Control of *Clostridium difficile* infection by defined microbial communities

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Summary

Each year in the United States, billions of dollars are spent combating almost half a million *Clostridium difficile* infections (CDI) and trying to reduce the ~29,000 patient deaths where *C. difficile* has an attributed role (1). In Europe, disease prevalence varies by country and level of surveillance, though yearly costs are estimated at €3 billion (2). One factor contributing to the significant healthcare burden of *C. difficile* is the relatively high frequency of recurrent *C. difficile* infections(3). Recurrent *C. difficile* infection (rCDI), i.e., a second episode of symptomatic CDI occurring within eight weeks of successful initial CDI treatment, occurs in ~25% of patients with 35-65% of these patients experiencing multiple episodes of recurrent disease(4, 5). Using microbial communities to treat rCDI, either as whole fecal transplants or as defined consortia of bacterial isolates have shown great success (in the case of fecal transplants) or potential promise (in the case of defined consortia of isolates). This review will briefly summarize the epidemiology and physiology of *C. difficile* infection, describe our current understanding of how fecal microbiota transplants treat recurrent CDI, and outline potential ways through which that knowledge can be used to rationally-design and test alternative microbe-based therapeutics.

History of *C. difficile* infection

A strictly anaerobic, spore forming, Gram-positive bacillus, *C. difficile* (originally named *Bacillus difficilis*) was first identified in healthy neonates in 1935 (6). It was not until the late 1970s however, that the role of *C. difficile* as an etiological agent of antibiotic-associated diarrhea and pseudomembranous colitis was described (7-10). Throughout the 1980s and 1990s the clinical importance of *C. difficile* remained limited. When encountered, disease was often mild and easily controlled with antibiotics (Table 1). In the early 2000s, a new lineage of *C. difficile* strains (equivalently known as ribotype 027, restriction endonuclease analysis group BI, or North American pulsed-field electrophoresis 1 (NAP1)) emerged that was linked to increased disease severity, morbidity and mortality (11, 12) (Figure 1). In the following years, the ribotype 027 lineage spread throughout North America and Europe (13, 14), paralleling increases in the overall incidence and severity of *C. difficile* infection. Strains of the ribotype 027 lineage have often been described as "hypervirulent" (11, 15). Studies have pointed to increased toxin production (16), production of a binary toxin (17), fluoroquinolone resistance (14), increased frequency of sporulation

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(18), enhanced ecological fitness (19) and a higher correlation with recurrent infection (20, 21) as potential mechanisms that led to increased incidence and/or severity of infections associated with ribotype 027 strains. However, some "hypervirulent" attributes of ribotype 027 vary across strains (e.g., degree of toxin production and sporulation (22)) or are found in ribotypes not typically associated with increased disease severity (e.g., fluoroquinolone resistance (23)), making it difficult to pinpoint specific mechanisms responsible for increased incidence and/or severity of infection with ribotype 027 strains. Correspondingly, some clinical studies (24-26) failed to reproduce statistically significant increases in severe CDI in patients infected with ribotype 027 observed in other studies (13, 27, 28). Finally, even as the frequency of ribotype 027-associated CDI has stabilized or decreased in some regions (29, 30), the overall frequency of CDI has remained high, with other *C. difficile* ribotypes increasing in prevalence (29- 32). The picture emerging from these diverse and sometimes conflicting studies is that although the spread of the ribotype 027 lineage significantly contributed to the initial increase in CDI, factors in addition to the ribotype of the infecting strain contribute to the continued prevalence of CDI, which remains a significant threat to public health.

Clostridium difficile carriage and disease

Traditionally, *C. difficile* infection has not been considered a risk to healthy individuals. Infection is most often preceded by disruption of the 'healthy' gut microbiome through antibiotic use (33). Meta-analyses examining the risk of CDI following antibiotic use found clindamycin to be the greatest hazard followed by carbapenems, fluoroquinolones and cephalosporins; more moderate risks were associated with macrolides, sulfonamides and penicillins; and no risk associated with tetracyclines (34-36). Use of proton pump inhibitors (37), inflammatory bowel disease (38), and advancing age (39) are also risk factors, likely due to changes to the gut microbiota (40, 41), and in the latter case, reduced efficacy of the immune system (39). However, increases in the rates of community-acquired *C. difficile* infection have also demonstrated that CDI can occur in relatively healthy populations previously thought to be at low-risk for infection, including people who had not been exposed to antibiotics within the previous 3 months (reviewed in (31, 42)), revealing that there remain additional, previously unrecognized risk factors for *C. difficile* infection.

Once a susceptible individual is exposed to *C. difficile*, multiple outcomes are possible (Table 1). Asymptomatic carriage occurs in a subset of susceptible individuals. Symptomatic *C. difficile* infection can manifest with increasing degrees of severity, which influences approaches used to treat disease. Following the resolution of symptomatic disease, asymptomatic shedding of spores in stool can persist for up to six weeks (43). A brief description of treatment modalities is described later in the review to provide context for the use of microbial-based therapies for treatment of *C. difficile* infection. However, as neither author is a clinician, we point to several recent reviews (44-46) written by clinicians experienced in diagnosis and treatment of *C. difficile* that provide excellent detailed descriptions of the diagnosis and treatment of CDI in adults.

