

# Gut microbiome and *Clostridioides difficile* infection: a closer look at the microscopic interface

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Ther Adv Gastroenterol

2021, Vol. 14: 1–9

DOI: 10.1177/  
1756284821994736

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**Abstract:** The pathogenesis of *Clostridioides difficile* infection (CDI) was recognized with its link to the use of antimicrobials. Antimicrobials significantly alter gut microbiota structure and composition, which led to the discovery of the association of this gut perturbation with the development of CDI. A number of factors implicated in its pathogenesis, such as advancing age, proton-pump inhibitors, and gastrointestinal diseases, are linked to gut microbiota perturbations. In an effort to better understand CDI, a multitude of studies have tried to ascertain protective and predictive microbial footprints linked with CDI. It has further been realized that CDI in itself can alter the gut microbiome. Its spore-forming capability poses as an impediment in the management of the infection and contributes to its recurrence. Antibiotic therapies used for its management have also been linked to gut microbiota changes, making its treatment a little more challenging. In an effort to exploit and utilize this association, gut microbial restoration therapies, particularly in the form of fecal microbial transplant, are increasingly being put to use and are proving to be beneficial. In this review, we summarize the association of the gut microbiome and microbial perturbation with initial and recurrent CDI.

**Keywords:** microbiota, dysbiosis, antibiotics, *Clostridium difficile*, colitis, diarrhea, infection

Received: 2 October 2020; revised manuscript accepted: 19 January 2021.

## What is the microbiome?

The human gastrointestinal system harbors approximately 100 trillion microorganisms whose collective genetic material makes up at least 100-fold more genetic diversity than the entire human genome.<sup>1</sup> These microorganisms include bacteria, viruses, fungi, and protozoa, which make up the microbiota.<sup>2</sup> The composition of the gut microbiome was previously evaluated by isolation and culturing of microorganisms in different growth media, but more recent studies analyzing 16s ribosomal RNA or shotgun sequencing are being utilized for the analysis of all the genomes in a sample to map the entire microbial genome.<sup>1</sup> Through these efforts, it has been established that the majority of the bacteria in the gastrointestinal system belong to these taxa: Bacteroidetes (23%), Firmicutes (64%), Proteobacteria (8%), Gram-negative bacteria such as *Escherichia coli* and *Helicobacter pylori*, Fusobacteria, Verrucomicrobia, and Actinobacteria (3%).<sup>3</sup>

The initial colonization of gut microbiota in early life is governed by bacteria present in the environment relying on factors such as mode of delivery, mother's vaginal, fecal, and skin microbiota, and environmental factors. These microbes in the gut are metabolically active and perform a number of functions such as digestion of soluble fiber, harvesting energy, producing vitamins, maturation of the immune system, and importantly, preventing colonization with pathobionts. Furthermore, these microorganisms constantly interact with the human host and a diverse microbiome has been associated with host health.

Perturbations in the composition, that is, decreased diversity and numbers along with an altered function of the gut microbiota, can modify the interaction with the host and the immune system.<sup>4</sup> This perturbation is associated with a number of diseases including colonization and infection with multidrug-resistant organisms, inflammatory bowel

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disease, irritable bowel syndrome, asthma, allergy, metabolic syndrome, and cardiovascular disease.<sup>4</sup> Gut perturbation has a role in the development of primary and recurrent *Clostridioides difficile* infection (CDI).

### What about the microbiome and CDI?

*C. difficile* has been established as the most common organism causing healthcare-associated infections in hospitals in the USA.<sup>5</sup> According to the Centers for Disease Control and Prevention, in 2017 there were 223,900 CDIs and 12,800 deaths associated with CDI. It also has had a tremendous economic impact with healthcare costs ranging from US\$8911 to US\$30,049 per hospitalized patient.<sup>6</sup> *C. difficile* was essentially considered to be a hospital-acquired infection, with increasing evidence of community-acquired cases, indicating that reservoirs of CDI outside a hospital setting play a significant role in its transmission. This was illustrated in a study by Eyre *et al.*<sup>7</sup> where they demonstrated that community acquisition of infection was the largest source of CDI and that person-to-person transmission in hospitals accounted for almost a third of infections.

