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Microbial and metabolic interactions between the gastrointestinal tract and *Clostridium difficile* infection

Casey M Theriot* and Vincent B Young

University of Michigan; Ann Arbor, MI USA

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Antibiotics disturb the gastrointestinal tract microbiota and in turn reduce colonization resistance against *Clostridium difficile*. The mechanism for this loss of colonization resistance is still unknown but likely reflects structural (microbial) and functional (metabolic) changes to the gastrointestinal tract. Members of the gut microbial community shape intestinal metabolism that provides nutrients and ultimately supports host immunity. This review will discuss how antibiotics alter the structure of the gut microbiota and how this impacts bacterial metabolism in the gut. It will also explore the chemical requirements for *C. difficile* germination, growth, toxin production and sporulation. Many of the metabolites that influence *C. difficile* physiology are products of gut microbial metabolism including bile acids, carbohydrates and amino acids. To restore colonization resistance against *C. difficile* after antibiotics a targeted approach restoring both the structure and function of the gastrointestinal tract is needed.

Introduction

Clostridium difficile is an anaerobic, spore-forming, gram-positive bacillus first isolated in 1935 by Hall and O'Toole.[1] Attention to this organism as a pathogen developed when *C. difficile* was recognized as the cause of antibiotic-associated pseudomembranous colitis in the 1970s.[2] Within the past decade, there has been a renewed focus on *C. difficile* infection (CDI) due to an increase in morbidity, mortality and health care costs.[3,4] In hospitals, CDI accounts for almost all cases of pseudomembranous colitis and 20% of nosocomial diarrhea cases.[5] CDI can manifest a range of clinical disease from mild diarrhea to severe pseudomembranous colitis and even death.[4]

Hospitalization, advanced age (greater than 65 years), and antibiotic treatment are main risk factors for CDI.[6,7] Antibiotics associated with CDI include clindamycin, quinolones, cephalosporins, and aminopenicillins.[8-10] The key role of antibiotics in the development of CDI has prompted an interest in how these drugs can reduce colonization resistance against pathogens.[11,12] Antibiotics, even at sub therapeutic levels, can have significant and long lasting effects on the gut microbiota.[13-15] By altering the community structure of the gut microbiome, antibiotics also alter the intestinal metabolome, which is composed of both host- and microbial-derived metabolites.[16-18]

How an antibiotic-altered microbiome and metabolome facilitates the development of CDI is not well understood. There are multiple chemical queues that *C. difficile* encounters and reacts to within the host. In vitro studies, and a limited number of in vivo studies, have shed light on chemical requirements for *C. difficile* germination, outgrowth, and toxin production.[19-22] This review will focus on how microbes shape the metabolic environment of the gastrointestinal tract and how this influences *C. difficile* pathogenesis.

*Correspondence to: Casey M Theriot; Email: caseythe@med.umich.edu
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Role of the Microbiome in Intestinal Metabolism

The indigenous gut microbiota is the complex community of microorganisms that populates the gastrointestinal tract. This community composes 70% of the total microbiota found on the human body (total 10^{14} bacterial cells).[23] It plays a critical role in human health by providing resistance to colonization and infection by pathogenic organisms.[12,24] It also has profound effects on homeostasis of the host, providing signals for epithelial maturation, shaping the immune response and participating in key metabolic transformations.[17] Bacteria carry out multiple metabolic processes that have a profound effect on the chemical composition of the gastrointestinal environment (**Fig. 1**).

Two bacterial phyla that make up the majority of the gut bacterial population are the Firmicutes and Bacteroidetes.[25] Much attention has been given to members of the Bacteroidetes phylum for their ability to breakdown host glycans and non-digestible carbohydrates including resistant starches and plant cell wall polysaccharides.[26-28] The Firmicutes phylum, specifically members from the Lachnospiraceae and Ruminococcaceae family, makeup 50–70% of the colonic bacterial population and are also important for polysaccharide degradation.[27] Additionally, *Clostridium* species are the most common amino acid fermenting bacteria found in the gut.[29]

It is estimated that 20 to 60 g of undigested carbohydrates enter the colon each day.[30,31] The colonic microbiota plays a major functional role by fermenting these complex carbohydrates and

amino acids into short chain fatty acids (SCFAs), which are important for colonic health and secondary bacterial fermenters in the gut.[32-34] SCFAs, especially butyrate, are the main energy source for the colonic mucosa. SCFAs also play key roles in the regulation of host gene expression, inflammation, differentiation and apoptosis.[35]