Asymptomatic carriage in infants

Infants (< 1 year of age) are frequently asymptomatic carriers of *C. difficile*, with a wide range of carriage rates reported (2-100% (47-51)). The mechanisms that mediate high levels of asymptomatic carriage in infants are unclear, though the immature microbiome likely plays a role. During the first year of life, the infant gastrointestinal microbiome undergoes successional maturation, transitioning from a low-complexity community, dominated by facultative anaerobes and aerobic bacteria, to a complex community dominated by obligate anaerobes and more similar to the adult gastrointestinal microbiome (52, 53). Hypotheses for how infant carriage rates remain on the whole asymptomatic include the failure of the immature infant immune system to activate an inflammatory response, protective maternal antibodies transferred in breast milk, and/or the inability of toxins to interact appropriately with receptors and cause disease (reviewed in (50, 54)). Additional studies, including those that utilize physiologically relevant models of the infant gastrointestinal tract, are needed to delineate mechanisms through which infants are asymptomatically colonized with *C. difficile*.

Asymptomatic carriage in adults

Carriage rates decrease with increasing age (49, 50), with asymptomatic carriage rates in healthy adults reported between 0 and 15% (50, 55-57). Patients in Long-Term Care Facilities have a higher average asymptomatic carriage rate at 14.8% (95% CI 7.6% - 24.0%), though it is hard to discern if this is due to a change in the cohort physiology (microbiota, immune response), the environment, or both (58). Asymptomatic carriage is often limited in duration (reviewed in (56)) and has been associated with a lower likelihood of developing symptomatic CDI (59, 60).

Potential mechanisms underlying asymptomatic carriage

Two small studies have suggested that differences in the host microbiota could be a potential mechanism that distinguishes between asymptomatic carriage and disease (61, 62). By comparing microbial community composition between four patients with CDI, four asymptomatically colonized patients and non-colonized controls, Vincent et al (62) found that organisms classified as members of the *Clostridiales incertae sedis* XI family, *Eubacterium* species, or *Clostrdium* species other than *C. difficile* were enriched in asymptomatically colonized patients compared to patients with symptomatic CDI. By comparing eight symptomatically infected patients, eight asymptomatically colonized *C. difficile* patients, and healthy controls, Zhang et al (61) observed trends towards increased levels of *Bacteroides, Lachnospiraceae incertae sedis*, and *Clostridium XIVa* species and statistically significant decreases in the levels of *Escherichia/Shigella* species in asymptomatically colonized patients compared to healthy controls. Both asymptomatically colonized patients and patients with CDI also had significantly decreased microbial diversity compared to healthy controls. Although the authors reached different conclusions as to the potential driving forces responsible for microbiome composition differences, both studies observed increased levels of specific *Clostridiales* family members in asymptomatically colonized patients relative to symptomatic CDI patients and healthy controls. Although preliminary, this data suggests that expansion of these *Clostridiales* members may limit *C.*

difficile pathogenesis, either by regulating levels of *C. difficile* and/or its toxin, or by blocking pathogenic interactions between *C. difficile* and the host epithelium and/or immune system.

Variation in immune response is another potential mechanism that distinguishes symptomatic from asymptomatic carriage of *C. difficile*. A study by Kyne et al (63) compared adults who became colonized with *C. difficile* upon admission to the hospital and either developed *C. difficile*-associated diarrhea (CDAD) or remained asymptomatic. Asymptomatic patients mounted a higher serum IgG response to Toxin A than patients who developed CDAD. This difference was only observed in patients newly exposed to *C. difficile*; patients in long-stay hospitalization that were either asymptomatically colonized or suffering from CDAD had similar levels of serum IgG to Toxin A (64), suggesting the timing of immune response following initial *C. difficile* infection may be more important than the ability to mount a serum IgG response. Furthermore, patients that mount a more robust anti-toxin immune response in the early stages of CDI are less likely to experience recurrent disease (65). This property has been exploited with the recent FDA approval of a monoclonal antibody to Toxin B, Zinplava (Bezlotoxumab), developed by Merck. In a 12-week trial, patients administered Zinplava in combination with Standard of Care antibacterial drugs (metronidazole, vancomycin or fidaxomicin) saw a 10% absolute reduction in CDI recurrence (40% relative risk reduction) compared to placebo. However, an increase (3x) in heart failure was also observed in patients with prior history of heart failure (http://www.fda.gov/Drugs/InformationOnDrugs/ucm528793.htm). Merck have also developed an antibody to toxin A (actoxumab) but it provided no additional benefit over Zinplava.

Mechanisms governing *Clostridium difficile* infection and disease

C. difficile life cycle

Two physiologically distinct cell types are important in mediating *C. difficile* infection and disease – vegetative cells and spores. Actively growing, vegetative cells are the primary mediators of disease. In this state, *C. difficile* cells are capable of producing two large exotoxins, Toxin A (TcdA, 308 kDa) and Toxin B (TcdB, 270 kDa) (66). Toxins A and B are both glucosyltransferases that inactivate Rho and Ras-family GTPases within target epithelial cells; the ensuing disruption of signaling pathways causes loss of structural integrity and cell death via apoptosis (reviewed in (67, 68)). Both toxins are also capable of inducing host pro-inflammatory signaling pathways (reviewed in (69, 70). At higher concentrations, Toxin B can induce production of reactive oxygen species (ROS) through induction of NADPH-oxidase complexes which leads to necrosis (71). Toxin A has also been shown to induce production of ROS (72, 73), though the mechanism of induction is unknown. A subset of *C. difficile* strains also produce a third toxin, binary toxin (CDT; composed of a 53 kDa enzymatic component CDTa and a 98.8 kDa binding component CDTb), which ADP-ribosylates actin leading to its depolymerization and may contribute to more severe disease (reviewed in (74)).