The pathogenesis of CDI is primarily linked to antibiotic use. The first outbreaks of *C. difficile* were associated with clindamycin use and subsequently have been linked to the use of broad-spectrum antibiotics such as fluoroquinolones and third-generation cephalosporins.<sup>8,9</sup> In the early 2000s, a hypervirulent fluoroquinolone-resistant strain of the *C. difficile* bacterium, known as North American pulsed-field gel electrophoresis type 1/restriction endonuclease analysis group B1/PCR ribotype (RT) 027 emerged and increased the incidence of CDI leading to outbreaks. The association of antibiotics with the development of CDI is due to the emergence of disturbances in the gut microbial ecosystem with the use of antibiotics. However, studies have also identified additional risk factors that can result in CDI. A prospective study conducted by McFarland *et al.*<sup>10</sup> recognized that stool softeners as well as gastrointestinal stimulants were implicated in the pathogenesis of the infection.

The gastrointestinal system is the natural habitat of *C. difficile* in neonates where it grows and proliferates.<sup>11</sup> *C. difficile* interestingly, does not lead to active infection in neonates despite frequently colonizing them. It is believed that with time as

the neonate grows, is exposed to the environment and begins consumption of an adult diet, other microbial species are introduced into the gut, become the predominant species, and outgrow *C. difficile*.<sup>12</sup> These acquired microbial species grow and constitute the gut microbiota, providing a protective environment against the colonization of *C. difficile*.<sup>13</sup> Any disturbance in the gut microbial system can potentially promote the growth of *C. difficile* if there is exposure to *C. difficile* during a period of gut microbial disequilibrium.

### Microbiome stability and composition affect the risk of developing CDI

The microbiota usually remains stable in healthy individuals; various factors tend to disturb the composition. Several studies have been conducted to understand the microbial composition and its variability. One study attempted to decode the human microbiome at various points in time and discovered that the composition varies temporally, which is dependent on a number of factors including types of foods, medication use, physical environment like travel, and even intrinsic factors including the immune system.<sup>14</sup> Research across animal and human models has suggested that resultant microbial disturbances can potentially predispose to acquiring infections<sup>15</sup> and conceivably be associated with disease states like obesity<sup>16</sup> and autoimmune diseases.<sup>17</sup>

The most commonly implicated factor in the disruption of the gut microbiota is antibiotic use. A decrease in the diversity of the gut microbiota is observed within days of antibiotic use with the changes in composition being dependent on the specific antibiotic class used and on the microbial structure of the individual.<sup>18</sup> The bacterial community tends to restore with time, but some bacterial taxa do not recover completely.<sup>18</sup> There is also a reduced resistance to colonization, which encourages the growth of pathogenic microbes such as *C. difficile* to change the structure of the microbiota in the individual.<sup>19</sup> Periodic use of antibiotics induces an increase in the reservoir of antibiotic-resistant genes in the gut microbiome.<sup>20</sup> Studies conducted on mouse models and *in vitro* models have demonstrated a decrease in obligate anaerobic bacteria and dominance by Enterobacteriaceae as a consequence of clindamycin usage; Pseudomonadaceae and Lactobacillaceae-dominated population in cephalosporin exposure and lower Bacteroidetes and higher Proteobacteria numbers with

tigecycline use; all of which have been associated with CDI.<sup>21</sup>

A human gut model used to study the effect of cefotaxime instillation caused modest decreases in Bifidobacteria and Bacteroidetes and subsequently resulted in *C. difficile* germination and toxin production at high levels.<sup>22</sup> A similar study demonstrated that although clindamycin had detrimental effects on the gut microbiota, the diversity re-established to pre-drug levels before CDI occurred, indicating that the role of gut flora in regulating *C. difficile* is more complex than previously believed.<sup>23</sup>

Another factor implicated in gut microbiota alteration is increasing age. The composition of the gut changes drastically during the early years of life but relatively stabilizes in adult life.<sup>24</sup> However, in the elderly, a lower microbial diversity and an altered composition has been observed. Studies have reported a decrease in protective species, including Bifidobacteria and some members of Firmicutes and Bacteroidetes, and an increase in harmful species such as Proteobacteria.<sup>25</sup> While these changes are likely conducive to CDI due to a decreased colonization resistance, increasing age is also linked with frequent use of antibiotics, more healthcare exposure, and development of comorbid conditions, all of which contribute to the susceptibility of acquiring *C. difficile*.<sup>26</sup>

In addition, proton-pump inhibitors (PPIs), which result in an increase in the pH of the stomach, have been seen to impact the distal gut microbiota as well. *In vitro* studies have demonstrated that PPIs affect the growth of *Lactobacillus*, which is a commensal of the mouth and gut.<sup>27</sup> Clooney *et al.*<sup>28</sup> also demonstrated that the gut microbial composition changed with PPI use with a decrease in Bacteroidetes and an increase in Firmicutes, which possibly led to an increased susceptibility to CDI.

