

Recent advances in the treatment of *C. difficile* using biotherapeutic agents

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Abstract: *Clostridium difficile* (*C. difficile*) is rapidly becoming one of the most prevalent health care-associated bacterial infections in the developed world. The emergence of new, more virulent strains has led to greater morbidity and resistance to standard therapies. The bacterium is readily transmitted between people where it can asymptotically colonize the gut environment, and clinical manifestations ranging from frequent watery diarrhea to toxic megacolon can arise depending on the age of the individual or their state of gut dysbiosis. Several inexpensive approaches are shown to be effective against virulent *C. difficile* in research settings such as probiotics, fecal microbiota transfer and immunotherapies. This review aims to highlight the current advantages and limitations of the aforementioned approaches with an emphasis on recent studies.

Keywords: antibiotics, fecal matter transfer, polyclonal adjuvants, *C. difficile*, biotherapeutic agents

Introduction

Approximately 10–35% of all cases of antibiotic-associated diarrhea in developed countries are caused by the gram-positive, spore-forming, toxin-producing anaerobe, *Clostridium difficile* (*C. difficile*).^{1–8} Exposure to antibiotics is recognized as the most important risk factor for *C. difficile* infection (CDI).^{9–13} In a recent study, >45% of the CDI patients had taken antibiotics in the 90d prior the development of symptoms, whilst in another case-control study, 48% of the patients were exposed to antibiotics in the 4-week time period to CDI onset.^{14,15} Fluoroquinolones (FQs), clindamycin (CLI) and cephalosporins (CFs) are the antibiotics commonly associated with CDI.^{16,17} Resistance to these antibiotics continues to play an important role in the emergence of new *C. difficile* clones.¹⁸ An investigation by Wasels et al showed that in CDI ribotype 27 (RT 027) FQ resistance is associated with a modest fitness cost; a trait linked to the presence of a favorable mutation (Thr82Ile) in the *gyrA* gene.¹⁹ In 2014, Lee et al reported on the emergence of 3 new ribotypes (RT) 014, 017 and 018 in a Korean hospital; all the strains carried the Thr82Ile mutation. Moreover, the same mutation was detected in isolates of some additional ribotypes genetically related or unrelated to RT027.^{20,21}

Increasing age >65 is another known risk factor associated with CDI, accounting for the majority of diarrheal cases in residential facilities.^{22–26} In the United States alone, near half a million cases have been reported with 29,000 fatalities attributed to CDI.²⁷ Patients in health care settings are particularly susceptible to infection and reinfection with a recurrence rate of over 20% and a mortality rate of over 9% within days of diagnosis. It is also estimated that up to 57% of the long-term care facility

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residents (LTCF) are asymptomatic carriers of *C. difficile*. Although CDI often occurs as a secondary infection, it can also occur in healthy adults with similar rates of recurrence.²⁸ An article detailing the various risks (1960–2010) associated with infection can be found in a review by Spigaglia et al.²⁹

Once a patient exhibits symptom, the first step in treatment is the discontinuation of antibiotics associated with CD risk. For the past 40 years, first-line treatments for mild, recurrent and severe CDI have been the drugs metronidazole (MET) and oral vancomycin (VAN). Unfortunately, in 27% of the cases, the drugs do not effectively treat the infection or prevent recurrence. If metronidazole and oral vancomycin treatments are ineffective, fidaxomicin (FDX) can be administered. This RNA polymerase inhibitor has been shown to reduce sporulation and toxin production in hundreds of *C. difficile* strains.^{30–33} Although recurrence and relapse rates for FDX are lower compared to VAN, fidaxomicin still fails in approximately 1 out of 8 patients treated with the antibiotics and in clinical trials.³⁴ Moreover, a recent report showed that vancomycin-resistant isolates are >250 times less susceptible to fidaxomicin compared to fidaxomicin-sensitive strains, even though these two antibiotics have different mechanisms of action.³⁵ Failure of FDX in these cases requires the development of novel cost-effective therapies for *C. difficile* infections, ensuring that new treatments do not promote reduced susceptibility to antibiotics in current use.

