefits to receiving unwanted inflammation (Kaiko and Stappenbeck 2014). The amount of ongoing research in the gut microbiome reflects its overall complexity and enormity.

The initial colonization of the human gut begins at birth. Until recently, the gut microbiome was thought to be a completely nonexistent at birth (Clemente et al. 2012). However, samples from umbilical cord blood and meconium have demonstrated the presence of bacteria in utero (Jimenez et al. 2008). It is still widely accepted that the primary colonization event occurs during birth when the neonate is exposed to a variety of new bacterial species depending on the route of delivery (Adlerberth & Wold 2009; Dominguez-Bello et al. 2010). For example, neonates born via cesarean section exhibit early colonization by species resident to the skin, while neonates born via vaginal delivery are typically colonized by bacteria resident to the mother’s vagina (Dominguez-Bello et al. 2010).

Following birth, the gut is low in both diversity of species and number of microbial cells (Clemente et al. 2012). Initially, the human gut is host to facultative anaerobes, such as Proteobacteria, given the highly oxidative environment (Sommer and Backhed 2013). These early aerotolerant species are eventually replaced with anaerobes
as the gut decreases in environmental oxygen (Palmer et al. 2007). With exposure to solid foods and other agents such as antibiotics, the microbiome grows and widely fluctuates in composition (Koenig et al. 2011). By 11 months old, an infant’s microbiome contains microbial species that can be very different than the mother’s (Vaishampayan et al. 2010). At 12 months, the gut microbiome begins to stabilize and at 2.5 years it fully resembles its adult profile (Palmer et al. 2007; Koenig et al. 2011).

In adulthood, the density of microbial cells increases with progression from the stomach to the colon, with the colon harboring 70% of the microbial content of the entire GI tract (Eckburg et al. 2005; Gill et al. 2006; Ley et al. 2006). The adult gut microbiome is comprised mainly of bacteria, though Archaea, microbial Eukaryotes, and viruses also exist (Haynes and Rowher 2011; Clemente et al. 2012). Anaerobic species of bacteria outnumber aerobic and facultative species 100:1 and 1,000:1, respectively (Clemente et al. 2012). In general, membership at the phylum level is stable over time with Bacteroidetes and Firmicutes dominating the gut, and Actinobacteria, Proteobacteria, and Verrumicrobia present in comparatively smaller numbers (Figure 7) (Qin et al. 2010; Human Microbiome Project Consortium 2012b). However, the microbial composition at the genera level continually evolves in response to a variety of factors (Eckburg et al. 2005; Human Microbiome Project Consortium 2012b).

The microbial composition varies between and within each histologically distinct area of the GI tract. This is termed longitudinal variation and latitudinal variation, respectively. Longitudinal variation exists due to the differences within the gut environment (e.g. substrate availability, pH, host secretions, flow rate) (Human
Lachnospiraceae class of Firmicutes and Bacteroidetes dominate the colon due to slow flow rates, mildly acidic pH, and their ability to ferment indigestible glycans (Frank et al. 2007; Human Microbiome Project Consortium 2012b; Lee et al. 2013; Ng et al. 2013). On the other hand, in the small intestine, the Bacilli class of Firmicutes and Actinobacteria dominate due to the higher oxygen concentrations, availability of short chain carbohydrates and amino acids, lower pH due to bile acid secretions, and faster flow rates (Booijink et al. 2010; Zoetendal et al. 2012; Human Microbiome Project Consortium 2012b).

Latitudinal variation results primarily from the mucus layer that lines the intestines, which creates both a physical and chemical barrier to its inhabitants. Via fluorescence in situ hybridization (FISH) experiments, Swidsinski et al. 2005 demonstrated that only particular species of bacteria were able to colonize the mucus layer. For example, species from the genera *Clostridium*, *Enterococcus*, and *Lactobacillus* were found in both feces and the mucus layer, however, species from other genera such as *Bacteroides*, *Bifidobacterium*, *Streptococcus*, and *Ruminococcus* were only found in feces. These findings suggest that sampling the stool is inadequate in determining the microbiota of the colonic mucosa, and vice versa. However, 16S rRNA analysis of the bacterial genomes in the colonic mucosa demonstrated similar proportions of bacterial sequences as ones obtained from stool (Eckburg et al. 2005; Wang et al. 2005; Durban et al. 2010; Hong et al. 2011; Stearns et al. 2011; Human Microbiome Project Consortium 2012b). For example, the Firmicutes to Bacteroidetes ratio
determined from mucosal samples ranged from 1.27 (50.9% to 40.2%) to 4.62 (76.2% to 16.5%), which were well within the ratios of stool samples displayed in Figure 5 (Eckburg et al. 2005; Wang et al. 2005; Durban et al. 2010; Hong et al. 2011; Stearns et al. 2011; Human Microbiome Project Consortium 2012b). At the time this paper is written, it appears that both types of samples are adequate in determining the bacterial composition of the gut at the phylum level, though discrepancies arise when analyzing at the genera level. It will become more important to discern between the types of samples and how they relate to metagenomes, as more is understood of how particular species impact the gut microbiome.

As people age, there are general differences in the microbial composition of the gut in comparison to younger adults. Claesson et al. 2011 compared the microbial composition of participants >65 years of age to participants between 18-58 years of age. This study found that the elder group demonstrated a shift in the Bacteroidetes:Firmicutes ratio, as well as altered levels of Clostridial groups (Figure 7) (Claesson et al. 2011). The authors proposed that these changes might result from increased usage of oral medications taken to treat various morbidities associated with advanced age. As the gut microbiota take part in metabolism of various drugs including acetaminophen, digoxin, and lovastatin, it is not unforeseeable that these drugs exert effects on the bacterial populations (Saad et al. 2012; Yoo et al. 2014).
The core fecal microbiota of elderly subjects (>65 years old) at the level of phylum, genera, and *Clostridial* cluster compared to the core microbiota of nine younger adult controls and other documented core microbiota. Elderly subjects demonstrate a higher Bacteroidetes:Firmicutes ratio and altered genera/*Clostridial* cluster distribution than younger adults. Adapted from Claesson et al. 2011.

### 1.4 The Immune System and Gut Microbiota

Over the past millions of years, mammals and their microbiota have evolved a symbiotic relationship (Ley et al. 2008). However, given the proximity of the microbes to the intestinal epithelium, there is always risk unwanted inflammation. The host immune system aids in the development of the microbial community and continually monitors its composition (McFall-Ngai, 2007; Chow et al. 2010; Kawamoto et al. 2014). The development of the gut microbiota in tandem with the gut immune system is imperative to gut homeostasis; failure to develop properly results in hypersensitivity or autoimmune disorders (Loftus, 2004; Halfvarson et al. 2006; Wen et al. 2008; Lee and Mazamanian 2010).

The mucus layer, a component of the gut’s innate immune system, is the predominant barrier between host tissue and residential microbiota (Johansson et al. 2011). As discussed above, the mucus layer partitions the gut microbiota since only...
particular species are able to colonize the mucosa (Swidsinski et al. 2005). The mucus layer is composed of an inner and outer layer made of sheets of mucin secreted by Goblet cells (Johansson et al. 2008; Johansson et al. 2011). Mucin 2 (MUC2), a highly glycosylated protein, is the predominant component of the mucus layer and provides antimicrobial protection (Johansson et al. 2011). For example, Muc2-deficient mice are shown to be more susceptible to spontaneous and chemically induced colitis (An et al. 2007; Petersson et al. 2011; Fu et al. 2011). Because of the scaffolding of mucin sheets, bacteria are unable to maneuver within the inner layer of the mucosa, though they can occupy the outer layer (Johansson et al. 2008; Juge, 2012; Ambort et al. 2012). There is evidence that the gut microbiota influences the structure of the mucus layer. In comparison to conventionally housed mice, germ-free mice displayed fewer Goblet cells on average, which corresponded to a thinner mucus layer and altered mucin content (Sharma et al. 1995). However, by introducing bacterial cell wall components such as peptidoglycan and lipopolysaccharide (LPS) in the germ-free mice, the mucus regained normal characteristics (Petersson et al. 2011). This suggests that the microbiota are closely involved in the development gut epithelium and mucus layer, though details of this process are still unclear (Peterson et al. 2011).