The gut microbiota also plays a role in many other host and bacterial metabolic reactions including regulating amino acid metabolism and protein digestion.[29] Host and bacterial proteases are important for breaking down exogenous protein into smaller peptides and amino acids. Members of the gut microbiota utilize amino acids and peptides as sources of nitrogen.[29] End products of amino acid fermentation can be both beneficial and toxic to the host. Beneficial end products include SCFAs (acetate, butyrate, propionate, valerate), BCFAs (isobutyrate and isovalerate), organic acids and gaseous compounds. Toxic end products include phenols, indoles, ammonia, amines, thiols and hydrogen sulfide.[29,32]

The gut microbiota also plays a role in lipid metabolism. Bile acids are amphipathic lipids that are important in fat and cholesterol metabolism.[36] Additionally bile acids modulate lipoprotein, glucose, drug and energy metabolism.[36,37] Specific members of the gut microbiota, including some of the spore-forming and anaerobic members of the *Clostridium* genus, are able to perform two-enzymatic reactions on bile acids: deconjugation and 7α-dehydroxylation.[36-39] Deconjugation of the glycine and taurine conjugates yields primary bile acids, which can then undergo 7α-dehydroxylation via gut microbial enzymes, yielding secondary bile acids.[36] Members of the gut microbiota are important for shaping metabolism in the gastrointestinal tract.

Effect of Antibiotics on Microbiome Structure and Function

Given the key role of antibiotics in CDI there have been multiple studies in mice and humans examining the relationship between antibiotics and the gut microbiome. Treatment of mice with an antibiotic cocktail consisting of ampicillin, gentamicin, metronidazole, neomycin and vancomycin resulted in a 10-fold reduction in fecal bacterial density.[40] Antibiotic treatment was associated with significant alteration of the gut community including decreased abundance of the Firmicutes phylum and increased persistence of Bacteroidetes and Proteobacteria. In agreement with Hill et al., Antonopoulos et al. demonstrated that treatment of mice with a cocktail of antibiotics (amoxicillin, metronidazole and bismuth or AMB) altered the gut microbiota with a persistent decrease in overall diversity.[41] After antibiotics the murine gut microbiota was dominated by the Proteobacteria phylum, Enterobacteriaceae family, whereas the Bacteroidetes and Firmicutes phyla only made up a small portion of the total population.[41] After AMB-treated animals were given 2 wk to recover off of antibiotics, the gut microbiota was restored to the original structure observed prior to antibiotic treatment. Mice treated with a broader spectrum antibiotic, cefoperazone, showed longer lasting alterations to the gut microbiota up to 6 wk after stopping antibiotics.[41]

There are fewer studies defining the structure of the gastrointestinal tract after antibiotic treatment in humans. We know that administration of the combination antibiotic amoxicillin-clavulanic acid (antibiotic and β-lactamase inhibitor) altered the gastrointestinal bacterial community structure and was associated with the development of antibiotic-associated diarrhea (AAD),[42] including a marked reduction of many butyrate-producing bacterial members from the Clostridiaceae family that are essential for colonic health.[42] Resolution of AAD and an increase in Clostridiaceae members was observed after cessation of antibiotic therapy.[42] In a separate study, a 5-d ciprofloxacin treatment resulted in a decrease in the richness and diversity of the gut microbial community.[43] Four weeks after antibiotic administration, the gut microbiota for one patient returned to the state prior to antibiotic therapy; however, the other patients' microbiota took up to six months to recover. These studies demonstrate the potentially long-lasting alterations to the structure of the human gastrointestinal tract microbiota following antibiotic use.

By altering the gut microbial communities, antibiotics affect the intestinal metabolome, which is the total number of metabolites in the intestine. Over 87% of murine gut metabolites changed after streptomycin treatment, although the most significantly altered play an important role in sugar, amino acid, fatty acid, steroid, bile acid and eicosanoid metabolism.[16] Additionally, streptomycin treatment resulted in an increase of the bile acids glycocholate, taurocholate, and taurochendeoxycholate and a decrease in chendeoxycholate and cholate.[16]

Zhao et al. demonstrated that gentamycin and ceftriaxone treatment resulted in a significant change to the fecal metabolome of mice.[44] This resulted in decreased levels of monosaccharides (glucose, fucose, xylose, and galactose) and SCFAs, as well as increased levels of oligosaccharides (sucrose, cellobiose, raffinose, and stachyose). Shifts in amino acid and bile acid metabolism, specifically cholate, taurocholate, and tauro-β-muricholate to deoxycholate, were observed after antibiotics. Similar results were observed in other studies following treatment with vancomycin or enrofloxacin (fluoroquinolone).[45,46] In a separate study, rats given a broad-spectrum β-lactam, imipenem/cilastatin sodium, specific metabolomic changes were observed.[47] Amino acids (tryptophan, tyrosine, phenylalanine, histidine, cysteine, methionine, valine, leucine, isoleucine, lysine, arginine, and proline), organic acid (SCFAs), oligopeptide, carbohydrate, purine, pyrimidine, and TCA cycle metabolites were all affected.[47]