During *C. difficile* infection, a subset of vegetative cells differentiate into endospores (75, 76). Metabolically dormant, spores are intrinsically resistant to prolonged oxygen exposure,

desiccation, low pH, bleach-free cleaning agents (such as ethanol hand wash commonly seen in hospitals), antibiotics and heat that would kill vegetative cells (77, 78). These characteristics make the spore the primary infectious and transmissible morphotype (79). Upon ingestion, spores pass undamaged through the low pH environment of the stomach and along the gastrointestinal tract to the lower intestine, the putative germination site (76, 80, 81). To resume active growth, spores germinate in response to multiple environmental signals.

The principal germinants of *C. difficile* are the primary bile salt, cholate, and its derivatives (taurocholate, deoxycholate, glycocholate), which act in combination with glycine, and potentially other amino acids (L-alanine, L-arginine, L-phenylalanine, L-cysteine, β-alanine, L-norvaline, and γ-aminobutyric acid (82)), to promote efficient germination (80, 83). Germination is competitively inhibited by a second primary bile salt, chenodeoxycholate, its derivative (lithocholate) and other analogs (84, 85).

Cholate and chenodeoxycholate are produced in the liver and conjugated to either glycine or taurine (reviewed in (86)). Following transport to the gall bladder for storage, bile salts are secreted into the duodenum to aid in digestion. The majority of bile salts are reabsorbed in the small intestine, though a portion (<10%) continues into the colon (87), where they are subject to microbiota-driven transformations (86). Bile salt hydrolases, encoded by several different members of the gastrointestinal community, cleave the taurine and glycine-conjugated primary bile salts into unconjugated bile salts (cholate and chenodeoxycholate) and free taurine or glycine (86). Enzymes for a second modification step, 7α-dehydroxylation of cholate and chenodeoxycholate to deoxycholate and lithocholate, are encoded by a limited number of bacterial species present in the colonic microbiome (86).

Potential mechanisms of colonization resistance

Patients receiving broad spectrum antibiotic treatment show marked alterations and loss of diversity in their gut microbiota (e.g.,(88, 89)). Concomitant to the loss of microbial diversity is a notable shift in bile salt composition from secondary bile salts, deoxycholate and lithocholate, to primary bile salts (90-92). Changes in the ratios of bile salts have important consequences for both germination and outgrowth of vegetative *C. difficile* cells. As described above, increases in the levels of cholate and taurocholate favor germination. Although increases in chenodeoxycholate could competitively inhibit germination, absorption of chenodeoxycholate by the colonic epithelium, which occurs at a rate ten times faster than cholate (93), likely reduces levels of chenodeoxycholate below that needed for inhibition (85). Both lithocholate and deoxycholate inhibit growth of vegetative *C. difficile* (80, 94, 95). Antibiotic treatment is known to reduce the levels of organisms that are capable of 7α-dehydroxylation (90, 95-97). Addition of *Clostridium scindens*, a member of the human microbiome that is capable of 7α-dehydroxylation of primary bile salts ((85), and references therein), partially restored the ability of an antibiotic-disrupted mouse microbiome to resist *C. difficile* infection (95).

In addition to disrupting bile salt metabolism, the large changes in microbial diversity that accompany antibiotic treatment significantly alter other features of the gastrointestinal environment that could help mediate colonization resistance. Studies in mice have

demonstrated that antibiotic treatment leads to profound shifts in the levels and types of metabolites present in the cecal contents and feces (90, 98). Loss of organisms important for consumption of simple sugars and sugar alcohols liberates carbohydrates that can be metabolized by *C. difficile* (90, 99, 100). In mouse, hamster, and chemostat-models of *C. difficile* infection, competition for nutrients appears to play a role in inhibition of *C. difficile* growth by the microbiome (90, 99, 101, 102). Decreased microbial fermentation following antibiotic treatment also lowers the levels of short chain fatty acids (SCFAs, (90, 103)) which may provide a more favorable environment for *C. difficile* growth. Physiologically-relevant butyrate levels have been shown to inhibit *C. difficile* growth *in vitro* (104) and higher SCFA levels correlate with growth inhibition in porcine (105) and murine (90) models. Bacteriocins targeting Gram-positive organisms, including *C. difficile* (e.g., nisin, lacticin 3147 (106, 107)), or narrowly targeting *C. difficile* (thuricin CD (108)) are another potential mechanism for preventing *C. difficile* colonization that could be encoded by antibiotic-susceptible members of the microbiome. Finally, the commensal microbiome plays a significant role in maintaining intestinal homeostasis, promoting barrier integrity, and educating the immune system (109, 110). Immune responses to *C. difficile* infection can play a role in limiting *C. difficile* disease (e.g., IL-25 mediated recruitment of eosinophils) or causing more severe disease outcomes (e.g., IL-23 mediated neutrophil recruitment and histopathology (111, 112)) and may also differentiate patients who develop *C. difficile* disease from asymptomatic carriers (reviewed in (70, 113)).

In summary, disruption of the gut microbiota by use of broad spectrum antibiotics causes dramatic changes that inhibit the ability of the host to resist colonization by *C. difficile*. Antibiotic treatment alters the bile salt composition in the intestinal lumen, leading to conditions that promote spore germination. Decreases in secondary bile salts, as well as the presence of fermentable carbohydrates and absence of potentially inhibitory molecules produced by the microbiome, provide a niche in which *C. difficile* can replicate and cause disease. Disrupted interactions between the microbiome, the host epithelium, and host immune system due to dysbiosis inhibit other host-encoded mechanisms to limit *C. difficile* infection and disease. In cases of recurrent CDI, it appears that colonization resistance cannot be restored without further intervention (Figure 2). One approach that has shown significant success in restoring colonization resistance and ameliorating CDI is Fecal Microbiota Transplantation (FMT). Better understanding how FMT ameliorates disease should provide insights into the rational design of defined communities for treatment of recurrent CDI.