Lastly, it has been speculated that microbial perturbation is also linked with diseases such as obesity, autoimmune and allergic diseases, diabetes, and inflammatory bowel disease (IBD).<sup>19</sup> By studying animal models using mice, a clear interplay between gut microbiota and obesity has been established, with a decrease in Bacteroidetes and an increase in Firmicutes.<sup>29</sup> However, a similar simplistic correlation has not been established in human studies and it is speculated that the

composition of these two bacterial taxa further depends on other factors such as diet, fasting, and eating patterns along with the use of probiotics and prebiotics.<sup>1</sup>

The gut microbes also interact with the immune system and mediate its maturation. It has been revealed that the gut microbiota interact with the immune system and modify the predisposition towards the development of diabetes in a mouse model.<sup>30</sup> Further supporting this finding, autoimmune diseases do not seem to develop in mouse models that are devoid of any microbes in their gut, known as germ-free mice.<sup>31</sup>

A study investigating the association between IBD and the gut microbiota using mouse models and humans has concluded that a complex relationship exists between the two, rather than a simplistic cause-effect relation.<sup>32</sup> They hypothesized that firstly, gut microbial perturbations might not be the initiating event for IBD but might develop later as the disease progresses and contribute to its chronicity. Secondly, if gut microbiota do have a role to play in inciting the disease, this might occur early in life in the form of cesarean section, childhood antibiotic exposure, and infant formula usage. These findings are relevant as patients with IBD have inherent microbial perturbation and IBD remains a clinically independent risk factor for CDI even in the absence of antibiotic exposure.

### Mechanisms responsible for *C. difficile* colonization

Extensive work has explored the possible molecular signaling mechanisms implicated in the causation of CDI. A study in gnotobiotic mice assessed the gene expression profile of *C. difficile* in response to dietary or microbial composition alterations.<sup>33</sup> It was discovered that *C. difficile* utilizes the succinate (a microbiota fermentation end-product) and metabolizes it into butyrate, in the presence of *Bacteroides thetaiotaomicron*. The concentration of succinate, which is lower in conventional raised mice, is temporarily increased after antibiotic treatment or induced intestinal motility disturbance, and is used by *C. difficile* to proliferate in the perturbed gut.<sup>33</sup>

Interestingly, another study proposed the idea that widespread use of disaccharide trehalose in the human diet might have been one of the factors

responsible for the emergence of epidemic and hypervirulent strains (RT027 and RT078) of *C. difficile*.<sup>34</sup> They discovered these two epidemic ribotypes acquired mechanisms to utilize low concentrations of trehalose, enabling *C. difficile* to colonize the human intestine.

Further, primary bile acids, such as taurocholic acid, are thought to promote *C. difficile* spore germination in the presence of glycine *in vitro* and conversely, secondary bile acids, such as deoxycholic acid and lithocholic acid, inhibit the vegetative growth and toxin of *C. difficile*.<sup>35</sup> Antibiotics are thought to disturb the commensal gut microbiota that produce secondary bile acids. Stool specimens collected from antibiotic-treated mice have been found to contain higher concentrations of primary bile acids, while untreated mice stool extracts have higher levels of secondary bile acids.<sup>36</sup> In this context, a human study by Allegretti *et al.* aimed to assess the levels of primary and secondary bile acids associated with CDI and found that antibiotics result in an abundance of primary bile salts by eradicating commensal bacteria which permits recurrent CDI.<sup>37</sup> They suggested the possible use of secondary bile salts as biomarkers for recurrence.

#### Gut microbial alterations due to *C. difficile*

Much like the way gut microbiota disruption can predispose to CDI, *C. difficile* in itself can lead to a perturbation in the gut microflora. Numerous studies have been conducted to investigate these microbial changes induced by CDI. One such study utilized cellular fatty acid profiles to identify bacteria from fecal samples of patients suffering from CDI.<sup>38</sup> It reported that patients had decreased *Bacteroides*, *Prevotella*, and *Bifidobacteria* and increased number of *Clostridium* and *Lactobacillus* spp. These findings were further confirmed by a similar study conducted by Rea *et al.*, and they additionally reported an increase in *Lactobacillaceae* and *Enterobacteriaceae* but a decrease in *Enterococcaceae* in patients who had CDI.<sup>39</sup> It remains to be determined if these changes are secondary to the risk factors that led to CDI or due to the presence of the *C. difficile* bacterium in the gut.