In mice and humans, antibiotics can cause a decrease in the bacterial load, bacterial diversity and a change in the bacterial community dynamics in the gut. Given that the most predominant members of the gut microbiota (Firmicutes and Bacteroidetes) are important for many metabolic processes including fermentation of carbohydrates and amino acids, it is expected that antibiotics will impact bacterial metabolism in the gastrointestinal tract. This was demonstrated in humans where clindamycin and ampicillin treatment decreased fecal SCFAs, functionally reflective of diminished bacterial fermentation in the gut.[48] Antibiotics alter bacterial fermentation in the murine and human gut, which results in a decrease in SCFAs and an excess in fermentation

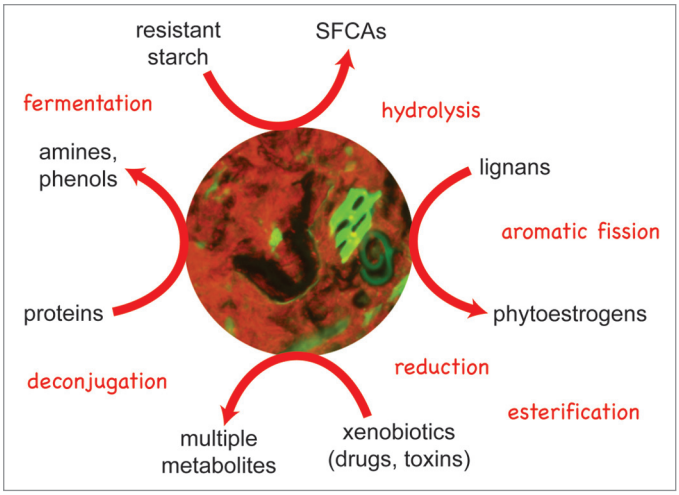

Figure 1. Functional role of the indigenous gastrointestinal tract microbiota. The gastrointestinal tract microbiota provides many metabolic functions that are able to convert luminal compounds into secondary metabolites. The chemical reactions (labeled in red) can produce metabolites that are both beneficial and harmful to the host. Fluorescence in situ hybridization (FISH) of the gastrointestinal tract microbiota in a wild type mouse is at the center (red, hybridized with Cy3-labeled Eub338) and was provided by Christine Bassis, PhD.

substrates including carbohydrates and amino acids. *C. difficile* can utilize many of the metabolites in the gastrointestinal tract that were altered after antibiotics including bile, carbohydrates and amino acids for germination and growth.[19,21]

The Influence of the Metabolic Environment of the Gut on *C. difficile*

C. difficile spores require a germinant for outgrowth into vegetative cells. Much insight on *C. difficile* germination has been accomplished with in vitro studies. In 1972 Wilson et al. first characterized *C. difficile* spore germination and discovered that sodium taurocholate supplemented media increased recovery of spores.[49,50] Since then, other researchers have revisited germination requirements for *C. difficile*. In a series of in vitro studies, Sorg and Sonenshein found that bile acids, and analogs made by the host, were able to both inhibit and support *C. difficile* spore germination and colony formation.[51] Based on in vitro germination assays, primary bile acid chendeoxycholate inhibited spore germination and colony formation and was able to out compete other bile acids including taurocholate, cholate and glycocholate.[19,52] *C. difficile* spores were able to use taurocholate and glycine as a co-germinant for maximal germination.[19] Moreover, the secondary bile acid deoxycholate was able to stimulate germination of *C. difficile* spores; however, like chendeoxycholate, it also inhibited growth of *C. difficile*.[19,52] Additionally, amino acids have the ability to stimulate germination of spores including histidine, another co-germinant in vitro.[53,54] Interestingly, certain *C. difficile* clinical isolates can utilize a wide range of bile acids for germination while others only require taurocholate and glycine for maximal germination.[55,56]