Fecal Microbiota Transplantation (FMT)

The historical treatment for primary, non-severe, CDI involves oral administration (over 10-14-days) of either metronidazole or vancomycin (45). In most cases, this is sufficient for full resolution of disease. For ~25% of patients, however, at least 1 recurrence of disease is observed and of those patients up to 35-65% will suffer multiple episodes, developing chronic, recurrent disease (rCDI) (5, 114). Relapses can be caused by the initial infective strain or by reinfection from another *C. difficile* strain acquired from the environment (115-119). Administering a tapering or pulsed course of vancomycin can reduce rCDI occurrences (120), presumably by killing off vegetative cells as they germinate from the

spore form. Recently, the narrow spectrum antibiotic fidaxomicin has been approved for CDI treatment. Fidaxomicin is non-inferior to vancomycin for CDI treatment and results in fewer relapses of disease (121). Whereas a course of metronidazole or vancomycin can be relatively inexpensive ($35 - $700 respectively), fidaxomicin can cost $3,360 for a 10 day course (122). This has led to some reluctance to prescribe fidaxomicin, however, it has been argued that compared to the expense (financial and to the health of the patient) associated with rCDI, fidaxomicin is the superior first line treatment option (123). Despite the increased efficacy of fidaxomicin, >10% of patients will develop recalcitrant rCDI (124).

Because of the limited options available for pharmaceutical treatment of rCDI, interest has increased in alternative treatments. FMT is one therapy that has risen in prominence since the early 2010s, due primarily to its startlingly high efficacy (over 90% success rate reported in some studies (125, 126)). FMT, however, is not a new idea. The earliest known roots can be traced back to the consumption of "yellow soup" in 4th century China by patients suffering with food poisoning or severe diarrhea (127) and FMT has been utilized by veterinarians since the 1600s when it was described by Italian anatomist, Fabricius Aquapendente, for treating ruminants (114). The first reported use of FMT in humans as a treatment for pseudomembranous colitis (potentially caused by *C. difficile*) occurred in 1958 (128) and its first definitive use against CDI was reported in the early 1980s (129).

Over the past five years, several controlled trials have been reported that evaluate the efficacy of FMT and explore how different variables impact outcome. We summarize some of the key outcomes of these studies below and discuss key variables (FMT donors, FMT recipients, and mode of administrations) with their potential to influence efficacy. These data are presented to provide context for discussion of therapeutic microbes and not intended as guidelines for treatment. More comprehensive evaluations of FMT, including guides for clinicians on how to implement FMT, have been recently published (130, 131).

Key variables with potential to influence success of FMT

FMT Donors—The goal of FMT is to cure patients with recalcitrant rCDI by reintroducing a diverse microbiome within the colon, restoring a state of colonization resistance. There is, however, inherent risk in the transfer of biological material between donor and patient. Because of the potential to transmit infectious agents, donor stool samples are often screened for *C. difficile* toxin, enteric bacterial pathogens, parasites, and rotavirus; whilst blood samples from potential donors are screened for hepatitis A, B, and C as well as HIV and syphilis; however, the regulatory requirements governing these tests vary (reviewed in (132, 133).

The role of the gastrointestinal microbiome has been increasingly studied in recent years, highlighting potential complications with wholesale transfer of microbial communities between individuals. Studies have linked the gut microbiome in numerous conditions including (but not limited to): depression (134) inflammatory bowel disease (135), and metabolic syndrome (136). An obese phenotype has been shown to be transmissible in animal studies (137) and at least one case study infers this can also take place in humans (138).

Donors can be split into two main groups: Universal and patient selected. Universal donors enable expedited treatment by providing a bank of processed fecal material that has already undergone a rigorous screening process (e.g., (139, 140)). This option requires pre-planning and protocols to be in place within the medical establishment (discussed in (141)), and has recently been standardized by Rebiotix in their formulation RBX2660 (142) which is currently advancing to Phase 3 clinical development following successful treatment in Phase 2B trials (78.8% treatment). Patient-selected donors are usually family members or close friends (e.g., (139)). There is also the potential for patients to acquire untested stool from friends or family and to self-administer using one of the methods easily obtainable online (e.g., http://thepowerofpoop.com/epatients/fecal-transplant-instructions/). This practice is unadvisable and considered especially risky. Even with well screened samples there is the possibility of low amounts of highly contagious infectious agents to slip through undetected (143).

FMT recipients—Patient criteria for FMT typically requires symptomatic, toxin-positive *C. difficile* disease that has recurred at least once following cessation of antibiotic treatment (125, 139, 140, 144, 145). Reports suggest that FMT can be both safe and effective in severely ill patients (Table 2), though further clinical enquiry is required. Contraindications for use of FMT differ by hospital though can often include: age <18 years, medical frailty (life expectancy from non-CDI disease <1 yr), prolonged compromised immunity because of recent chemotherapy, the presence of HIV infection with CD4 count of less than 240, neutropenia ($<0.5 \times 10^9$/L), peripheral WBC ($>30 \times 10^9$/L), toxic megacolon, pregnancy, and corticosteroid use (125, 139, 144). A more complete understanding of patient populations most likely to benefit from FMT is continuing to develop as new studies are completed. The fact that some groups have reported successful FMT to children (146) and patients with severe disease (Table 2), criteria used by other groups as contraindications to treatment, indicate that these treatment considerations are continuing to evolve and outlines the need for increased controlled trials.