To a similar effect, antimicrobial peptide (AMP) secretion is stimulated by direct contact with microbial cells. These innate effector molecules are considered “natural” antibiotics and are produced by almost every plant and animal (Zasloff, 2002). AMPs vary by type, body niche, secretory cell, and cellular target (Table 1). In the gut, AMPs are primarily produced and secreted in the crypts of Lieberkuhn by Paneth cells, though
intestinal epithelial cells and some immune cells also participate along the length of the GI tract (Gallo and Hooper, 2012). The secretion of AMPs is stimulated by the gut microbiota. Studies using a murine model demonstrated that germ-free mice displayed significantly lower levels of β-defensins and RNases in comparison to their conventionally raised littermates, suggesting the presence of the microbiota is the trigger for AMP release (Hooper et al. 2003). Several molecular pathways govern AMP expression. For example, Reg3-γ (C-type lectin) expression is triggered through a toll-like receptor (TLR) dependent manner whereas other AMPs are dependent on activation of intracellular nucleotide binding domains (NODs) (Brandl et al. 2006; Petnicki-Ocwieja et al. 2009). NODs and TLRs are activated by a variety of stimuli including microbial antigens, such as cell wall components and nucleic acid, as well as non-microbial antigens such as food particles (Gallo and Hooper, 2012). This suggests that the local microbiota and gut environment play a pivotal role in activating of these molecular pathways, impacting the secretion of AMPs and gut homeostasis.
Table 1. Major antimicrobial peptides of the skin and intestines

<table>
<thead>
<tr>
<th>Family</th>
<th>Representative proteins</th>
<th>Mechanism of action</th>
<th>Tissue sites of expression</th>
<th>Cellular sites of expression</th>
<th>Target organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-defensins</td>
<td>ANG4, angiogenin 4; BD, β-defensin; CRAMP, cathelin-related antimicrobial peptide; DEFA, α-defensin; GAL, galectin; HIP/PAP, hepatointestinal pancreatic/pancreatitis-associated protein; NA, not applicable; PGLYRP, peptidoglycan recognition protein; REG3γ, regenerating islet-derived protein 3γ; RNase, ribonuclease. Adapted from Gallo and Hooper, 2012.</td>
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</tr>
<tr>
<td>β-defensins</td>
<td>BD1, BD2, BD3</td>
<td>Membrane disruption; lipid II binding (BD3)</td>
<td>Large intestine, skin, respiratory tract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calponerin (SERAAR-SKOA)</td>
<td>NA</td>
<td>Membrane disruption</td>
<td></td>
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<td></td>
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<tr>
<td>Cathelicidin</td>
<td>LL37 (in humans); CRAMP (in mice)</td>
<td>Membrane disruption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>HIP/PAP (in humans); REG3γ (in mice)</td>
<td>Membrane disruption; lipid II binding (BD3)</td>
<td>Large intestine, skin, respiratory tract, pancreatic/pancreatitis-associated protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Galectin</td>
<td>GALα, GALβ</td>
<td>Unknown</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lipocalin</td>
<td>Lipocalin 2</td>
<td>Secretion of iron-laden siderophores</td>
<td>Intestine, broad expression, including intestine and lung</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Lysozyme     | NA                                                                                      | Enzymatic attack on bacterial cell wall peptide

The adaptive immune system is also linked to the gut microbiota. IgA is the principal component of the adaptive immune system of the gut mucosa (Gutzeit et al. 2014). IgA is transcytosed across the intestinal epithelium and secreted into the intestinal lumen, where it opsonizes bacteria for destruction. The gut microbiota influence the secretion of IgA in the intestines. For example, germ-free mice display marked decreases in IgA secretion compared to conventionally raised mice (Moreau et al. 1978). This immunological defect is reversible when the germ-free mice are colonized with bacteria from a normal mouse colon, suggesting that the gut microbiota are responsible for inducing IgA secretion in the intestines (Hapfelmeier et al. 2010). IgA secretion is
initiated by the migration of activated dendritic cells from the intestinal epithelium to secondary lymphoid structures, where antigens are presented to naïve B cells for activation and class switching (Macpherson and Uhr 2004; Wesemann et al. 2013). Through this mechanism, the gut microbiota “educate” the adaptive immune system to target luminal antigens, increasing IgA specificity (Wesemann et al. 2013).

The secretion of IgA affects the density and diversity of the gut microbiota. By opsonizing bacteria within the intestinal lumen, IgA aids in the elimination of microbial cells, thus preventing overgrowth. Without IgA secretion into the gut, there is an increased density of bacterial cells in the lumen, increased translocation of select species across the intestinal epithelium, and increased numbers of bacteria draining from the mesenteric lymph nodes (Johansen et al. 1999; Wei et al. 2011). The diversification of the gut microbiota by the IgA secretion is carried out in a positive feedback manner. Kawamoto et al. 2014 demonstrated that the diversity of the gut microbiota is dependent on the variety of IgA repertoires of intestinal B cells. In this study, Foxp3+ regulatory T cells (Tregs) were transferred into T cell deficient mice, which expanded the IgA specificity of B cells in Peyer’s patches (Figure 8). This in turn increased the diversity of the gut microbiota in terms of representative species, particularly in the Firmicutes phylum, which induced Treg differentiation. This positive feedback loop illustrates the symbiotic relationship between the host and gut microbiota and also the critical role the adaptive immune system has in maintaining gut homeostasis. Alterations to either the gut microbiota or the immune system can have negative effects on the host, as is in the case of inflammatory bowel disease (IBD).
Figure 8. Diversified community of gut microbiota expand IgA repertoire in regulatory loop mechanism
IgA secretion into the lumen of the intestines modulates the diversity of the gut microbiota. Increased diversity and expansion of Firmicutes results in Treg (Foxp3+) differentiation. Migration of the differentiated Tregs to germinal centers induces IgA selection, generating a pool of plasma cells with a diverse IgA specificity. Adapted from Kawamoto et al. 2014.

1.5 Inflammatory Bowel Disease and the Gut Microbiome

Inflammatory bowel disease is a chronic relapsing inflammatory condition of the intestinal mucosa, which is characterized by disruption of the normal gut homeostasis.
The two major forms of IBD, Crohn’s disease (CD) and ulcerative colitis (UC), differ in clinical manifestations and symptoms (Targan et al. 2003). CD affects the entire length of the GI tract and patients generally present with fatigue, diarrhea (no bloody stools), fever, diffuse abdominal pain, and/or weight loss (Targan et al. 2003). Divergently, UC typically affects only the colon and patients present with rectal bleeding, tenesmus, mucousy stools, lower abdominal pain, and/or frequent stooling (Targan et al. 2003).