There have been a limited number of studies on the requirement for germination in vivo. Recently, Giel et al. demonstrated that filtered gastrointestinal contents from antibiotic treated mice were able to stimulate colony formation of *C. difficile* spores; however, samples from non-antibiotic treated mice from the small intestine also supported germination.[22] Furthermore, Giel et al. demonstrated that primary bile acids were the predominant bile acid in the mouse gut after antibiotic treatment. Additionally, taurocholate, when supplemented into unfiltered, non-antibiotic treated samples, was converted to secondary bile acids, a result not observed when supplemented into antibiotic treated samples. Antibiotics alter the bacterial community capable of deconjugating and 7α-dehydroxylating bile acids in the gastrointestinal tract resulting in a decrease in secondary bile acids and an increase in primary and conjugated bile acids.[22,57] *C. difficile* can use both primary (taurocholate) and secondary bile acids (deoxycholate) for germination, although deoxycholate can inhibit *C. difficile* growth in vitro.[19] Biotransformation of bile acids by the gut microbiota could play an important role in *C. difficile* germination in vivo.[22]

Many gaps still exist in our understanding of how *C. difficile* germinates within a host and how this contributes to disease onset. The physiologically relevant concentration of bile acids that spores encounter in the human and mouse intestine is unknown and most germination assays are done in a pure culture in vitro environment for only a 30 min period, and it is unknown to what extent this mimics that of the host, especially in the gastrointestinal tract. Furthermore, germination is only the first step of the *C. difficile* lifecycle, and the metabolic environment leading to germination may also contribute to downstream events during disease development, including outgrowth of vegetative cells, toxin production and ultimately sporulation.

C. difficile has a wide repertoire of energy producing pathways. *C. difficile* is heterotrophic, saccharolytic, proteolytic, and has recently been discovered to be autotrophic due to its ability to utilize carbon dioxide and hydrogen.[58-60] In a defined minimal media, *C. difficile* requires amino acids (cysteine, isoleucine, leucine, proline, tryptophan, and valine) and vitamins (biotin, pantothenate, and pyridoxine) for optimal growth.[60,61] Additionally, *C. difficile* is able to ferment many carbohydrates including fructose, glucose, mannitol, mannose, melezitose and sorbitol.[21] Expression of toxin A and B, the primary virulence factors of CDI, are induced during the stationary growth phase when nutrients become limited and is affected by amino acids, butyrate, butanol, glucose and other carbon sources.[62-66] More specifically, repression of *C. difficile* toxins has been observed when proline, cysteine, butanol or glucose is supplemented into growth media. Alternatively, toxin is induced when growth media is supplemented with butyrate[67] and or limited in biotin[68] suggesting a relationship between virulence and metabolism. Many of the nutrients that support *C. difficile* growth and toxin production were present in the gastrointestinal tract metabolomic studies highlighted in this review.

Taking advantage of the *C. difficile* genetic system has given researchers insight into *C. difficile* metabolism and pathogenesis. *C. difficile* encodes many genes important for regulating toxin expression and sporulation, many of which are directly linked to

metabolism and availability of nutrients. CodY, a global regulatory protein that monitors nutrient availability, represses *C. difficile* toxin gene expression during growth in rich media.[69,70] The direct target of CodY is the *tcdR* gene, which encodes the sigma factor required for the transcription of toxin genes,[69] which lies with in the 19.6-kb pathogenicity locus (PaLoc). The PaLoc also includes toxin genes, *tcdA* and *tcdB*, as well as *tcdR*, *tcdE*, and *tcdC* genes.[71,72] Other genes regulated by CodY are involved in amino acid biosynthesis, nutrient transport, fermentation, membrane components, and surface proteins. Another global regulator in *C. difficile* that controls transcription in response to carbohydrate availability is CcpA, a carbon catabolite control protein.[73] CcpA binds to the regulatory region of the *tcdA* and *tcdB* genes.[74] CcpA directly regulates genes important for sugar uptake, fermentation, amino acid metabolism, sporulation and toxin, suggesting a link between carbon metabolism and toxin production.

Studies defining the *C. difficile* transcriptome in two different animal models (germ-free mouse and pig ileal ligated loop) have shed light on what may be required for in vivo colonization and infection.[75,76] Recently, Janoir and colleagues defined the *C. difficile* transciptome during early and late infection in a germ-free mouse model.[75] Genes differentially expressed in vivo, compared with in vitro growth, were involved in *C. difficile* metabolism (fermentation, amino acids, and lipids), regulatory processes, cell process, stress response, pathogenicity and sporulation.[75] Expression of genes responsible for degradation of polysaccharides and fermentation of carbohydrates and amino acids increased during infection in vivo. Furthermore, increased expression of genes responsible for butyrate biosynthesis and the production of ethanol and butanol, which are important for fermentation, were observed. Similarly, genes important for the biosynthesis of leucine and D-proline reductase increased in expression, suggesting *C. difficile* could be using the Stickland reaction to generate ATP in vivo.[61] Expression of ethanolamine and N-acetylglucosamine utilization genes were found to have increased expression during late infection, suggesting there are potential carbon sources for *C. difficile* in the germ-free mouse gut even late in infection.[77]