Another study used the analysis of 16S rRNA encoding gene sequences and compared the fecal composition of controls with patients who had initial or recurrent CDI and demonstrated that patients with CDI had lesser microbiome diversity

compared with controls.<sup>40</sup> A similar study compared healthy individuals with patients suffering from CDI and non-*C. difficile* diarrhea, which interestingly reported that both these groups of patients with diarrhea, regardless of the diarrhea being from *C. difficile* or not, have lesser diversity, particularly decreased Firmicutes.<sup>41</sup> In addition, they observed that controls were enriched with *Bacteroidetes* species, *Lachnospiraceae*, and *Ruminococcaceae*, while patients with diarrhea had *Enterobacteriaceae*, *Erysipelotrichaceae*, and *Lachnospiraceae*.

Recurrent CDI continues to be a concern in a large number of patients. A study carried out by Chang *et al.*<sup>40</sup> aimed to differentiate gut microbiota in different patient populations. They found that controls and patients with an initial episode of *C. difficile* diarrhea had a majority of organisms belonging to *Bacteroidetes* and *Firmicutes* taxa, while patients with recurrent CDI varied from this normal composition. Species richness of patients with an initial episode was similar to that seen in controls but richness in patients with recurrence of infection was lower. Interestingly, they also observed that in two out of three patients with recurrent infection, *C. difficile* had become a dominant member of the gastrointestinal community. However, another study investigated the burden of *C. difficile* relative to the overall gut microbiome and found that while *C. difficile* had a higher presence in cohorts of patients with CDI, the relative abundance in healthy individuals in comparison with other members of the microbiota was significantly lower, indicating that it is not usually a dominant member in the gut of healthy individuals.<sup>42</sup>

Furthermore, in an effort to establish a microbiota pattern for CDI prediction, a study found that higher levels of *Bifidobacterium longum* was the most important predictor associated with a negative *C. difficile* status compared with positive assays.<sup>43</sup> They also analyzed fungal associations with *C. difficile* and observed that *Candida albicans* and *Candida glabrata* were associated with the presence of *C. difficile*. The best predictive score included *Enterobacteriaceae* and *Candida glabrata*. They finally concluded that the likelihood of acquiring CDI does not solely depend on the presence or absence of a particular species, but more on the combination of gut microbes.

A prospective cohort study that intended to identify microbiome markers to predict the response to treatment in patients with *C. difficile* infection

was carried out.<sup>44</sup> In this study, *Faecalibacterium*, Ruminococcaceae, *Bacteroides*, and Rikenellaceae (all of which are associated with colonization resistance), were present in greater numbers in primary responders to treatment. The presence of *Faecalibacterium prausnitzii* and members of the Ruminococcaceae, that are butyrate producers, may be associated with an improvement in the response to treatment either by providing nutritional competition or by a direct inhibitory effect on *C. difficile*. Moreover, the taxa found in patients with recurrent CDI were different from those found in nonresponders. An increase in Enterobacteriaceae, *Veillonella*, and *Parabacteroides* was seen in patients with recurrent infection.

### **C. difficile treatment and its effect on the gut microbiota**

The therapy for CDI has been rapidly evolving over the past few years. Metronidazole and oral vancomycin were previously used as the main antibiotic agents. While these agents are effective against *C. difficile*, there were growing concerns regarding their undesirable impact on the native intestinal microbial population.<sup>45</sup> For instance, oral vancomycin suppresses anaerobic organisms such as Bacteroidetes species as it attains high concentrations in the intestinal tract.<sup>45</sup> This disturbance in the native microbiota could predispose individuals to recurrent CDIs and promote colonization by healthcare-associated organisms such as vancomycin-resistant enterococci (VRE), and even species of *Candida* as demonstrated in mouse studies.<sup>46,47</sup> To address this concern that both these agents were potentially associated with VRE, a prospective observational study was performed where stool samples before, during, and after 90 courses of therapy were collected and cultured for VRE.<sup>48</sup> This study observed that patients who had a pre-existing colonization with VRE had a promotion of this colonization that decreased only after discontinuation of treatment with either of the two drugs. These findings highlighted the fact that both these antibiotics caused unnecessary disruptions in the gut microbiota and that there was a need for the development of a therapeutic option that specifically suppressed *C. difficile* with minimal gut microbial interruptions.

The landscape for treatment of *C. difficile* changed as randomized clinical trials demonstrated that oral vancomycin had higher cure rates and was superior to metronidazole. Since then, the use of