One of the most cost-effective alternative therapies to treat *C. difficile* is FMT. Recent reports suggest that FMT has the potential to dominate recurrent and severe CDI treatments^{36–38} and in some cases primary CDI as well.³⁹ The impact of FMT and alternative therapies on CDI is yet to be fully realized. In this review, we briefly visit the infection cycle and roles of CDI genes in toxin production, and then discuss several bio-therapeutic options under investigation, highlighting those which have the potential to replace FDX and VAN in the treatment of initial, recurrent and severe CDI. In this regard, in-vivo studies and clinical trials conducted using known bio-therapeutic options are discussed. Finally, we close by looking at the challenges that emerging CDI biotherapeutic treatments currently face.

Infection cycle and the roles of *C. difficile* genes in toxin production

Transmission of the *C. difficile* occurs via the fecal-oral route in the form of highly resistant spores. Once passed

the acidic pH of the stomach, the spores germinate in the presence of certain bile acids within the intestine. The active cells then progress to the colon where they out-compete the host bacteria for residence in the hypoxic folds and nutrient-rich crypts. As the colonies form and localized resources decline, a quorum threshold is reached initiating toxin production. The amount of toxin produced determines the severity of the infection. Once outside the localized influence of the CD film or crypt, some cells or spores migrate to the anus and are defecated by the host.⁴⁰ A summary of the CDI cycle is shown in [Figure 1](#).

Most *C. difficile* (CD) clinical isolates produce two high molecular weight-related toxins, namely TcdA (308 kDa) and TcdB (270 kDa). TcdA and TcdB expression can fluctuate depending on the bacteria's exposure to various physical (temperature) and chemical (iron and carbon availability) stressors and the types of strains used in trials.⁴¹ The proteins are part of the large clostridial toxin (LCT) family which includes *C. perfringens* cytotoxins, *C. sordelli* hemorrhagic toxins (TcsL) and (TcsH) as well as *C. α-novyi* toxin. CD toxin expression is dependent on the regulation of *tcdA* and *tcdB* genes located on the pathogenicity locus PaLoc. The PaLoc locus also contains 3 accessory genes *tcdC* (negatively regulates *tcdA* and *tcdB*) *tcdE* (encodes a putative holin necessary for toxin release) and *tcdR* (RNA polymerase sigma factor). The roles of *tcdC* and *tcdE* remain controversial as toxin production barely differs between *tcdC* mutant and wild type strains, whilst reports suggest *tcdE* holin protein may play a role in toxin release.⁴² Furthermore, several studies show *tcdC* expression levels do not diminish during the stationary phase of growth, suggesting that *tcdC* may adopt a modulatory role rather than a repressive one. In addition to *tcdA* and *B*, some strains (RT027 type III, RT251) also express a third toxin, binary toxin (CDT_a and CDT_b). CDT is structurally related to iota toxin and C2 toxins of *C. perfringens* and *C. botulinum* and is thought to upregulate TcdA and TcdB production.^{43–45}

The roles of these three toxins in *C. difficile* disease remain controversial. Over the years, several studies using hamster models and isogenic *C. difficile* strains have shown that both TcdA and TcdB mutants are capable of causing fulminant disease and death. Recent studies using hamster and mouse models exposed to wild-type, double toxin knockout and isogenic single strains induced innate and pro-inflammatory immune responses. Strains expressing only TcdB resulted in significant weight loss and severe systemic disease in both models, implying the