The pig ileal loop model has also been used in *C. difficile* infection studies.[76] Gene expression profiles of *C. difficile* were similar to those seen in the germ-free mouse during infection, and expression of genes encoding amino acid and carbohydrate transport and metabolism significantly increased in vivo compared with in vitro grown cultures.[76] The pig ileal loop model also exhibited differences compared with the germ-free mouse model. Of note, toxin expression increased early during infection in the pig ileal loop model. Additionally, the observed decrease in expression of glucose degradation pathways and increase in degradation of mannose, xylose and glycogen was not observed in the germ-free mouse model discussed above.[75] These differences likely reflect the ability of *C. difficile* to adapt to different host environments.

Animal Models and Human Studies of *C. difficile* and/or Gut Microbiome Interactions

Several animal models have been developed to study CDI.[78-80] Bartlett et al. developed the first rodent model that was used to study pathogenesis of CDI.[81] Hamsters were administered clindamycin followed by *C. difficile* challenge five days later, resulting in pseudomembranous colitis and death within 3 d.[81,82] In 2008, Chen et al. developed a mouse model that approximates human CDI using a pretreatment of five antibiotics (gentamicin, kanamycin, colistin, metronidazole and vancomycin), followed by an intraperitoneal injection of clindamycin before challenge with *C. difficile*.[78] This model of CDI is unlike the uniformly fatal hamster model because disease severity was dependent on the bacterial inoculum administered, and treatment with vancomycin prevented death. Additionally, when vancomycin was discontinued, relapse occurred, which resembles human disease. In addition to studying CDI pathogenesis in the hamster model, the mouse model of CDI is a valuable tool to explore the interplay between antibiotics, the gut microbiota, the host and colonization of *C. difficile*.

Reeves et al. used this murine model of CDI to analyze the microbiome in response to antibiotics and determined that antibiotic pretreatment resulted in a decrease in the relative abundance of Firmicutes and Bacteroidetes phyla, and an increase in Proteobacteria, specifically members of the Enterobacteriaceae family.[83] Mice treated with broad-spectrum cephalosporin, cefoperazone, were also susceptible to *C. difficile* infection.[83] In particular, cefoperazone treatment resulted in significant and long-lasting alterations to the mouse gut microbiota.[41,83] An increased abundance of the Firmicutes and Proteobacteria phyla (specifically Lactobacillaceae and Pseudomonadaceae family members) was observed in cefoperazone-treated mice.[83] Similarly, Buffie et al. demonstrated that clindamycin treatment alone resulted in *C. difficile* susceptibility, and observed a decrease in microbial diversity and long-lasting effects on the gut microbiota.[84] After clindamycin treatment, bacterial members from the Proteobacteria phylum (Enterobacteriaceae family) were dominant. Thus, specific changes to the murine indigenous gut microbiota have been associated with loss of colonization resistance against *C. difficile* (**Table 1**).[83-87]

Only a handful of studies have detailed the structure of the human gut microbiota after *C. difficile* infection, and human samples prior to CDI are limited (**Table 2**).[88-93] The most recent study comes from Antharam et al. 2013 who compared the fecal microbiota of healthy subjects (n = 40) to those with antibiotic-associated diarrhea (AAD, n = 36) and *C. difficile* infection (CDI, n = 39).[88] Decreased microbial diversity and species richness was observed in the fecal microbiota of AAD and CDI cases compared with healthy controls. Additionally, a decrease was seen in butyrate-producing bacteria from the Ruminococcaceae and Lachnospiraceae family and from Clostridia clusters IV and XIVa. Furthermore, CDI cases had a gut microbiota profile enriched in *Enterococcus, Veillonella*, and *Lactobacillus*, and members from the Gammaproteobacteria class.[88]

In another study by Manges and colleagues, fecal samples were collected from 599 patients after 72 h of admission to a Montreal hospital.[90] Twenty-five patients developed *C. difficile* associated diarrhea (CDAD) and their fecal DNA was analyzed by 16S rRNA-gene encoding microarrays. CDAD patients had