The incidence of IBD is increasing worldwide and varies depending on region (Desail and Gupte 2005; Zheng et al. 2005; Molodecky et al. 2012;). It is estimated that over 5 million individuals are affected by IBD including 1.6 million from the United States and 3 million from Europe (Burisch et al. 2013). Changes in overall diet and hygiene correlate with the increased prevalence of IBD (Molodecky et al. 2012). For example, in comparison to the general population of North American and Western European countries, immigrants moving from developing countries to these modernized countries display a higher incidence of IBD (Probert et al. 1992; Barreiro-de Acosta et al. 2011). With yearly expenditures per patient costing more than diabetes, stroke, coronary artery disease, chronic obstructive pulmonary disease, or multiple sclerosis, it is clear that the rising prevalence of IBD is a major burden on health care costs (Kappelman et al. 2007; Gunnarsson et al. 2012).

The definitive etiology of IBD is unclear, though the current pathological model describes a “multihit” phenomenon that precipitates the disease (Figure 9) (Bringiotti et al. 2014). Factors contributing to the onset and pathology of IBD include genetic, environmental, and immunological triggers (Targan et al. 2003). Large-scale genetic
studies have identified 163 loci related to gut homeostasis and IBD susceptibility (Khor et al. 2011; Jostins et al. 2012). *Nod2*, a gene coding for a type of NOD, was the first gene implicated in host susceptibility to IBD and is still considered to be the strongest genetic determinant, though there are now a handful of genes identified as known markers for IBD (Hugot et al. 2001; McCauley and Abreu, 2012; Connelly et al. 2015).

Most genetic markers for IBD are mutations in proteins linked to the immune system, which influence the state of the gut microbiome. For example, NOD2 is a protein expressed on several innate immune system cells including intestinal epithelial cells, dendritic cells, and Paneth cells (McCauley and Abreu, 2012). NOD2 detects muramyl dipeptide (a component of LPS) and triggers downstream activation pathways such as mitogen-activated protein kinase and nuclear factor κβ (NF-κβ) (Abraham and Cho, 2009). In IBD, there is increased expression of NOD2 on Paneth cells, resulting in dysregulated inflammatory cytokine expression and impaired activation of NF-κB, ultimately decreasing AMP secretion and contributing to a pro-inflammatory state. (Bonen et al. 2003; Lala et al. 2003; Tan et al. 2015).

The altered microbial composition of the gut in IBD is well studied. The term used to characterize a shift in the normal levels of representative microbiota is dysbiosis. The dysbiosis and pro-inflammatory state in IBD are related. However, it is unclear if the dysbiosis in IBD is a direct cause of inflammation or a reflection of the inflammatory processes inherent to the disease pathology (Carding et al. 2015). The general changes in the composition of the gut microbiota are well documented (Frank et al. 2007). Some studies have aimed to identify select species responsible for the induction of IBD,
however, there has been no conclusive evidence supporting a causal relationship between a singular species and IBD pathogenesis (Darfeuille-Michaud et al. 2004; Rosenfeld and Bressler, 2010). Interestingly, the loss of a several key species appears to influence the disease. For example, a reduction in the abundance *Faecalibacterium prausnitzii* has been implicated in enhancing inflammation in IBD as these bacteria demonstrate anti-inflammatory effects *in vitro* and *in vivo* (Packey and Sartor, 2009; Qiu et al. 2013). Collectively, the changes in the gut environment that result from the dysbiosis contribute to the pathogenic inflammation seen in IBD.

**Figure 9. The intestinal epithelium in inflammatory bowel disease**
Genetics, gut microbiota, and imbalanced inflammatory response disrupt homeostasis at the intestinal epithelium. This results in mucosal inflammation and tissue damage. Adapted from Coskun, 2014.
1.6 CDI and the Gut Microbiome

*Clostridium difficile* infection (CDI) is a major health care concern as it represents 10-35% of all antibiotic-induced diarrheal illness, costs the United States an estimated $3.2 billion dollars in health care spending, and kills close to 14,000 people a year (Ricciardi et al. 2007; O’Brien et al. 2007; Centers for Disease Control and Prevention 2013). Since its pathogenic role was discovered in 1978, CDI has been the leading cause of nosocomial-related diarrhea (Bartlett, 2006). CDI is primarily a nosocomial disease with 80% of its cases starting in a hospital or long-term care facility setting (Khan and Elzouki 2014). Because of its increased incidence, severity of disease, and poor outcomes, CDI has gained interest from the scientific community (Kuijper et al. 2006). As a result, since 2003, there has been a 3-fold increase in number of published articles studying *C. difficile* (Le Monnier et al. 2014).

*C. difficile* is a Gram-positive spore-forming anaerobe that infects the colon of mammals, causing illness ranging in severity from mild diarrhea to fulminant colitis (Kutty et al. 2010). However, *C. difficile* is considered to be a normal member of the gut microbiota (Bien et al. 2013). In a healthy gut, colonization resistance from the resident microbial community prevents colonization of *C. difficile* (Figure 10) (Pechine et al. 2007). However, following short-term changes in the microbial diversity and distribution of the gut, *C. difficile* is able to colonize the gut readily (De La Cochetiere et al. 2008). Pathogenesis of CDI is initiated after ingestion and germination of *C. difficile* spores (Rupnik et al. 2009). The germination process is not well understood and the current knowledge of the process is based on studies of other *Clostridial* species (Edwards and
McBride, 2014). Following germination, vegetative *C. difficile* cells release exotoxins A and B, which cause the symptoms of CDI by inducing a pro-inflammatory state and disrupting the integrity of the intestinal epithelium (Voth and Ballard, 2005; Kelly and Kyne, 2011). The pro-inflammatory state ultimately causes migration of neutrophils to the affected site, leading to pseudomembrane colitis, the hallmark characteristic of CDI (Bobak et al. 2008).

**Figure 10. Proposed mechanism of the colonization resistance imparted by the gut microbiota**

(Left) Microbial metabolites and host factors inhibit the germination and growth of *C. difficile*. A healthy and diverse community of gut microbiota also competes for resources, limiting the growth of *C. difficile*. The normal gut microbiota also modulates inflammatory responses of the host tissue and stimulates IgA and AMP secretion. (Right) Perturbation of the normal gut microbiota caused by antibiotics or other factors alters metabolite production and host factor secretion, facilitating the germination of *C. difficile* spores. The dysbiotic state in combination with toxin release by vegetative *C. difficile* alters the immune response of the gut, promoting a pro-inflammatory state and resulting in damage to the epithelium. Adapted from Seekatz and Young, 2014.
There are many factors linked to the development of CDI (Table 2). These risk factors facilitate the pathogenesis of *C. difficile* by increasing exposure to spores or altering the composition of the gut microbiota. For example, the 3 primary risk factors for developing CDI are recent hospitalization, advanced age, and antibiotic usage within the previous 3 months (Stanley and Burns, 2010). Although these risk factors are found in a majority of CDI cases, they are not mandatory in the induction of disease. For example, roughly 60% of IBD patients diagnosed with CDI are treated with antibiotics preceding diagnosis (Issa et al. 2007). This suggests that the development of CDI typically involves a combination of factors and not a singular entity.