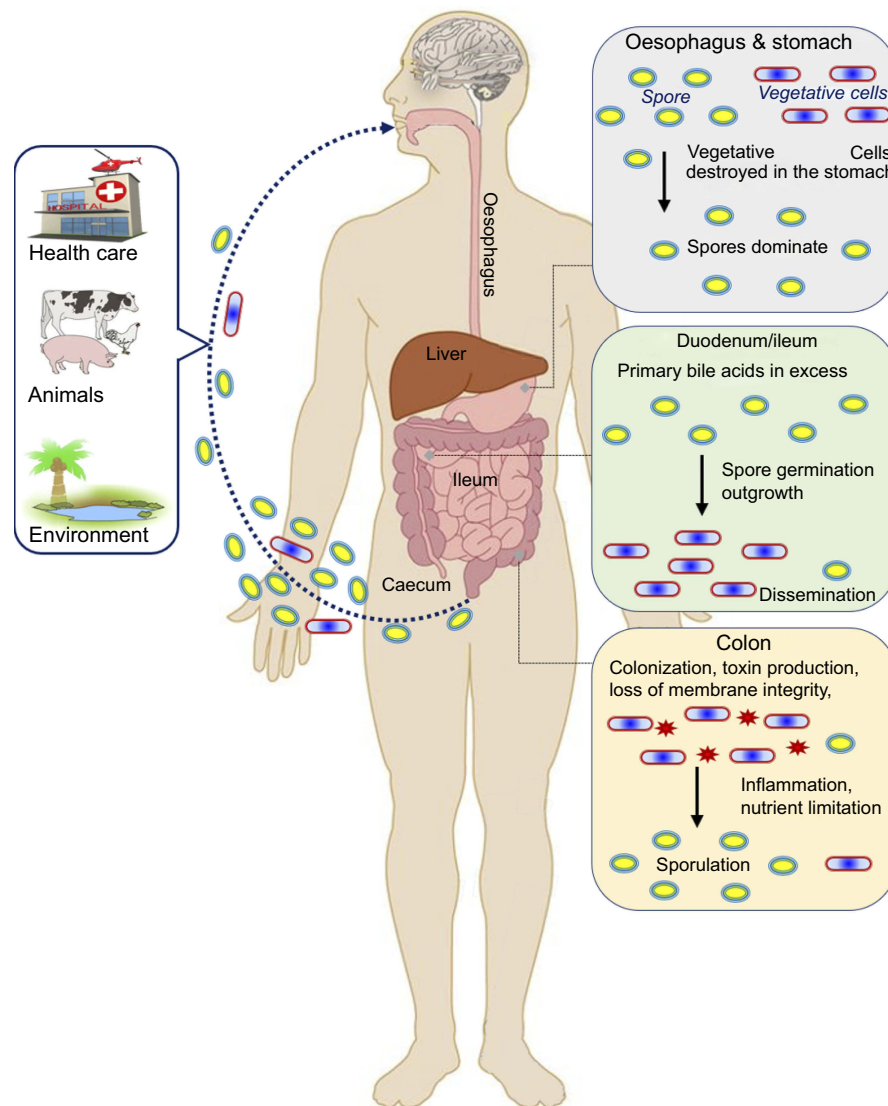


Figure 1 Infection cycle of toxigenic *Clostridium difficile* in the human gastrointestinal track. As *C. difficile* is an obligate anaerobic bacterium, transmission occurs primarily via spores. Three sources of infection (health care, animal and community residences) are indicated. Spores and some vegetative cells (most of which are eliminated in the hosts stomach) are ingested. Once past the stomach a range of metabolic factors (primary to secondary bile acid ratio, short chain fatty acids) encourages spore germination in the duodenum. After germination, the cells disseminate to the anaerobic folds of the ileum and caecum, forming colonies (assuming dysbiosis). Once in the colon, some cells enter sporulation, others produce toxins. As toxin levels increase, the epithelial barrier is challenged, this in turn initiates the inflammatory response and upregulates the production of anti-toxin antibodies in the host.

severe aspects of the disease might be attributable to TcdB rather than TcdA.⁴⁶ Interestingly, the majority of these strains produce a modified form of TcdB (B) toxin that shares enzyme-GTPase substrate site homology with *C. sordelli* hemorrhagic toxins, allowing it to carry out glucosylation events in the absence of TcdA.⁴⁷ Furthermore, the observation that A⁻B⁺ strains are virulent in infected individuals indicates that B toxin is sufficient for pathology in humans.