Table 1. Animal studies of the gut microbiota and *C. difficile* infection

Host	Antibiotics (route/dose)	Microbiome analysis	Structural changes to the gut microbiota	Strain of *C. difficile*	Reference
Female CF-1 mice	Subcutaneous injections saline, tigecycline (0.05 mg/day), clindamycin (1.4 mg/day), or piperacillin-tazobactam (8 mg/day) for 4 d.	Culture based: plating onto brucella agar and *Bacteroides* bile-esculin agar	• Tigecycline did not suppress total anaerobes or Bacteroides spp. in comparison to saline controls and did not allow for *C. difficile* colonization. • Clindamycin and piperacillin-tazobactam did suppress Bacteroides spp.and allowed for *C. difficile* colonization.	ATCC 43593 VA 17 VA 11	86
Male and female C57BL/6 mice	-Antibiotic cocktail in drinking water: kanamycin (0.4 mg/ml), gentamicin (0.035 mg/ml), colistin (850 U/ml), metronidazole (0.215 mg/ml), vancomycin (0.045 mg/ml) for 5 d followed by clindamycin (10 mg/Kg) intraperitoneal injection. -Cefoperazone (0.5 mg/ml) in drinking water for 10 d.	Non-culture based: 16S rRNA-encoding gene clone libraries	• Increased abundance of the Proteobacteria phylum (Enterobacteriaceae family) and decreased abundance of the Firmicutes phylum (Lachnospiraceae family) was associated with *C. difficile* colonization. • Increased abundance of the Firmicutes and Proteobacteria phyla specifically members of the Lactobacillaceae and Pseudomonadaceae family were also associated with *C. difficile* colonization.	VPI 10463	83
Female C57BL/6 mice	Single dose of clindamycin (200 ug) by intraperitoneal injection.	Non-culture based: Roche-454 pyrosequencing (V1-V3 primers)	• Loss of Lachnospiraceae family members and *Barnesiella* populations and expansion of the Enterobacteriaceae species was associated with *C. difficile* colonization.	VPI 10463	84
Female C57BL/6, C57BL/6 p402/2, C3H/HeN and C3H/HeJ mice	Clindamycin (250 mg/L) in drinking water for 1 wk.	Non-culture based: 16S rRNA-encoding gene clone libraries	• Increased abundance of facultative anaerobes including members of the Enterobacteriaceae family and Enterococci was associated with *C. difficile* colonization. • Supershedder microbiota contained 16S rRNA gene clones derived from *Blautia producta* and included 16S rRNA gene sequences of *Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, Parabacteroides distasonis* and *Enterococcus faecalis*.	BI-7 M68 630	85
Male golden syrian hamsters	Single dose of clindamycin (50 mg/Kg) by subcutaneous injection.	Non-culture based: Roche-454 pyrosequencing (V1-V2 primers)	• Reduction in the abundance of Bacteroidetes and Firmicutes and increase in Proteobacteria was associated with *C. difficile* colonization. • Temporary suppression of Bacteroidales and the fungus Saccinobaculus was also associated with *C. difficile* colonization. • Inoculation with *C. difficile* was associated with increases in Clostridiales on days 1 and 2 with a smaller increase in *Burkholderiales* and *Pasteurellales* species.	ATCC 43596	87

increased probe intensities for the Firmicutes, Proteobacteria and Actinobacteria phylum and decreased for Bacteroidetes; however, only Firmicutes and Bacteroidetes were significantly correlated after accounting for epidemiologic factors. CDAD patients also had an increased abundance of the Lactobacillaceae and Enterococcaceae family members.[90]

Another study by Rea et al. in 2012 compared the fecal microbiota of elderly subjects who were asymptomatic (n =