<table>
<thead>
<tr>
<th>Primary risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age greater than 65 years</td>
</tr>
<tr>
<td>Antibiotic use within the last 3 months</td>
</tr>
<tr>
<td>Hospitalization</td>
</tr>
<tr>
<td>Secondary risk factors</td>
</tr>
<tr>
<td>Female gender</td>
</tr>
<tr>
<td>Double occupancy rooms</td>
</tr>
<tr>
<td>ICU admission</td>
</tr>
<tr>
<td>Admission to a long-term care facility within the last year</td>
</tr>
<tr>
<td>Postpyloric tube feedings</td>
</tr>
<tr>
<td>Acid reducing therapy</td>
</tr>
<tr>
<td>Gastrointestinal procedures</td>
</tr>
<tr>
<td>Hypoalbuminemia</td>
</tr>
<tr>
<td>Renal disorders</td>
</tr>
<tr>
<td>Organ transplantation</td>
</tr>
<tr>
<td>HIV</td>
</tr>
<tr>
<td>Autoimmune disease</td>
</tr>
<tr>
<td>Malignancy or chemotherapy</td>
</tr>
<tr>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>Smoking</td>
</tr>
</tbody>
</table>

*Table 2. Risk factors for Clostridium difficile infections*

Adapted from Stanley et al. 2013.
SPECIFIC AIMS

As IBD is a risk factor for the development of CDI, the increased incidence of CDI in the IBD population is well documented (Issa et al. 2007; Bossuyt et al. 2009; Goodhand et al. 2011). Disruption of the gut microbiota clearly plays a role in altering the gut environment of IBD patients. However, it is unclear to how this dysbiosis influences susceptibility to CDI. This study aims to highlight the GI features of IBD that result from its dysbiosis, and how these features facilitate colonization by C. difficile and symptom development.
2.0 Dysbiosis in IBD

In general, the dysbiosis associated with IBD is defined by an overall decrease in diversity of microbial species within the gut, correlating with a decrease in number of non-redundant bacterial genes as well (Ott et al. 2004; Frank et al. 2007). There has been a multitude of studies analyzing the gut microbiota of IBD patients (Table 3) (Seksik et al. 2003; Ott et al. 2004; Swidsinski et al. 2005; Scanlan et al. 2006; Bibiloni et al. 2006; Manichanh et al. 2006; Gophna et al. 2006; Sepehri et al. 2007; Frank et al. 2007; Wang et al. 2007; Dicksved et al. 2008; Takaishi et al. 2008; Swidsinski et al. 2008; Sokol et al. 2008; Willing et al. 2009; Andoh et al. 2009; Fyderek et al. 2009; Nishikawa et al. 2009; Willing et al. 2010; Verma et al. 2010; Schweitz et al. 2010; Kang et al. 2010; Noor et al. 2010; Frank et al. 2011; Andoh et al. 2011; Walker et al. 2011; Joossens et al. 2011; Lepage et al. 2011; Thomazini et al. 2011). In general, alterations of the gut microbiota in IBD include abnormal patterns of Clostridial groups, increases in Escherichia coli and members of the Bacteroidetes phylum, and decreases in members of the Firmicutes phylum (Sokol et al. 2006; Takaishi et al. 2008; Chassaing and Darfeuille-Michaud 2011; Walker et al. 2011). However, there are differences in the diversity and microbiology between CD and UC. For example, CD-specific shifts in the gut microbiota consist of increases in Ruminococcus and Enterobacteriaceae, with decreases in Faecalibacterium and Roseburia (Manichanh et al. 2006; Willing et al. 2009; Willing et al. 2010; Kang et al. 2010; Walker et al. 2011). On the other hand, UC-specific shifts in the microbiota include increases in species from the Prevotellaceae family, Actinobacteria, and Proteobacteria (Lepage et al. 2011).
<table>
<thead>
<tr>
<th>Changes in Microbiota</th>
<th>Disease</th>
<th>Sample Source</th>
<th>Method</th>
<th>Year</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑Escherichia coli</td>
<td>CD, UC</td>
<td>Tissue</td>
<td>Culture</td>
<td>2011</td>
</tr>
<tr>
<td>↑Enterobacteriaceae, Ruminococcus gnavus</td>
<td>CD</td>
<td>Tissue</td>
<td>CI</td>
<td>2011</td>
</tr>
<tr>
<td>↑Clostridium</td>
<td>CD, UC</td>
<td>Feces</td>
<td>T-RFLP</td>
<td>2011</td>
</tr>
<tr>
<td>↑Bacteroidetes</td>
<td>CD</td>
<td>Feces</td>
<td>T-RFLP</td>
<td>2011</td>
</tr>
<tr>
<td>↑Firmicutes</td>
<td>CD, UC</td>
<td>Tissue</td>
<td>CI, qPCR</td>
<td>2011</td>
</tr>
<tr>
<td>↑Bacteroidetes</td>
<td>CD, UC</td>
<td>Tissue</td>
<td>CI, qPCR</td>
<td>2011</td>
</tr>
<tr>
<td>↑Enterobacteriaceae</td>
<td>CD, UC</td>
<td>Tissue</td>
<td>CI, qPCR</td>
<td>2011</td>
</tr>
<tr>
<td>↑Bacteroides vulgaris</td>
<td>CD</td>
<td>Feces</td>
<td>DGGE</td>
<td>2011</td>
</tr>
<tr>
<td>↑Dialister invisus, Clostridium cluster XIVa, Faecalibacterium prausnitzii, Bifidobacterium adolescentis</td>
<td>CD</td>
<td>Feces</td>
<td>DGGE</td>
<td>2011</td>
</tr>
<tr>
<td>↑Ruminococcus gnavus</td>
<td>CD</td>
<td>Feces</td>
<td>qPCR</td>
<td>2010</td>
</tr>
<tr>
<td>↑Actinobacteria, Proteobacteria</td>
<td>UC</td>
<td>Tissue</td>
<td>Pyro</td>
<td>2010</td>
</tr>
<tr>
<td>↑Faecalibacterium, Roseburia</td>
<td>CD</td>
<td>Feces</td>
<td>Pyro</td>
<td>2010</td>
</tr>
<tr>
<td>↑Enterobacteriaceae, Ruminococcus gnavus</td>
<td>CD</td>
<td>Feces</td>
<td>Pyro</td>
<td>2010</td>
</tr>
<tr>
<td>↑Clostridium</td>
<td>UC</td>
<td>Tissue</td>
<td>qPCR</td>
<td>2010</td>
</tr>
<tr>
<td>↑Eubacterium</td>
<td>CD</td>
<td>Tissue</td>
<td>qPCR</td>
<td>2010</td>
</tr>
<tr>
<td>↑Methanobrevibacter</td>
<td>CD, UC</td>
<td>Tissue</td>
<td>qPCR</td>
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</tr>
<tr>
<td>↑Sulfur reducing bacteria (SRB)</td>
<td>CD, UC</td>
<td>Tissue</td>
<td>qPCR</td>
<td>2010</td>
</tr>
<tr>
<td>↑Ruminococcus, Lactobacillus, Bifidobacterium, Bacteroidetes</td>
<td>CD, UC</td>
<td>Tissue</td>
<td>qPCR</td>
<td>2010</td>
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<tr>
<td>↑Faecalibacterium prausnitzii, Bifidobacteria</td>
<td>CD</td>
<td>Feces</td>
<td>qPCR</td>
<td>2010</td>
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<tr>
<td>↑Escherichia coli</td>
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<td>Feces</td>
<td>qPCR</td>
<td>2010</td>
</tr>
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<td>↑Bifidobacteria</td>
<td>UC</td>
<td>Feces</td>
<td>qPCR</td>
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<td>↑Eubacterium rectale, Bacteroides fragilis group, B. vulgatus, Ruminococcus albus, R. collidus, R. bromii, Faecalibacterium prausnitzii</td>
<td>CD</td>
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<td>PMA, qPCR</td>
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<td>↑Enterococcus sp., Clostridium difficile, Escherichia coli, Shigella flexneri, Listeria sp.</td>
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<td>Feces</td>
<td>PMA, qPCR</td>
<td>2010</td>
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<tr>
<td>↓Bacteroides vulgaris, B. ovatus, B. uniformis, Parabacteroides sp.</td>
<td>UC</td>
<td>Feces</td>
<td>DGGE</td>
<td>2010</td>
</tr>
<tr>
<td>↑Streptococcus</td>
<td>CD</td>
<td>Tissue</td>
<td>Culture</td>
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</tr>
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<td>↑Lactobacillus</td>
<td>UC</td>
<td>Tissue</td>
<td>Culture</td>
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<td>↑Bifidobacteria</td>
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<td>Tissue</td>
<td>Culture</td>
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</tr>
<tr>
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<td>CD</td>
<td>Tissue</td>
<td>T-RFLP, CI, qPCR</td>
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</tr>
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<td>Tissue</td>
<td>T-RFLP, CI, qPCR</td>
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<td>↑Clostridium cluster IV, XIVa, Bacteroides fragilis, B. vulgatus, B. ovatus, Clostridium cocoides, C. leptum, Atopobium, Bacteroidaceae, Bifidobacteria, Veillonella</td>
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<td>Feces</td>
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<td>Culture</td>
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<td>Tissue</td>
<td>ARISA, T-RFLP</td>
<td>2007</td>
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<td>Tissue</td>
<td>ARISA, T-RFLP</td>
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<td>Tissue</td>
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Table 3 (continued). Documented microbial alterations in IBD. Adapted from Nagalingam and Lynch, 2012.
Table 3. Documented microbial alterations in IBD