However, the role of the CDT toxin in disease pathogenesis remains unclear. Several studies have shown that CDT production in addition to TcdA and B is associated

with an elevated risk of recurrence, disease severity and mortality.⁴⁸ Moreover, reports of blood, inflammation and fluid retention in infected hamster and rabbit models indicate that CDT could be enterotoxic.⁴⁹ It has also been demonstrated that TcdA/CDT-producing strains are more virulent in hamsters than isogenic TcdA⁺TcdB⁻CDT⁻ strains. Additional studies in mice also showed the host eosinophilic response is suppressed in the presence of CDT.⁵⁰ Recent work by Kaplan et al demonstrated AB toxin production in hypervirulent and non-hypervirulent strains is under the control of a novel thiolactone quorum-signaling peptide which is independent of tcdC-mediated

regulation.⁵¹ Furthermore, Lyras et al demonstrated that CdtR may act as a global regulator of virulence in epidemic 027 strains and not others, suggesting that each epidemic strain has its own regulatory mechanism.⁵² These initial experiments highlight the potential roles of CDT and quorum-signaling peptides in pathogenic virulence and toxin production. A schematic depicting the organization of the toxin genes can be found in Figure 2. For the rest of the review, TcdA and TcdB will be used interchangeably with toxin A and toxin B.

Probiotics

Research has shown that probiotics can confer a wide range of health benefits especially those directly related to the human gut.⁵³ For example, probiotics can improve your immune system,⁵⁴ regulate gut microbiota as well as prevent gastrointestinal infections in animals and humans.⁵⁵ For many years, various species of probiotics have been studied as preventative therapies for CDI, with the most common being within the *Bifidobacterium* and *Lactobacillus* genus. Recently, a decade-long study examining the efficacy of a three-strain probiotic mixture involving 45,000 patients was reported.⁵⁶ The probiotic mixture BioK+® (containing *Lactobacillus acidophilus* CL1285, *Lactobacillus rhamnosus* CLR2 and *Lactobacillus casei* LBC80R) was administered 2–12 hrs after antibiotic

treatment and continued for at least 30 days or until treatments were discontinued. In the patients who received the mixture, the CDI rate decreased from 18.0 cases per 10,000 patient days to an average of 2.3 cases per 10,000 patient days. In addition to *Bifidobacterium* and *Lactobacillus*, the yeast *Saccharomyces* has been utilized as preventive treatment for CDI. Of note is the medicinal yeast *Saccharomyces boulardii* CNCM I-745 which has been approved for the treatment and prevention of diarrhea of various causes.⁵⁷ *Saccharomyces boulardii* CNCM I-745 secretes a 54-kDa protease, which is capable of inactivating *C. difficile* toxins A and B resulting in its efficacy being evaluated in several clinical trials. In 2000, Surawicz et al conducted a randomized, double-blind, placebo-controlled study utilizing *S. boulardii* for the treatment of recurrent CDI (n=168).⁵⁸ Patients were given either vancomycin 500 mg daily, vancomycin 2 g daily, or metronidazole 1 g daily for 10 days. On day 7 of the antibiotic course, *S. boulardii* 500 mg or placebo were administered and continued for a total of 28 days. Results of the study showed that those treated with *S. boulardii* in addition to vancomycin 2 g daily had a 16.7% recurrence rate versus a 50% recurrence rate in individuals treated with vancomycin 2 g daily and placebo. Unfortunately, a follow-up trial found that *S. boulardii* was not effective at preventing AAD in elderly patients.⁵⁹ Additional smaller trials using mixtures of *Lactobacillus rhamnosus* GG