Route of Administration—The majority of reported FMT procedures have been performed with fresh stool suspensions, though there appears to be no significant differences in infection clearance for fresh versus frozen samples (144). FMT for rCDI has been reported successful whether administered via a top-down nasogastric (147) or capsule (140) route; or bottom-up colonoscopy (148) or enema (149); though a slight increase in efficacy has been observed with lower GI delivery (See Table 3) (150, 151). Self-administered enemas can also be highly efficacious (152) and may be preferred by some patients as they can be performed at home (ideally in consultation with a medical professional and with a sample that has been screened for infectious agents). Adverse effects associated with FMT route of delivery (reviewed in (153); summarized in Table 3) should be taken into account along with success rates. One problem, regardless of administration route, is the potential for injury caused by endoscopic tools. Encapsulated FMT (shown to be successful in limited studies) may therefore be an attractive option, although use is limited in patients susceptible to esophageal reflux (140).

Recolonization of gastrointestinal tract (GIT) after FMT

Human stool is a complex mixture of Bacteria, Archaea, human colonocytes, Fungi, Protists, viruses (human, bacterial, and archaeal), metabolites, antibodies, and other proteins (reviewed in (154)). The *Firmicutes* and *Bacteroidetes* are the dominant phyla in healthy individuals (though this is not the case for some traditional hunter gathers (155)), yet their ratios differ between people (156). Despite microbial community variation, metabolic pathways present within the microbial gene pool remain evenly distributed across healthy individuals (157). Patients with rCDI, in contrast, have a markedly reduced microbial diversity that differs significantly from healthy samples (158). At the phylum level, the microbiota of rCDI patients is dominated by *Proteobacteria* with an abundance of *Enterobacteriaceae* often observed (159-161). Following FMT, there is an immediate shift in the patient gut microbiota to look more like the donor. Post-transplant samples are enriched for *Bacteroidetes* whilst the overall abundance of *Proteobacteria* is significantly reduced. This suppression of *Proteobacteria* populations may play an important role in resetting intestinal immune homeostasis, as several species of *Proteobacteria* are capable of thriving in the inflamed intestine and perpetuating continued inflammation (reviewed in (162)) The stability and similarity of recipients' microbiota following transplant when compared to donor can vary, suggesting that some microbiota compositions may be more stable (160). In a recent study utilizing shotgun metagenomic sequencing to examine microbial colonization following FMT, Lee *et al* (163) observed a significant increase in *Bacteroidetes* post-transplant and, interestingly, a negative correlation between genes involved in sporulation and successful colonization of the FMT recipient. This suggests that alternative treatments that rely heavily or solely on spore forming bacteria (such as Seres Therapeutics SER-109, described in detail below) may suffer from limited colonization efficiency.

Alternatives to FMT

Despite its efficacy, the mechanisms that underlie successful FMT are not well understood. FMT mediates restoration of a diverse microbial population within recipients, but it is not clear whether the curative effect of FMT is due solely to the bacteria present. Although very few serious adverse events have been reported following FMT (noted above and Table 3), it is unclear what long-term impacts fecal microbiota transplantation may have upon the recipient. For these reasons, several alternative microbiota-driven approaches are being investigated.

Tvede and Rask-Madsen described the use of a defined consortia of 10 bacterial isolates to treat rCDI in 5 patients (164). They observed 100% efficacy with loss of *C. difficile* and its toxin and no recurrence during the 1 yr follow-up period. The authors proposed mechanism, based upon *in vitro* antagonism studies and stool cultures before and after treatment, was that *Blautia productus, Escherichia coli* and *Clostridium bifermentans* present in the consortia could directly antagonize *C. difficile*, which itself was actively inhibiting the survival of *Bacteroides* strains. In the absence of *C. difficile*, the levels of *Bacteroides* increased which may be important for maintenance of colonization resistance. Follow up studies from this group have not been reported, although a consortia based upon their work is being evaluated in an ongoing clinical trial (NCT01868373).

In 2013, Elaine Petrof and her colleagues reported the successful use of a consortia of 33 human fecal isolates to treat two patients with rCDI (165). They analyzed the change in microbiome composition before and after treatment by Ion Torrent sequencing of the V6 region of the 16S rRNA gene and observed that 50-70% of sequence reads were identical to the 16S genes present in the organisms in their consortia in the first two weeks following treatment. Although the persistence of sequences identical to the treatment consortia declined over time (both patients were treated with antibiotics for other conditions), neither patient experienced a disease recurrence. Follow-up studies using mouse model of *C. difficile* colitis, indicated that their synthetic consortia, now named MET-1, limits CDI by inhibiting toxigenicity of TcdA (166). Somewhat surprisingly, MET-1 had no impact on levels of *C. difficile* in the mouse model. A larger human clinical trial (NCT01372943) is ongoing and will provide additional insights into the efficacy of MET-1 when tested against a larger patient population.