<table>
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<th>Changes in Microbiota</th>
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<th>Sample Source</th>
<th>Method</th>
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</thead>
<tbody>
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<td>Enterobacteriaceae, Bacteroides fragilis, Faecalibacterium prausnitzii-like &quot;Butyrate-producing bacterium&quot; L2-6, Pseudomonas aeruginosa</td>
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<td>Tissue</td>
<td>Cl</td>
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<td>FISH, Cl</td>
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<td>DGGE</td>
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<td>FISH</td>
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<td>Cl</td>
<td>2006</td>
</tr>
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<td>Tissue</td>
<td>Cl</td>
<td>2006</td>
</tr>
<tr>
<td>Clostridia</td>
<td>UC</td>
<td>Tissue</td>
<td>Cl</td>
<td>2006</td>
</tr>
<tr>
<td>Eubacteria rectale</td>
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<td>Tissue</td>
<td>FISH</td>
<td>2005</td>
</tr>
<tr>
<td>Bacteroides fragilis, Bacteroidetes-Provetella</td>
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<td>Tissue</td>
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<td>2005</td>
</tr>
<tr>
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<td>Tissue</td>
<td>Cl</td>
<td>2004</td>
</tr>
<tr>
<td>Clostridium cocoides</td>
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<td>Feces</td>
<td>TGGE</td>
<td>2003</td>
</tr>
<tr>
<td>Enterobacteria</td>
<td>CD</td>
<td>Feces</td>
<td>TGGE</td>
<td>2003</td>
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</tbody>
</table>

↑ Increase in bacteria compared to healthy controls. ↓ Decrease in bacteria compared to healthy controls. UC, ulcerative colitis; CD, Crohn’s disease; qPCR, quantitative PCR; Cl, clones; FISH, fluorescent in situ hybridization; Pyro, 454 pyrosequencing; DGGE, denaturing gradient gel electrophoresis; TGGE, temperature gradient gel electrophoresis; T-RFLP, terminal restriction fragment length polymorphism; ARISA, automated ribosomal spacer analysis; PMA, phylogenetic microarray. Adapted from Nagalingam and Lynch, 2012.

The loss of particular bacteria in the gut of IBD patients has profound effects on the gut environment and host. 16S rRNA analysis of fecal samples demonstrates a reduction of Clostridial cluster XIVa in IBD (Frank et al. 2007). Species of this cluster are from Clostridium, Ruminicoccous, Eubacterium, Coprococcus, Dorea, Lachnospira, Roseburia, and Butyrivibrio genera (Collins et al. 1994). However, this cluster does not include the pathobiont species Clostridium difficile, which is delegated to Cluster XI (Bien et al. 2013). Clostridial species are some of the first bacteria to colonize the gut during development (Roberts et al. 1992). In adulthood, Clostridial clusters XIVa predominates in the colon, roughly comprising 10-40% of the total bacteria in the gut microbiota (Hold et al. 2002; Frank et al. 2007; Manson et al. 2008). Given the presence
of Clostridial cluster XIVa early in life and its large abundance in the adult colon, these bacteria likely play a crucial role in shaping the gut microbiome (Lopetuso et al. 2013).

2.1 Clostridial Cluster XIVa Functions in the Colon

Clostridia colonize specific areas of the colonic mucosa. Using high-power magnification and laser capture microdissection, bacteria from Clostridial cluster XIVa were found to densely populate areas between the mucosal folds in the colons of mice (Nava et al. 2011). The plica semilunaris is an anatomical structure of the human colon that is analogous to the mucosal folds observed in mice (Nava et al. 2011). This suggests that members of Clostridial cluster XIVa occupy a specific region of the colonic mucosa, close to the host epithelium, where they influence a variety of physiological and biochemical processes (Lopetuso et al. 2013).

Clostridial cluster XIVa participates in a variety of functions that help maintain gut homeostasis (Figure 11). As Clostridia colonize in close proximity to the gut epithelium, this suggests they strongly influence the host immune system (Lopetuso et al. 2013). These bacteria have been shown to promote the development of αβ T-cell receptor intraepithelial lymphocytes and (IgA)-producing cells in the large intestine (Umesaki et al. 1999). Additionally, Clostridial cluster XIVa have been shown to induce the differentiation of Tregs in the colon and promote increased expression of anti-inflammatory mediators such as IL-10, matrix metalloproteinases, TGF-β, and indoleamine 2,3- dioxygenase (D’Angelo et al. 2001; Atarashi et al. 2011). Interestingly, Clostridial cluster XIVa has also been implicated in generating free catecholamines, suggesting their role in systemic and local processes influenced by nervous system such
as cognition, mood, immune reactions, motility, and water reabsorption in the colon
(Barry et al. 1994; Barry et al. 1995; Eisenhofer et al. 2004; Sarkar et al. 2010; Nakano et
al. 2011; Asano et al. 2012).

**Figure 11. Regulation of gut homeostasis by Clostridial cluster XIVa**

*Clostridial* species occupy the interfolds of the colon in close proximity to the epithelial surface. They promote the development of αβ T-cell receptor intraepithelial lymphocytes and IgA-producing cells through the induction of IL-6, IL-7 and TGF-β. They are also able to induce colonic T regulatory cell (Treg) accumulation through the activation of Dendritic cells (DCs) and the induction of indoleamine 2,3-dioxygenase (IDO), matrix metalloproteinases (MMPs) and TGF-β in colonic epithelial cells. Butyrate produced by *Clostridial* species provides energy to colonocytes and inhibits the activation of NF-kβ, providing anti-inflammatory effect. Increased production of IL-10 in Treg cells also contribute to anti-inflammatory effects. Finally, Clostridia, enriched in β-glucuronidase activity, could be responsible for generating free catecholamines, including norepinephrine (NE) and dopamine (DA). Adapted from Lopetuso et al. 2013.