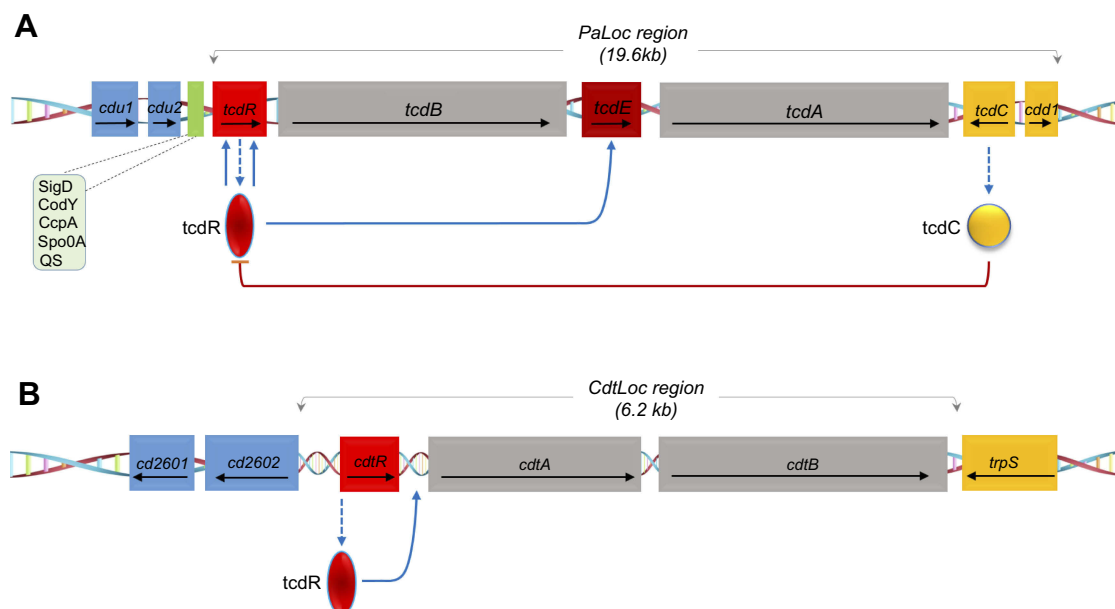


Figure 2 Schematic representation of the toxin genes and regulatory proteins. **(A)** Pathogenicity locus (PaLoc) region containing the following genes: *tcdR*, *tcdB*, *tcdE*, *tcdC* and *tcdA*. The arrows indicate the direction of transcription. TcdC negatively regulates AB toxin expression. Other regulators Sigma D (SigD), the nutritional repressor CodY (known as GTP-sensing transcriptional pleiotropic repressor CodY), catabolite control protein A (CcpA), Stage 0 sporulation protein A (Spo0A) and quorum sensing (QS) that affect toxin gene transcription (boxed) mostly act via expression of the *tcdR* gene. **(B)** Schematic of the binary toxin locus (CdtLoc) and flanking regions with regulatory interactions. CdtR positively regulates the transcription of *cdtA* and *cdtB*. CdtR also regulates the production of AB toxins in various 027 strains but not in biotypes 078 and 012.

and *Lactobacillus plantarum* did not demonstrate efficacy of probiotic treatment over the placebo for the prevention of AAD. Similarly, a multicenter, randomized, double-blind, placebo-controlled trial found no benefit to probiotic (mixture of two *Lactobacillus* and two *Bifidobacterium* strains) administration in the prevention of CDI in more than 2,941 elderly patients >65 years. Several meta-analyses combining data from studies of different probiotic strains in different patient populations produced results that were largely inconclusive.⁶⁰ Despite the potential benefit to the host microbiota, long-term safety concerns remain. Among those concerns is the transfer of antibiotic-resistant genes between gut microflora and opportunistic pathogens via mobile genetic elements. Another concern is the resistant profiles of commercial and medicinal probiotics are unavailable.⁶¹ Clinical guidelines suggest that for the prevention of AAD, probiotics may be considered based on the evaluation of individual cases. At present, the recommended strain for CDI is *S. boulardii* CNCM I-745 although the quality of evidence is low.⁶²

Immunotherapies

Given the prevalence of CD within the general populous, the ease with which it is transmitted and the propensity for infection recurrence, a long-term treatment strategy that invigorates the host's immune response maybe considered the most prudent or cost-effective approach. There are many reviews that document the host's immune response to *Clostridium difficile* infection,^{63–66} but perhaps the most insightful work is by Solomon⁶⁷ in which the adaptive response to AB toxins, surface-layer proteins (SLPs, Cwp66 and Cwp84) and flagella proteins (FliC and FliD) were addressed. It was found that patients who can generate an enhanced anamnestic systemic immune response to these toxins and proteins are more likely to remain asymptomatic. In addition, symptomatic patients who can mount a rapid immune response early on are less likely to have recurrent CDI. There is now considerable evidence to show that host immune and inflammatory responses contribute in large part to patient outcomes. In the next part of this review, we examine the different types of immunotherapies that can be applied in the treatment and prevention of rCDI and severe CDI.