Seres Therapeutics has developed preparations of *Firmicutes* spores isolated from human fecal donors (SER-109) to treat rCDI. These preparations, which vary from donor to donor, are composed of 50 ± 8 distinct operational taxonomic units (97% identity) based upon Illumina sequencing of the V4 region of the 16S rRNA genes (167). Results from Phase II clinical trials, demonstrated a 96.7% success rate at eight weeks post treatment (no CDI in 29 of 30 patients), although three successfully-treated patients had recurrence of CDI (one following antibiotic treatment) in the 8-24 week follow-up period (167). Microbial community analyses of patient samples before and after treatment demonstrated significant changes. In addition to an overall increase in microbial diversity and the expected increases in the proportion of *Firmicutes* (present in the SER-109 consortia), the authors observed increases in the proportion of *Bacteroidetes* sequences and decreases in the number of *Klebsiella* sequences in treated patients. Interesting parallels can be drawn with the work of Tvede and Rask-Madsen who proposed that suppression of *C. difficile by* two species in the *Firmicutes* phylum (*Blautia producta* and *Clostridium bifermentans*) allowed re-emergence of *Bacteroides* species which were being suppressed by *C. difficile*. The 89-subject Phase II trial of SER-109, however, did not show a reduced relative risk of recurrence of CDI. Subsequent re-analysis of the data by Seres Health suggests there may have been false positive CDI diagnosis of patients at time of enrollment and potentially during putative relapse due to Nucleic Acid Amplification Test (NAAT) based testing without ensuing cytotoxin assays. In these cases, positive result indicates that *C. difficile* cytotoxin genes are present, but not necessarily that *C. difficile* is the source of clinical symptoms. (Although there is some support for diagnosis based solely upon a positive NAAT in the presence of symptoms, other experts recommend use of a multi-step approach to distinguish asymptomatic from symptomatic colonization (reviewed in (45)). The analysis also suggested that the 1×10^8 spores received by patients in the Phase II trial (Phase I recipients received doses from 3×10^7 to 2×10^9 spores) may have been suboptimal – possibly due to the limited colonization efficiency of spore forming bacteria (163). Despite the setback, Seres Health recently announced (June 12, 2017) the initiation of a Phase 3 SER-109 clinical study (ECOSPOR III) in patients with multiply recurrent *C. difficile* infection. This study will aim to enroll 320 patients and utilize a cytotoxin assay to diagnose *C. difficile* rather than PCR as well as an increased treatment dose.

In 2014 as a follow-up to a smaller 2009 study, Johan Bakken reported on the combined use of probiotic kefir with tapered reduction in vancomycin or metronidazole as an alternative treatment for rCDI (168, 169). In Bakken's study, a commercially available preparation of kefir produced by Lifeway (11 bacterial and 1 yeast species) was self-administered by patients three times per day for 6 weeks during staggered vancomycin/metronidazole withdrawal. Kefir self-administration continued for 2 months following the end of antibiotics. All 25 patients were free of symptoms at the end of the staggered antibiotic withdrawal and 21 of 25 patients remained asymptomatic for 9 months following the end of the study. Despite no reported adverse effects in CDI patients in either study, the same probiotic kefir when used in a mouse model of CDI was found to exacerbate disease, highlighting the difficulty in studying translational therapies in animal models (170).

Gerding and colleagues have also evaluated the ability of non-toxigenic *C. difficile* administration to prevent and/or resolve rCDI (171). In their study, patients who had a primary episode of CDI or a single recurrence of CDI were recruited and treated with preparations of non-toxigenic spores for 7-14 days (based upon treatment group). Recurrence rate in patients receiving non-toxigenic *C. difficile* spores was 11% compared to 30% for placebo-treated patients. Treatment with non-toxigenic *C. difficile* holds promise, and success may be improved by changes in administration or by coupling with other microbe based therapeutics to more fully restore colonization resistance. One concern with this approach, however, is the ability of non-toxigenic strains to acquire the pathogenicity locus (PathLoc) encoding toxins A & B from pathogenic strains via horizontal gene transfer (172).

Many microbially-based treatments for *C. difficile* target rCDI but there is also an opportunity for these treatments to be used for *C. difficile* prophylaxis. A number of commercially available probiotic preparations have been used for *C. difficile* prophylaxis in several different institutions, with reported decreases in the overall rate of CDI (reviewed in (173)), although there is still not sufficient consensus to recommend widespread use of probiotics in CDI prophylaxis (reviewed in (174)) .

Conclusions

An intact gut microbiome exerts wide ranging influence on both the host and its constituent members. Complex carbohydrates are fermented to produce SCFAs that can both inhibit certain bacterial species and provide energy to the cells that line the intestine; epithelial barrier integrity is promoted; bile salts are modified; and a milieu of communication signals and bacteriocins are produced. These factors, and others, result in a state of colonization resistance that prevents CDI. Upon significant disruption, some, or all, of these factors are lost leaving the host susceptible to *C. difficile* infection. For most patients, antibiotic treatment is sufficient to cure CDI and the gut microbiome eventually returns to a state of restored functionality and colonization resistance. However, for a significant minority the microbiota fails to recover and recurrent disease occurs. For these patients, the transfer of an exogenous source of microbes via FMT has proved to be extremely efficacious. The discovery and application of specific consortia of microbes that can be administered in a

drug like fashion has proven to be more elusive but by targeting those microbes that can recapitulate the microbiome functionality there is great promise for the near future.