### 2.2 Clostridial cluster XIVa and Loss of Colonization Resistance

As a part of their role in maintaining gut homeostasis, bacteria from *Clostridial*
cluster XIVa also contribute to the colonization resistance of the gut. In general, the loss
of colonization resistance to *C. difficile* is attributable to the decreased diversity of the gut microbiota. Several mechanisms describe how the normal gut microbiota provide resistance to *C. difficile* such as competition for nutrients, ecological competition, and niche exclusion (Britton and Young, 2012). While many factors have been identified as disruptors of the gut microbiota (Table 2), antibiotic therapy is most commonly associated with the development of CDI (Seekatz and Young, 2014). Antibiotic-induced perturbation of the gut microbiota can be detected within a few days of usage, though long-term effects of these drugs are also demonstrated (Detlefsen et al. 2008; Antonopoulos et al. 2009). The effects of antibiotics vary depending on the type of antibiotic used and the composition of gut microbiota prior to administration (Table 4) (Antonopoulos et al. 2009).

16S rRNA analysis and metagenomic sequencing demonstrated a reduction of *Clostridial* cluster XIVa in the feces of CDI patients, suggesting that the loss of these bacteria plays a role in development of CDI (Perez-Cobas et al. 2014). Additionally, since depletion of *Clostridial* cluster XIVa is also a characteristic of IBD-associated dysbiosis, it is likely that this loss contributes to the susceptibility IBD patients have towards developing CDI (Frank et al 2007). To understand how the loss of *Clostridial* cluster XIVa governs this susceptibility, factors such as bile acid metabolism and butyrate fermentation are considered as these two metabolic processes are modified by *Clostridial* cluster XIVa and demonstrate inhibitor effects towards *C. difficile* (Smith et al. 2013; Buffie et al. 2014)
Table 4. Changes in the gut microbiota by different types of antibiotics identified by 16S rRNA analysis
Adapted from Lankelma et al. 2015.

<table>
<thead>
<tr>
<th>Author</th>
<th>Antibiotic regimen</th>
<th>Subjects</th>
<th>Short-term results (days, weeks)</th>
<th>Long-term results (months, years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>De la Cochetiere et al., 2005</td>
<td>Amanacin, orally, 3 days</td>
<td>6 adult healthy volunteers</td>
<td>Major shift in dominant species after 2.4 hours, average similarity (compared with pre-treatment) 74% after 4 days</td>
<td>1.2 month; average similarity 88-89%</td>
</tr>
<tr>
<td>Penders et al., 2006</td>
<td>Mostly amoxicillin</td>
<td>28 pediatric patients (1 month old)</td>
<td>Decreased counts of Bifidobacteria and E. fragilis species compared with untreated children</td>
<td>None reported</td>
</tr>
<tr>
<td>Jernberg et al., 2007</td>
<td>Clindamycin, orally, 7 days</td>
<td>4 adult healthy volunteers (vs. 4 controls)</td>
<td>Day 7: 21% large and persistent shift in composition</td>
<td></td>
</tr>
<tr>
<td>Dethlefsen et al., 2008</td>
<td>Ciprofloxacin, orally, 5 days</td>
<td>3 adult healthy volunteers</td>
<td>Abundance of about a third of the bacterial taxa in the gut affected, decreased taxonomic richness, diversity, and evenness of the community</td>
<td>1 and 6 months: richness, diversity and evenness comparable to pre-antibiotic state; some long-term losses</td>
</tr>
<tr>
<td>Dethlefsen et al., 2010</td>
<td>Ciprofloxacin, orally, 5 days; after 6 months again 5 days</td>
<td>3 adult healthy volunteers</td>
<td>Loss of diversity and shift in composition within 3-4 days; 7 days after end of course, communities start returning to initial state; often incomplete</td>
<td>10 months: composition stabilised but altered; long-term losses of some taxa</td>
</tr>
<tr>
<td>Jakobsson et al., 2010</td>
<td>Metronidazole + clarithromycin, orally, 7 days</td>
<td>5 adult healthy volunteers (vs. 3 controls)</td>
<td>Dramatic decline in diversity, especially loss of Actinobacteria, in both throat and faeces</td>
<td>1 year: diversity levels recovered to pre-treatment state</td>
</tr>
<tr>
<td>Feusby et al., 2012</td>
<td>Parenteral antibiotic treatment with ampicillin and gentamicin (within 6 h of birth)</td>
<td>9 paediatric patients (plus 9 untreated infants)</td>
<td>4 weeks after treatment, antibiotic treated infants had higher proportions of Proteobacteria and lower proportions of Actinobacteria as well as the genus Lactobacillus</td>
<td>2 months: Proteobacteria levels remained higher, but Actinobacteria and Lactobacillus levels had recovered</td>
</tr>
<tr>
<td>Panda et al., 2014</td>
<td>Different broad-spectrum antibiotic regimens, orally</td>
<td>21 adult patients</td>
<td>Fluoroquinolones and b-lactams decreased microbial diversity by 25% and reduced the number of taxa from 29 to 12; Increase in proportion Bacteroidetes taxa; B-lactams increased the average microbial load two-fold</td>
<td>None reported</td>
</tr>
</tbody>
</table>

2.3 Bile Acid Metabolism

The gut microbiota plays an important role in the metabolism of bile acids. Bile acids are saturated/hydroxylated C-24 cyclopentanephenanthrene sterols that are produced from cholesterol in the liver. Bile acids are conjugated with amino acids
(typically taurine or glycine) in the liver to form bile salts, which are secreted into the lumen of the small intestine to aid in digestion and absorption of dietary lipids. After passing through the small intestine, colonic bacteria modify bile salts by deconjugating and dehydroxylating the sterols in order to promote passive reabsorption along the ascending colon (Figure 12) (Ridlon et al. 2006). Deconjugation occurs when the amide bond between the bile salt and amino acid is hydrolyzed by bile salt hydrolases (BSHs), enzymes produced by colonic bacteria. Dehydroxylation, on the other hand, is a complex process that is carried out by enzymes named hydroxysteroid dehydrogenases, encoded by the bile acid-inducible (*bai*) operon (Ridlon et al. 2006).
Figure 12. Bacteria biotransformation of bile salts in the colon
Hydroxy carbons of cholic acid are labeled. Dehydroxylation of cholic acid (CA) at carbons 3,7, or 12 produce various secondary bile acids. BSH, bile salt hydrolase; HSDH, hydroxysteroid dehydrogenase. Adapted from Ridlon et al. 2006.

*In vitro* studies have demonstrated the effects bile acids exert on *C. difficile* (Wilson, 1983; Sorg and Sonenshein, 2008). The growth of vegetative *C. difficile* is inhibited by bile acids such as chenodeoxycholate (CDCA) (Figure 13) (Wilson, 1983; Sorg and Sonenshein, 2008). However, other bile acids such as cholic acid (CA), as well as the conjugated bile salt taurocholate and the free amino acid glycine, stimulate the germination of *C. difficile* spores (Sorg and Sonenshein, 2008; Ridlon et al. 2006). Interestingly, deoxycholic acid (DCA) both inhibits the growth of vegetative *C. difficile* and stimulates the germination of *C. difficile* spores (Sorg and Sonenshein, 2008). This suggests that the positive and negative effects exerted by bile acids on *C. difficile* depend on the composition of the bile acids along the GI tract.
Bile acid composition differs in the gall bladder and feces (Figure 14) (Ridlon et al. 2006). Major alterations of the bile acid composition occur in the distal ileum and colon where 95% of bile acids are reabsorbed and subjected to dehydroxylating activity of colonic bacteria, respectively (Ridlon et al. 2006). As the composition of bile acids differ in the different segments of the GI tract, the germinating and inhibiting effects of bile acids must also vary. For example, in the small intestines CA is the most abundant bile acid, suggesting that *C. difficile* spores are largely germinated in this area (Thomas et al. 2001; Sorg and Sonenshein, 2008). However, in the colon, DCA is the predominate

Figure 13. Bile acid metabolism and *C. difficile*

Conjugated bile salts produced by the liver are metabolized in the intestine, primarily by activities carried out by members of the gut microbiota. Primary bile salts as well as their metabolites can have direct effects on *C. difficile*, primarily affecting germination of spores as well as the activity of vegetative cells. Adapted from Britton and Young, 2012.
bile acid and largely restricts the growth of any vegetative *C. difficile* (Ridlon et al. 2006; Sorg and Sonenshein, 2008). This suggests that even though spores may germinate in the small intestine due to effects of CA, any vegetative *C. difficile* that arises in the colon is unable to grow due to DCA. The balance between CA and DCA is critical in the germination and inhibition of *C. difficile*. Thus, the bacteria that carry out the conversion of CA into DCA play a crucial role in providing the inhibitory effects imparted by DCA.