Traditional vaccines

Over the years, many antibody-based approaches, namely, intravenous (IV) immunoglobulin therapy and polyclonal antibody preparations^{68–70} have shown efficacy in treating CDI in animals. Commercially pooled polyclonal human

intravenous immunoglobulin G (IVIG) is a standard therapeutic preparation made from a plasma pool sourced from 10,000 to 50,000 healthy donors.⁷¹ In 1995, Torres et al found that a *C. difficile* culture filtrate inactivated with formalin was effective in protecting hamsters from CD-induced diarrhea and death.⁷² Since then, other groups have reported the advantages of using toxoid vaccines to treat CDI and rCDI. Of note is the Sanofi Pasteur (SP) Institute vaccine which has been fast tracked by the food and drug administration (FDA). During phase I trials, the dual toxin (A&B) vaccine induced a complete seroconversion for toxin A at all doses in adults, and at the highest vaccine dose in the elderly. Toxin B seroconversion was lower, both in adults and elderly groups reaching 75%. The antibody response appeared persistent only for toxin A in adult groups, whereas the toxin B response declined 6 months after vaccination,⁷³ suggesting the need for a booster dose. The vaccine also passed phase two trials in which the immunogenicity in adults for primary prevention (NCT01230957) and infected adults for prevention of recurrent disease (NCT00772343) was tested. Phase three trials (NCT01887912) launched by Sanofi in 2013 involved 15,000 people; 10,000 received the vaccine and 5,000 a placebo was terminated in late 2017 when it was determined that the probability of reaching its primary endpoint was low.⁷⁴ However, phase three trials of a similar competing vaccine created by Pfizer are still currently underway (NCT03090191).

The immunogenicity of vaccines based on polysaccharide (PS) glycans found on the surface of *C. difficile* cells, namely PSI, PSII, PSIII and lipoteichoic acid-based glycoconjugates has been extensively reported in the literature^{75,76} and shall not be covered herein. For those readers whose research focus is the development of vaccines against cell-surface components (sortase anchor proteins and cell wall proteins), the recent review by Fagan et al is recommended.⁷⁷

Recombinant vaccines (RV)

The large-scale production of highly toxic antigens can be a challenging and costly process. Vaccines based on non-toxic fragments of genetically engineered versions of the toxins alleviate some of these concerns including issues of safety.^{78,79} Karczewski et al investigated the potential of a recombinant vaccine composed of 2 separate fragments of toxin B against *C. difficile*.⁸⁰ A combination of toxin B fragments and toxin A were administered to Golden Syrian hamsters. The recombinant vaccine protected animals

against a lethal dose of *C. difficile* spores, with an efficacy equivalent to traditional toxoid vaccines. Other groups have also demonstrated recombinant toxin A and toxin B fragments protect hamsters against *C. difficile*. The study by Spencer et al noted the glucosyltransferase domain of toxin B induced a greater immune response compared to the binding domain of the whole toxin.⁸¹ A similar vaccine preferentially expressing the glucosyltransferase domain of toxin B and the C-terminal receptor binding domain (RBD) of toxin A was reported by Leuzzi et al. The antibodies generated against the glucosyltransferase domain provided more protection in a mouse infection model when used in conjunction with toxin A antibodies.⁸² While limited protection was observed with some combinations, co-administration of a cell-binding domain fragment of toxin A (toxin A-B1) and the glucosyltransferase moiety of toxin B (toxin B-GT) induced systemic IgGs which neutralized both toxins and protected vaccinated animals from death following challenge with two strains of *C. difficile*. Further characterization revealed that despite high concentrations of toxin in the gut lumens of vaccinated animals during the acute phase of the disease resulting in minimal toxicological damage.

The size and domain complexity of native recombinant toxins A and B make it a challenge to use them as vaccine candidates. A simple solution is to generate a smaller chimeric vaccine that retains the major neutralizing epitopes from both toxins. In 2012, Wang et al used a non-pathogenic *Bacillus megaterium* expression system to generate glucosyltransferase-deficient holotoxins.⁸³ The atoxic holotoxins induced potent antitoxin neutralizing antibodies showing little cross-immunogenicity or protection between toxin A and toxin B. The researchers subsequently generated a glucosyltransferase-deficient toxin chimera, cTxAB. Parenteral immunization of mice or hamsters with cTxAB induced rapid and potent neutralizing antibodies against both toxins. Complete and long-lasting disease protection was conferred by cTxAB vaccinations against both laboratory and hypervirulent *C. difficile* strains.