Acknowledgments

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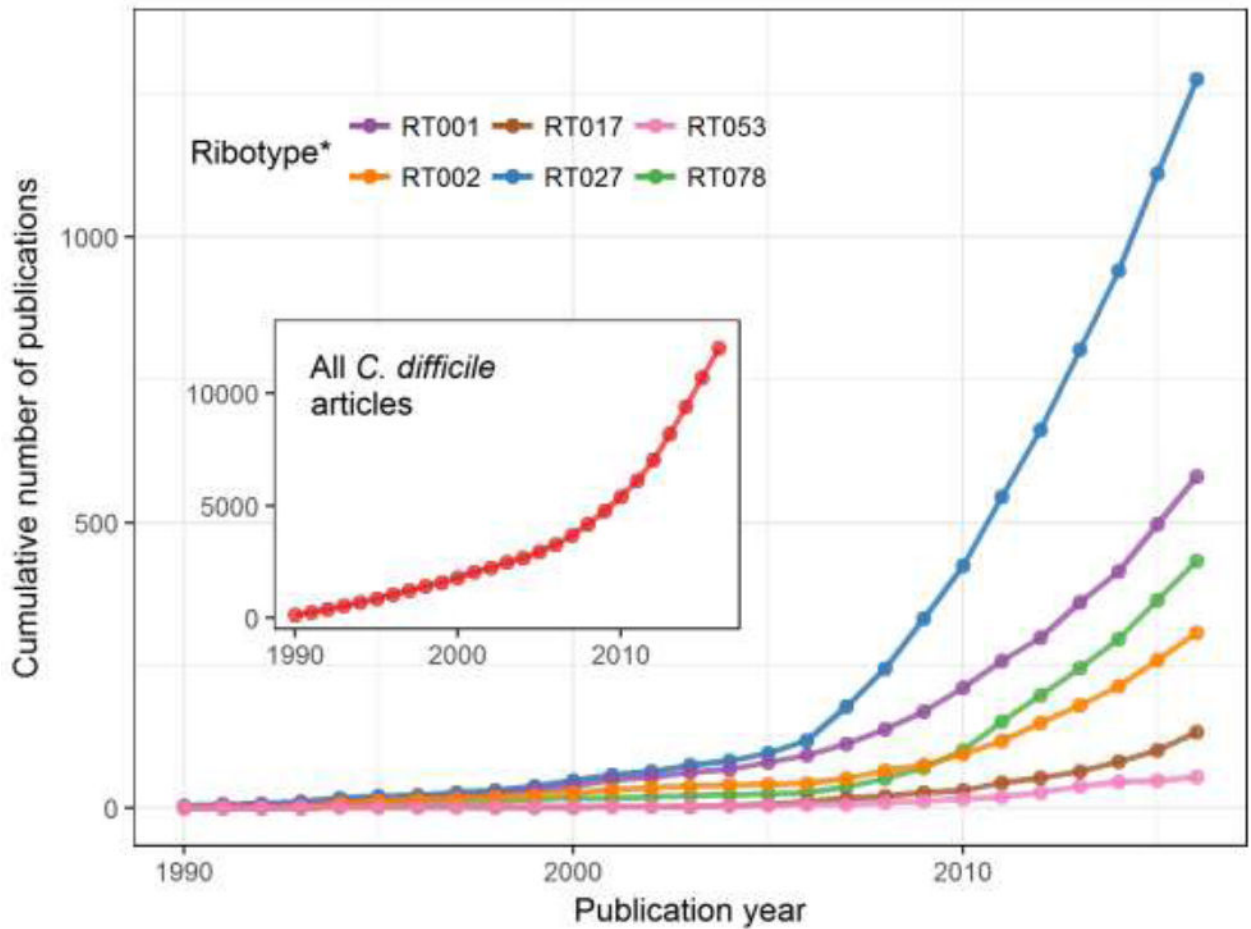
Figure 1. Cumulative number of *C. difficile* articles in PubMed
Total article number includes all articles with 'difficile' in either the title or abstract;
*Ribotype articles are those that have 'difficile' and the ribotype (or alternative
nomenclature) in the title or abstract e.g. RT027 articles were classified as such if they had
'difficile' AND 'Ribotype 027' OR 'RT027' OR 'Sequence Type 1' OR 'NAP1' etc.
Although not a definitive measure of global ribotype abundance, this data can serve as a
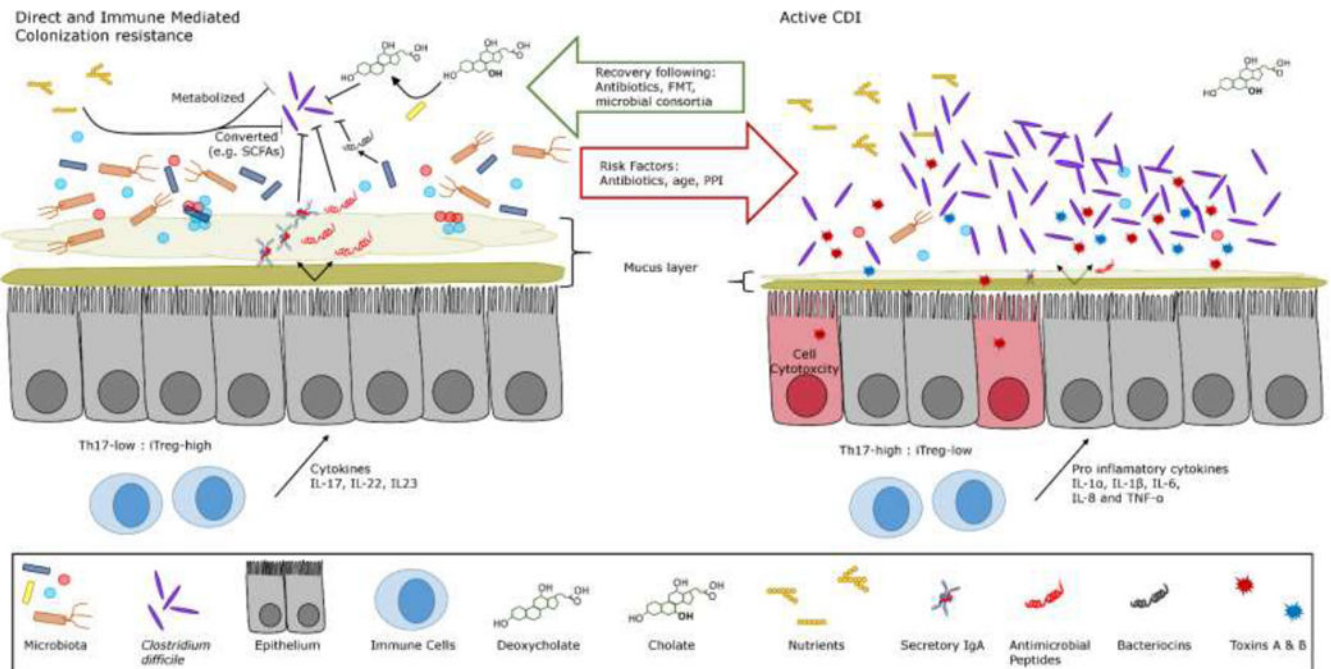proxy for the relative frequency of outbreaks associated with specific ribotypes.