Members of *Clostridial* cluster XIVa demonstrate 7α-dehydroxylating activity and are capable of converting CA into DCA (Ridlon et al. 2006; Kang et al. 2008). Buffie et al. 2014 demonstrated the bile acid-mediated effects of *Clostridial* cluster XIVa *in vivo*. In their study, rats were challenged with *C. difficile* spores following antibiotic administration (Buffie et al. 2014). 16S rRNA analysis of feces from CDI-susceptible and
CDI-resistant rats identified 11 bacterial species that conferred resistance to CDI. The 11 species comprised a small portion of the overall microbial composition (6%), but were predominantly members of *Clostridial* cluster XIVa (Buffie et al. 2014). *Clostridium scindens* (member of *Clostridial* cluster XIVa), the species that conferred the strongest resistance to CDI, was then transferred into CDI-susceptible rats, which decreased *C. difficile* content in feces and increased survival rate, confirming the CDI-resistance of *C. scindens*. To test the mechanism of resistance of *C. scindens*, secondary bile acid content was measured in the feces of CDI-susceptible and CDI-resistant rats and was significantly increased in the latter (Buffie et al. 2014). PCR-based assay for 7α-hydroxysteroid dehydrogenase (encoded for by the *bai* operon) correlated *C. scindens* with the increased secondary bile acid content and CDI-resistance (Buffie et al. 2014).

To extend their study to humans, Buffie and colleagues analyzed the microbiota obtained from feces of patients undergoing allogeneic hematopoietic stem-cell transplantation (allo-HSCT), a cohort recently given antibiotics as a prophylactic measure (Buffie et al. 2014). Of the 24 allo-HSCT patients, 12 were diagnosed with CDI and the other 12 were carriers of *C. difficile* without clinical diagnosis of CDI. By comparing the species that demonstrated strong inhibition against *C. difficile* in both experiments, *C. scindens* was identified as conferring resistance in both humans and rats, corroborating their rodent-based correlation analysis (Buffie et al. 2014).

**2.4 Metabolite Imbalance**

In addition to metabolism of bile acids, members of gut microbiota also participate in fermentation of short chain fatty acids (SCFAs). SCFAs are the products of
bacterial fermentation of indigestible dietary fiber and primarily include propionate, acetate, and butyrate (Figure 15) (Cummings 1983; Mortensen and Nordgaard-Andersen 1993; Cummings et al. 1996). These metabolites have different effects on the host. For example, propionate and acetate are mostly absorbed into the bloodstream and exert their effects systemically, whereas butyrate is the primary energy source of colonocytes and exerts local effects in the gut (Topping and Clifton 2001; Maslowski et al. 2009; Smith et al. 2013). As many bacteria in *Clostridial* cluster XIVa are butyrogenic, the loss of these species as seen in IBD, negatively affects the butyrate concentrations in the gut (Clausen and Mortensen 1995; Den Hond et al. 1998; Bien et al. 2013; Smith et al. 2013). The depletion of butyrate alters the host in several ways and ultimately weakens the host’s colonization resistance and ability to tolerate the toxin-mediated effects of *C. difficile.*
Figure 15. Schematic of the gut microbiota involved in the metabolism of short chain fatty acids

Acetate and lactate are intermediates, but also exist as end products. The phyla represented in the figure are limited to cultured species. CH4, methane; SO4, sulfate; H2S, hydrogen disulfide; CoA, coenzyme A. Adapted from Knudsen, 2015.

Butyrate contributes to the host’s colonization resistance by altering the pH of the luminal contents. Given the pKa of butyrate (~4.8) and luminal pH of the colon (5.6 in the proximal colon and 6.3 in the distal colon), approximately 90% of the butyrate in the colon exists in its dissociable anionic form (Cummings et al. 1987; Velazquez et al. 1997). The ionized butyrate lowers the pH of the colon, which is shown to inhibit the growth and toxin production of vegetative *C. difficile* (May et al. 1994; Wong et al. 2006). However, the lowered pH also decreases the solubility of free bile acids and denatures 7α-hydroxysteroid dehydrogenase (Thorton, 1981). This effectively prevents formation of secondary bile acids such as DCA, which ironically also weakens the
colonization resistance to *C. difficile* for reasons discussed above. While the lowered pH appears to have contrasting effects on colonization resistance, the result of this antagonistic relationship is unclear.

In addition to altering the pH, the decreased butyrate in the colon also weakens the colonization resistance against *C. difficile* by weakening the barrier defense imparted by the mucus layer. As discussed above, the mucus layer is the first line defense against the bacteria living in the lumen of the intestines. In comparison to non-IBD controls, IBD patients have a thinner mucus layer that is more permeable to colonic bacteria (Pullan et al. 1994; Einerhand et al. 2002; Fyderek et al. 2009; Swidsinski et al. 2007). From *in vitro* and *ex vivo* studies, butyrate is known to stimulate MUC2 production, suggesting that the decrease in butyrate observed in IBD contributes to the thinner mucus layer and weakened barrier defense (Finnie et al. 1995; Willemsen et al. 2003; Augenlicht et al. 2003; Gaudier et al. 2004; Hatayama et al. 2007). Additionally, the thinner mucus layer has recently been shown to inflict negative affects on the gut’s adaptive immune system. In a study by Olson and colleagues, the mucus layer was shown to work synergistically with IgA in preventing epithelial injury due to *C. difficile* toxins by protecting IgA from proteolytic cleavage, thus conserving its structural integrity and function (Olson et al. 2013). Although IgA is somewhat resistant to cleavage in the GI environment, these results suggest that IgA in the colon of IBD patients is more prone to cleavage due to the thinned mucus layer. The increased cleavage of IgA ultimately dampens the adaptive immune system’s ability to opsonize vegetative *C. difficile* and its toxins, resulting in increased susceptibility to toxin-mediated inflammation.
As a result of decreased butyrate fermentation, AMP secretion is also altered in the gut of IBD patients. LL-37 is a type of AMP (cathelicidin) secreted by colonic epithelial and macrophages (Bals, 2000). While LL-37 has demonstrated antibacterial effects against Gram-negative bacteria, it also has been shown to have anti-inflammatory effects (Bowdish et al. 2006; Gombart et al. 2009). In a study by Hing et al. 2013, exogenous LL-37 decreased tissue damage and inflammation in C. difficile colitis in mice. The authors demonstrated that the reduced inflammation was the result of decreased secretion of tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine released in response to C. difficile toxin (Rocha et al. 1997; Pothoulakis and Lamont, 2001; Hing et al. 2013;). As in vitro studies have demonstrated that butyrate stimulates the expression of LL-37 in colonic epithelial cells, it is suggested that the decreased butyrate also limits the expression of LL-37 in the gut (Schauber et al. 2003). Collectively, this indicates that patients with IBD are less able to control the toxin-mediated inflammation associated with CDI, making them more susceptible to symptom development.