In 2015, Sun's group generated a chimeric protein designated mTcd138, comprising the glucosyltransferase and cysteine proteinase domains of toxin B and the receptor-binding domain of toxin A.⁸⁴ Parental immunizations of mice and hamsters with mTcd138 induced protective antibodies to both toxins and provided protection against infection with the hypervirulent *C. difficile* strain UK6. Many hypervirulent strains also secrete a binary toxin, namely, CDT. CDT is composed of 2 active components,

CDTa and CDTb. Vaccines generated against hypervirulent strains sometimes include attenuated forms of the binary toxin.⁸⁵ Recently, a novel tetravalent vaccine was generated via a high yield insect-baculovirus system. The immunogenicity of bivalent and tetravalent vaccines was compared in immunized (21 days prior spore challenge) hamsters. Investigations revealed that bivalent and tetravalent vaccines induced similar neutralizing antibody titers to toxin A in prototypic strains VPI10463, BI17 and 8864. Only hamsters receiving the tetravalent and binary vaccines alone had elevating neutralizing titers to the binary toxin.⁸⁶

Monoclonal antibodies

There are many types of antibody-based approaches that have shown efficacy in treating CDI such as intravenous immunoglobulin therapy and polyclonal antibody preparations.⁸⁷ The toxin pair A/B are the primary targets for therapeutic antibodies against CDI while minor virulence factors such as CDT, surface layer proteins (SLPs) and flagella are sometimes targeted depending on the virulence of the strain under investigation. Initial studies using a mouse rCDI model indicated that the treatment of mice with antitoxin antibodies significantly protects against the morbidity and mortality associated with CDI induced by both historical (VPI 10463, in the case of the toxin challenge models) and hypervirulent strains of *C. difficile*.^{88,89}

As 85–95% of the clinical isolates test positive for toxin A & B (A+B+), it makes sense to target both toxins. Consequently, a number of antitoxin A/B combinations are already in the initial stages of development.⁹⁰ In a recent study, more than 20 monoclonal antibodies (mAbs) with neutralizing potential against toxin A and more than 50 with neutralizing potential against toxin B were evaluated.⁹¹ Of those 20 mAbs screened, CA 997 was the best at neutralizing toxin A strongly binding the toxin approximately 12 times. A combination of CA1125 and CA1151 mAbs demonstrated a binding valency of 3 for toxin B. Using an established experimental model, individually housed hamsters were then separately dosed on 4 consecutive days with 50 mg/kg of anti-toxin A and 50/kg of anti-toxin B before being orally challenged with *C. difficile* spores/vegetative cells. A tri-antibody mixture (UCB mAb) offered very high levels of protection (82%) with 9/11 of the hamsters surviving for 28 days. It worth noting that CDA1 exhibited negligible neutralizing activity against toxin A, a finding confirmed in a study by Marozsan et al.⁹²

The intended clinical use of mAb mixtures is for the prevention of recurrent diseases when administered in conjunction with standard-of-care antibiotics. Monoclonal antibody (mAb) and single-domain antibody (sdAb)-based therapies currently dominate the immunotherapeutic pipeline with bezlotoxumab leading the way. Bezlotoxumab (known as 124–1152, MK-3415, CDB1 and MDX-1388) recognizes the C-terminal receptor binding domain of toxin B exhibiting a binding valency of 3. Actoxumab (previously named 3D8, MK-3415, CDA1, MDX-066) is one of the first fully human mAbs to potently neutralize toxin A93. In a landmark study involving 2,655 adults receiving oral standard-of-care antibiotics for primary or recurrent *C. difficile* infection showed the sustained cure rates (initially clinically cured without recurrence of infection within 12 weeks) with 64% bezlotoxumab alone, 58% with actoxumab-bezlotoxumab and 54% for the placebo group, respectively.⁹³ The abundances of the *Clostridium* XIVa clade and *Holdemania* bacteria in the placebo group prior to treatment were not reported. *Akkermansia* is another bacterium that is frequently associated with CDI and rCDI as it is thought to contribute to infection by facilitating the access of luminal antigens to the intestinal immune system my mucin degradation.⁹⁴ Interestingly, a recent CDI study which analyzed the bacterial diversity of the guts of mice under different treatments including MK-3415, vancomycin and vancomycin combined with MK-3415 showed *Akkermansia* levels to be quite resilient, persisting in high amounts in both vancomycin groups. The authors suggested higher proportions of *Lactobacillus* and *Blautia* as well as changes in mucosal composition might attenuate the inflammatory role of *Akkermansia*.⁹⁵