Figure 2.

Under normal circumstances the gastrointestinal tract is able to resist infection by *Clostridium difficle*. This is thought to be acomplished by a combination of factors mediated by the host and colonization resistance due to the indigenous microbiota. These mechanisms, expanded upon in the main text, include: 1)competition for nutrients and their conversion into metabolites inhibitory to *C. difficile*, 2)microbial conversion of primary to secondary bile salts such as deoxycholate which can induce germination of *C. difficile* spores but prevent the growth of vegetative *C. difficile*, 3)production of antimicrobial peptides and bacteriocins by the host microbiota, and 4)a balanced host immune response that includes production of immunoglobulins, accumulation of protective iTreg cells in the lamina propria, and release of anti-inflammatory cytokines. Upon disruption of these resistance mechanisms, primarily through antibiotic use, there is an accumulation of pro-inflammatory Th17 cells and reduction in bacterial diversity. In this state *C. difficile* is able to invade and proliferate, causing toxin mediated damage to the epithlium. In many cases following suitable antibiotic treatment for CDI the indiginous microbiota is able to recover and reestablish colonization resistance. However, in a significant number of cases this does not occur and patients are liable to suffer relapse. FMT has been shown to be remarkably successful for treating these patients, likely because multiple facets of colonization resistance are restored.

Table 1

Stratification of disease severity associated with *C. difficile* colonization (44, 45).

Classification	Diagnostic criteria
Asymptomatic carriage/colonization	• Positive identification of *C. difficile* (toxigenic or non-toxigenic) without clinical symptoms
Mild to moderate *C. difficile* infection (CDI)	• 3 unformed stools in 24 hours • Positive identification of toxigenic *C. difficile* • White blood cell (WBC) count <15,000 and serum creatinine levels <1.5X pre-morbid level
Severe CDI	• Positive identification of toxigenic *C. difficile* and/or evidence of pseudomembranous colitis • White blood cell (WBC) count 15,000 cells/μl, serum albumin <3 g/dl and/or serum creatinine level 1.5X premorbid level

Severe, complicated CDI (sometimes referred to as "fulminant") (45)	• Hypotension, shock, ileus, or megacolon
	• Other systemic signs of severe infection (145) including:
	   – Admission to intensive care unit
	   – Altered mental status
	   – Fever 38.5°C
	   – WBC <2000 or >30,000/μl
	   – Lactate >2.2 mmol/L, or
	   – Evidence of end-organ damage
	• Positive identification of toxigenic *C. difficile*

Recurrent CDI (rCDI)	• Recurrence of symptoms within 8 weeks of successful *C. difficile* treatment

Table 2

Results of FMT in severely ill patients

Complication/extenuating factors	Success rate	Citation
Severe CDI: Hypoalbuminemia (<3 g/dL) with peripheral white blood cell (WBC) count >15,000/µl and/or abdominal tenderness.	91% (Primary cure rate)	(145)
Complicated CDI: Occurrence of 1 or more of the following as a consequence of CDI: admission to the intensive care unit, altered mental status, hypotension, fever >38.5°C, ileus, WBC <2000 or >30,000/µl, lactate >2.2 mmol/L, or evidence of end-organ damage.	66% (Primary cure rate)	(145)
Fulminant, life-threatening CDI	case report	(175)
Fulminant CDI in an allogeneic stem cell transplant patient	case report	(176)
Fulminant, life-threatening CDI with Toxic megacolon	case report	(177)

Table 3

Comparison of rates of success and adverse events as a function of route of delivery for FMT

Delivery	Success rate[*]	Adverse Events[†]	Serious Adverse Events[††]
Upper GI	83.5%	43.6%	2.0%
Lower GI	85.1%	17.7%	6.1%

[*] Success rate indicates 90-day cure rate of rCDI based upon multi study data in Furuya-Kanamori L et al (151).

[†] Adverse events include any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with FMT.

[††] Serious Adverse Events include: death, life-threatening experience, inpatient hospitalization or prolongation of existing hospitalization, persistent or significant disability or incapacity, congenital anomaly or birth defect, or an important medical event (178). Adverse event data includes multiple, rCDI & non-rCDI related, FMT studies.