**DISCUSSION**

The current review highlights evidence that suggests the decreased abundance of Clostridial cluster XIVa in the gut microbiota of IBD confers susceptibility to developing CDI by promoting colonization by C. difficile and perpetuating its toxin-mediated inflammation. Given the multifactorial etiology of IBD and CDI, it is difficult to fully assess how the loss of Clostridial cluster XIVa affects bile acid metabolism and butyrate fermentation. Complicating this notion is the fact that other bacteria in the gut, such as
Bacteroidetes species, carry out both bile acid metabolism and butyrate fermentation (McFarlane and McFarlane, 2003; Ridlon et al. 2006; Antharam et al. 2013). Additionally, as Clostridial cluster XIVa influences many cellular and biochemical processes in the gut, the loss of this taxa likely alters multiple other factors, thus further complicating how bile acid metabolism and butyrate fermentation are ultimately affected in vivo. However, in describing how Clostridial cluster XIVa provides colonization resistance to C. difficile, it is clear that the gut microbiota have profound effects on its host, both physiologically and biochemically. Also, there is corroborating evidence that the loss Clostridial cluster XIVa facilitates C. difficile pathogenesis. As noted above, advanced age (>65 years old) is one of the primary risk factors for CDI (Stanley and Burns, 2010). As the gut microbiota of elderly individuals is marked by a decrease in Clostridial cluster XIVa, this suggests that increased incidence of CDI in the elderly may be due to a similar mechanism as discussed previously (Figure 7) (Claesson et al. 2011).

The loss of Clostridial cluster XIVa observed in IBD and CDI provides a targeted approach in curing these diseases. Recently, fecal microbiota transplantation (FMT) has emerged as a treatment that restores the normal diversity and abundance of the gut microbiota. FMT has demonstrated great success in treating recurrent CDI as a systematic review discovered 89% (246/275) of patients diagnosed with recurrent CDI had no relapse of disease after undergoing FMT (Brandt and Reddy, 2011). However, the success of FMT in treating IBD has demonstrated mixed results (Damman et al. 2012). In knowing that the colonization resistance to C. difficile is positively influenced by members of Clostridial cluster XIVa, donor stool to be used in FMT should be screened
for inclusion of species from this taxa in order to assure efficacy. This may also improve the efficacy of FMT in treatment of IBD as restoration of the normal abundance and diversity of *Clostridial* cluster XIVa would likely restore the anti-inflammatory effects imparted by these bacteria (D’Angelo et al. 2001; Atarashi et al. 2011).

The loss of *Clostridial* cluster XIVa observed in IBD exerts strong local effects in the gut environment, though systemic effects may also exist. For example, *Clostridial* species have recently been implicated in the production of free catecholamines (Asano et al. 2012). In this study, gnotobiotic mice that were associated with 46 different *Clostridial* species, including members from cluster XIVa, demonstrated significantly higher levels of norepinephrine and dopamine in the lumen of the gut in comparison to germ-free mice (Asano et al. 2012). From this, it is suggested that the *Clostridial* species containing β-glucuronidase activity increase the luminal concentrations of norepinephrine and dopamine by converting these products from their biologically inactive, glucuronide-conjugated form (Asano et al. 2012). As the gut is densely innervated with noradrenergic and dopaminergic neurons, the loss of *Clostridial* bacteria would have detrimental effects on the brain-gut axis due to decreases in luminal catecholamines (Li et al. 2006; Asano et al. 2012). However, there is still much to learn regarding this mechanism and its effects on the host (Lopetuso et al. 2013).

Interestingly, the findings of this review contradict data reporting IBD as a risk factor for colorectal cancer. Colorectal cancer is thought to evolve in IBD from the recurrent/chronic inflammation and ulceration of host tissue (Jawad et al. 2011). Due to its hydrophobicity, DCA is able to passively diffuse across cell membranes and activate
signaling pathways, ultimately inducing cancer formation by exerting selective pressure for the generation of apoptosis-resistant epithelial cells (Qiao et al. 2001; Rao et al. 2002; Im and Martinez, 2004; Bernstein et al. 2005). However, due to decreased accumulation of DCA that results from the loss of 7α-dehydroxylating activity, it should be expected that IBD patients would exhibit a decreased risk of colorectal cancer. While this appears to contradict evidence supporting the increased risk of colorectal cancer in IBD, there are still many uncertainties involving this mechanism. However, this finding has large implications as recently, it was suggested that the risk of colorectal cancer in the population is much lower than previously thought (Kassem et al. 2014).

As *Clostridial* cluster XIVa demonstrates protective effects against *C. difficile*, further understanding of how these species are altered will provide insight into the role that the gut microbiota plays in CDI and IBD. However, there has been a lack of studies capable of assessing the physiology of these bacteria *in vivo*. One reason for this is that molecular techniques used to study microbial communities are ill equipped for the task. For example, while 16S rRNA analysis and metagenomic sequencing enable scientists to determine the structure and functional capacity of microbial communities, these techniques provide little information regarding which species of bacteria are active, damaged, or responsive to a given compound in these environments (Maurice et al. 2013). This suggests that studies should combine sequencing methods with flow cytometry and fluorescence-activated cell sorting, as these single-cell methods have been used to characterize the physiological structure of environmental and host-associated microbial communities in the past (Joux and Lebaron, 2000; Shapiro, 2000; Ben-Amor et
al. 2005). In a study using such methods, Maurice and colleagues demonstrated that the physiological responses of the gut microbiota to antibiotics varied between individuals, over time, and with the type of drug (Maurice et al. 2013). In knowing how the physiology of the gut microbiota differs from person to person, medicine can take a personalized approach, thus increasing the efficacy of treatments and limiting adverse events such as unexplained drug toxicity (Maurice et al. 2013).

Though many details are yet to be discovered, it is clear that the gut microbiome has a major influence on host physiology. As there are a variety of diseases linked to the gut microbiome such as IBD, CDI, colorectal cancer, irritable bowel syndrome, obesity, diabetes, allergies, rheumatoid arthritis, Parkinson’s disease, and multiple sclerosis, understanding how short-/long-term effects influence the function and shape of the gut microbiota will advance medicine (Backhed et al. 2004; Frank et al. 2007; De La Cochetiere et al. 2008; Jawad et al. 2011; Kverka and Tlaskalova-Hogenova, 2013; Crouzet et al. 2013; Abrahamsson et al. 2014; Hu et al. 2015). With continued research of the gut microbiota and its relationship with the human host, more will be understood of its role in human disease.

**CONCLUSION**

The funding of major projects such as the HMP has initiated a new era in human medicine. Not only are researchers discovering more about the roles bacteria have in disease, but also how these same bacteria actually promote, and are essential for, human health. The amount of research that has been conducted on the human microbiome is significant. From the collected data, it is clear that body habitats, such as the human gut,
are highly complex. The interactions between the host and microbes are pertinent in maintaining homeostasis within such environments. In the case of IBD, disruption of this homeostasis is detrimental and facilitates complications such as unwanted inflammation and susceptibility to infections such as CDI. With the rising prevalence of IBD and CDI, continued research into the gut microbiome is necessary. From this, novel therapies that manipulate the gut microbiota will hopefully improve diagnosis and treatment of such diseases.
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