Table 2. Human studies of the gut microbiota and *C. difficile* infection

Sample collection	Microbiome analysis	Structural changes to the gut microbiota	Reference
Fecal samples were collected from three subject groups: healthy young adults, aged 21–34 y (n = 7); healthy elderly people, aged 67–88 y (n = 4); and elderly patients with *C. difficile* associated diarrhea (CDAD), aged 67–73 years (n = 4).	Characterization of cellular fatty acid (CFA) profiles	• CDAD patients had a greater diversity of facultative species, Lactobacilli and Clostridia, and reduced numbers of Bacteroides, Prevotella and Bifidobacteria. • Enterobacteria and Enterococci increased in CDAD patients.	91
Fecal samples of patients with CDAD (both initial and recurrent episodes) were obtained from 10 individuals—patients with CDAD (n = 7) (initial *C. difficile,* ICD n = 3 and recurrent *C. difficile,* RCD n = 4) and control subjects (n = 3).	16S rRNA-encoding gene clone libraries	• Species richness in the patients with ICD was similar to the controls. • Species richness in the RCD patients was consistently lower than both the patients with ICD and the controls. • RCD is associated with decreased overall diversity of the gut microbiota.	93
Fecal samples from 599 patients, hospitalized from September 2006 through May 2007 in Montreal, Quebec, were obtained within 72 h after admission. Twenty-five developed CDAD, and 50 matched controls were selected for analysis.	16S rRNA-gene encoding microarrays	• Probe intensities were higher for Firmicutes, Proteobacteria, and Actinobacteria in CDAD patients, compared with controls, whereas probe intensities for Bacteroidetes were lower. • After epidemiologic factors were controlled for, only Bacteroidetes and Firmicutes remained significantly and independently associated with development of CDAD.	90
Fecal samples were collected from elderly subjects recruited from the community; including outpatient, short-term respite, and long-term hospital stay subjects. The carriage rate for *C. difficile* ranged from 1.6% (n = 123) for subjects in the community, to 9.5% (n = 43) in outpatient settings, and increasing to 21% (n = 151) for patients in short- or long-term care in hospital.	Culture-independent Roche/454 pyrosequencing (V4 region)	• *C. difficile* positive subjects had a decrease in Enterococcaceae but an increase in Lactobacillaceae and Enterobacteriaceae. • The dominant 072 ribotype was carried by 43% (12/28) of subjects, while the hypervirulent strain R027 (B1/NAP1/027) was isolated from 3 subjects (11%), 2 of whom displayed CDAD symptoms at the time of sampling. • Emerging ribotypes (078 and 018) were also isolated from two asymptomatic subjects.	89
Fecal samples (n = 208), of which 171 were routine samples and 37 were from healthy volunteers were collected. Of the 171 routine samples, 105 were *C. difficile* positive and 66 were *C. difficile* negative. From all 105 positive fecal samples *C. difficile* was isolated and strains were assigned to 22 different *C. difficile* PCR ribotypes. The five most frequent ribotypes were 027, 014/020, 081, 002 and 023.	Denaturing high-pressure liquid chromatography (DHPLC) and machine learning methods	• *C. difficile* positive samples showed lower levels of bacterial taxons from *Bifidobacterium longum*, Prevotella sp. and Bacteroides sp.. • *Bifidobacterium longum* was the most important predictor for the *C. difficile* negative status. • *C. difficile* positive samples had increases in *Ruminococcus bromii*, the family Peptostreptococcaceae and Streptococcus sp./Enterococcus sp. 2. • Healthy donors had higher frequencies of *Methanobrevibacter smithii* compared with *C. difficile* negative samples sent for routine testing and to *C. difficile* positive samples.	92
Fecal samples were collected from fecal microbiota transplant patients or FMT (n = 3) and their healthy donors (n = 3).	High-throughput 16S rRNA gene sequencing (V6 region)	• Post FMT samples from patients showed an increase in the abundance of Firmicutes and Bacteroidetes. Proteobacteria and Actinobacteria were less abundant (< 5%) than that found in patients prior to FMT. • Bacteroidetes phylum was represented by family members Bacteroidaceae, Rikenellaceae and Porphyromonadaceae, and were largely comprised of *Bacteroides, Alistipes and Parabacteroides* genera. • Firmicutes phylum was represented by family members Ruminococcaceae, Lachnospiraceae, Verrucomicrobiaceae and unclassified Clostridiales and members of the Firmicutes.	96
Fecal samples were collected from individuals with *C. difficile* infection (CDI) (n = 39), subjects with nosocomial diarrhea not attributed to *C. difficile* (CDN) group (n = 36), and healthy controls (n = 40).	Culture-independent Roche/454 pyrosequencing (V1-V3 primers)	• CDI and CDN subjects were accompanied by a marked decrease in microbial diversity and species richness driven by a decrease in phylotypes within the Firmicutes phylum. • CDI and CDN subjects were depleted of Ruminococcaceae and Lachnospiraceae family members and butyrate-producing C2-C4 anaerobic fermenters.	88

20) to patients that were culture negative for *C. difficile* (n = 252).[89] *C. difficile* positive subjects had a decrease in *Bacteroides, Prevotella* and *Bifidobacteria* and an increase in members from the Lactobacillaceae and Enterobacteriaceae family.[89] Using a culture-dependent method, Hopkins and MacFarlane observed similar results, including an increase in the diversity of facultative species such as Lactobacilli and Clostridia in four CDI cases.[91]

Standard treatment of CDI in humans is oral administration of either metronidazole or vancomycin. Unfortunately, after successful treatment more than 20% of patients experience one or more relapses of disease.[94] Patients that have failed traditional treatment with severe CDI have had success with fecal bacteriotherapy, which is the restoration of colon homeostasis by reintroducing normal bacterial microbiota from stool obtained from a healthy donor.[95] In 2008, Chang et al. found that patients with recurrent CDAD had decreased diversity of the fecal microbiota with highly variable bacterial composition.[93] A more recent study following successful fecal bacterial transplantation of three subjects observed an increase in Firmicutes and Bacteroidetes (Bacteroidaceae, Rikenellaceae and Porphyromonadaceae and Ruminococcaceae, Lachnospiraceae, Verrucomicrobiaceae, and unclassified Clostridiale members) and a decrease in Proteobacteria and Actinobacteria members.[96] Interestingly, an increased abundance of the Enterobacteriaceae family was observed in one patient requiring antibiotics during the study.[96]