metronidazole is now limited to an initial episode of non-severe infection when other therapeutic options are either not available or are contraindicated.<sup>49</sup> The current regimen that is widely accepted and used includes vancomycin or fidaxomicin. A few studies have investigated the impact of these antibiotics on the gut microbiota. One of the first studies to evaluate the effect of vancomycin and fidaxomicin in a 10-day treatment course for patients suffering from CDI concluded that while both drugs were effective in clearing out the infection, fidaxomicin did not suppress Bacteroidetes.<sup>50</sup> In a murine model exposure to either vancomycin or fidaxomicin led to a decrease in microbial diversity and a modification in the composition.<sup>51</sup> They discovered that mice that were exposed to vancomycin had a greater number of enterococci than those exposed to fidaxomicin. Further, vancomycin promoted colonization by VRE and *Klebsiella pneumoniae*, whereas fidaxomicin caused negligible gut microbial perturbation. Fidaxomicin preserves taxa that resist *C. difficile* overgrowth by preserving colonization resistance which mediates a faster microbial recovery and leads to lower rates of recurrence after treatment. Analyses of a subset of samples from a randomized clinical trial that assessed fidaxomicin versus vancomycin for *C. difficile* treatment demonstrated that fidaxomicin does not suppress the Bacteroidetes group and was not as likely as vancomycin to promote colonization by VRE or *Candida* species.<sup>52</sup>

### **Microbial modulation for C. difficile**

Due to the growing body of evidence revealing a strong association between the gut microbiota and CDI, the use of microbial modulation as a therapeutic option for treatment and management is increasingly gaining more ground. The goal with such therapies is to restore the gut microbiota in order to prevent future infection. One such therapy that is currently in use is fecal microbiota transplantation (FMT), which has shown promise in the prevention of recurrent infection by restoring the gut microbiome.<sup>53,54</sup>

Microbiome restoration can directly impact *C. difficile* by increasing the competition for nutrition, which results in resistance to its colonization and the production of bacteriocins (that are antimicrobial peptides).<sup>55</sup> A study was carried out using high-throughput 16S rRNA gene sequencing on patients who received FMT, which reported that the gut

microbial composition pre-FMT in these patients was drastically different compared with the healthy donors and the follow-up post-FMT samples.<sup>56</sup> They observed that donor and post-FMT patient samples had an increase in Firmicutes and Bacteroidetes strains, a finding that is consistent with a healthy gut composition. The change in composition after receiving FMT was observed as soon as 3 days along with a resolution of symptoms and recovery from CDI. A longitudinal study by Hamilton *et al.*<sup>56</sup> investigated the dynamics of the gut community up to 4 months post-FMT in three patients and found that while the gut environment was similar to the donor immediately post-transplant, this change was only transient. This suggests that FMT possibly provides an environment that encourages the growth of normal gut microbiota including Bacteroidetes and Firmicutes that inhibits the growth of *C. difficile*, thereby protecting from recurrent CDI. There has also been increasing interest in the study of metabolites, particularly bile acids, to determine the efficacy of FMT for CDI treatment. The conversion of primary to secondary bile acids occurs in the gut by the enzymes (bile salt hydrolase and 7- $\alpha$ -hydroxylase) that are produced by the gut microbiota. Consequently, a number of mice and human studies revealed that a pre-FMT stool has a higher concentration of primary bile acids and a post-FMT stool has higher levels of secondary bile acids, that is in accordance with healthy donors.<sup>57</sup> Recent studies have also revealed that restoration of microbial bile salt hydrolase is essential for successful FMT treatment for recurrent CDI and that this restoration reduced *C. difficile* load in the stool of mice that had CDI.<sup>58</sup> In addition, the success of FMT for recurrent CDI is also associated with the stimulation of farnesoid X receptor signaling, which seemingly affects the bile acid environment and hence the gut microbiota.<sup>59</sup>

A more novel therapeutic treatment option that has emerged is bezlotoxumab, which is a human monoclonal antibody that is directed against toxin B produced by *C. difficile*. Bezlotoxumab not only prevents intestinal epithelial damage but has also been seen to promote the reconstitution of a healthy gut microenvironment thereby preventing recurrence of infection.<sup>60</sup>

### Conclusion

*C. difficile* infection is typically a result of bacterial perturbations in the gut with the use of antimicrobials resulting in gut microbial disturbances being

at the core of its pathogenesis. The gut microbiota performs a fundamental role in resisting the colonization of *C. difficile* and hence, perturbation of this microbial structure creates an environment conducive to colonization and infection with *C. difficile*. In addition, infection with *C. difficile* adversely impacts the gut microbiome diversity. While there is now an established association between gut microbial perturbations and CDI, more information is still required on the exact bacterial strains that contribute to acquiring CDI. Prediction and therapy of recurrent CDI has been a challenge. The need for data on microbial patterns that could possibly predict response to treatment and CDI recurrence remains an avenue that needs more exploration, which could potentially aid in the appropriate management of CDI.

### Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

### Conflict of interest statement

SK received research grants from Rebiotix, and consulting fees from Shire, Premier, Facile Therapeutics, and Probiotech International, outside of the submitted work.

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