Structural changes to the gut microbiota in humans resemble changes in mice after antibiotics (as seen in **Tables 1 and 2**); however no specific structural profile has been correlated with decreased colonization resistance against *C. difficile*. Commonalities among studies that allow for *C. difficile* colonization in both **Tables 1 and 2** include a decrease in bacterial diversity, a decrease in bacteria from the phylum Bacteroidetes and an increase in Proteobacteria. A decrease in bacterial diversity and a shift in the predominant members of the gut microbiota could alter bacterial metabolism in the gut, potentially allowing for *C. difficile* colonization. Future studies will need to investigate which microbes inhabit the gastrointestinal tract during different disease states, as well as determine the metabolic role of each specific commensal member.

Targeted Interventions and Treatment Options

Based on the structural studies detailed in **Tables 1 and 2**, a healthy, diverse gut microbiota is necessary for colonization resistance against *C. difficile*. Targeted interventions that will preserve or reestablish the structure and more importantly the function of the gut microbiota (ie. non-antibiotics) are needed. In order to restore colonization resistance in the gut after multiple failed courses of antibiotics, patients have turned to fecal transplantation, which is recolonizing the gut with healthy donor stool. Fecal bacteriotherapy has a 90% success rate although the long-term consequences of this treatment are still unclear.[97,98] Repopulating the gut with healthy bacteria is not a new concept, and both human and murine models have demonstrated effective recovery of colonization resistance against *C. difficile*.[85,96] In 1989 this approach was applied to patients by Tvede and Rask-Madsen where a cocktail of ten bacteria was given to six patients suffering from recurrent CDI.[99] After rectal installation of the bacteria, patients' stool had decreased levels of *C. difficile* and toxin and an increase in *Bacteroides* sp. More recently, Petrof et al. used a synthetically prepared stool transplant comprised of 33 bacterial isolates from a healthy donor stool.[100] This cocktail, consisting of a community of bacteria, was able to treat two patients for up to 6 mo post-transplant at which point they were symptom free. In 2012, Lawley et al. designed a bacterial cocktail that was able to reestablish colonization resistance in mice against *C. difficile* 027/BI strain.[85] The cocktail of six bacteria isolated from the mouse gut consisted of *Staphylococcus, Enterococcus, Lactobacillus*, *Anaerostipes, Bacteroidetes,* and *Enterorhabdus*. It will be important to determine what the bacteria are doing in the gut that is creating functional resistance to *C. difficile*. There has also been some success using individual strains of bacteria in a murine model to prevent or minimize *C. difficile* infection with non-toxigenic *C. difficile*, *Escherichia coli*, *Bifidobacterium bifidum,* and members of the Lachnospiraceae family.[101-103]

Alternate ways to manipulate the gut microbiota that could restore colonization resistance against *C. difficile* include diet changes and the use of pre- or probiotics. Prebiotics are non-digestible food ingredients that promote the growth of beneficial microorganisms in the intestines.[104] Probiotics are microorganisms that are believed to counteract disturbances in the gut made by antibiotics, thus restoring colonization resistance against pathogens.[105] A recent review examining 20 trials of probiotics for the prevention of CDAD estimated they prevent 33 episodes per 1000 persons.[106] However, the mechanism for which probiotics prevent CDAD is unknown at this time and requires further study. A combination of diet, pre- and probiotics represents a promising strategy for reengineering the gastrointestinal tract environment to be functionally resistant to CDI.

Future Directions

It is well documented that antibiotics alter the structure of the gut microbiome,[43,83] but it is unknown how this impacts bacterial metabolism in the gut. New "omics" techniques including metagenomics, transcriptomics, proteomics and metabolomics are now available to help define function in the gut. Many things can cause an imbalance in the gut microbiota including antibiotic usage, changes in diet, medications, inflammation, and pathogens. Molecular tools, including metabolomics, will be critical in the future for understanding these imbalances and could contribute to diagnosis, biomarker discovery and aid in personalized medicine. A more targeted approach to alter the gut functionally and potentially restore colonization resistance to *C. difficile*, such as diet, pre- and probiotics is needed. Unlocking how *C. difficile* is able to overcome colonization resistance in the gut has major implications for the development of therapeutics for prevention and treatment of human CDI.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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