To date, PA-50 and PA-41 are two of the most potent mAbs currently under investigation. PA-50 is a humanized anti-toxin A mAb, that targets toxin A RBD at multiple sites and has been shown to neutralize toxin A from a broad range of *C. difficile* ribotypes.⁹⁶ The mAb is significantly more potent than actoxumab in-vitro, possibly due to its multivalent interactions with toxin A. PA-41 is a humanized anti-toxin B mAb and is significantly more potent compared to bezlotoxumab. In addition, PA-41 is capable of inhibiting toxins from the same range of *C. difficile* ribotypes stated previously. In a hamster model for CDI, 95% of the animals treated with a combination of humanized PA-50 and PA-41 showed long-term survival relative to 0% survival of animals treated with standard antibiotic or comparator mAbs.⁹⁷

Probiotic expression vehicles and single domain antibodies (sdAbs)

The ability to produce antibodies or antibody fragments in a self-limiting manner at the site of infection would be most advantageous in the treatment of CDI. A potential way to accomplish this is to use a probiotic sdAbs expression vehicle.^{98,99} Of recent note is the work by Andersen et al in which four VHHs (heavy domain only) were expressed on the surface of *Lactobacilli*.¹⁰⁰ Two strains of the probiotic delayed the death of hamsters challenged with AB toxin B and *C. difficile* spores, with 50% of the hamsters receiving the probiotic surviving until the end of the experiment. More recently, Shkoporov et al expressed two VHHs in *Bifidobacterium longum* demonstrating toxin A neutralization in vitro.¹⁰¹ The group administered the probiotic bacteria to mice and confirmed the in-vivo expression (secretion) of both single domain antibodies in the guts of mice. In a study by Unger et al,¹⁰² recombinant VHHs were generated against the subunit and the binding component. Three out of five CDTa and two from four CDTb specific antibodies were found to neutralize the cytotoxicity of CDTa and CDTb. Surprisingly three of the nanobodies selected for binding to CDTa also indirectly neutralized the binding component (CDTb) by restricting the translocation of CDTa into the cytosol. In other investigations, hamsters immunized with *Bacillus subtilis* spores expressing a carboxy-terminal segment of toxin A remained resistant to colonization when challenged with *C. difficile* strain. Anti-toxin A mucosal antibodies obtained following immunization with recombinant *B. subtilis* spores were able to reduce the adhesion of *C. difficile* to mucus-producing intestinal cells.¹⁰³ More recently, Sulea et al utilized the affinity maturation platform “Assisted Design of Antibody and Protein Therapeutics (ADAPT)” to develop a set of mutant sdAbs (camelid sdAb A26.8, a VHH) that bind to toxin A. The designer mutants showed enhanced affinity to toxin A, with the A26.8 double-mutant T56R, T103R neutralizes TcdA cytotoxicity with an IC50 of 12 nM.¹⁰⁴ While certainly a consideration for severe CDI, immune-based treatment and prevention of *C. difficile* infection^{91,105–112} that have been widely studied (Table 1).

Fecal microbiota therapy

The best therapeutic option for recurrent CDI is FMT. The therapy involves the transfer of suspended (saline or water) fecal matter from a healthy donor to a recipient

Table 1 Immune-based treatment and prevention of *Clostridium difficile* infection

Model	Antigen	Antibody	Route of administration	Treatment method	Outcome	Reference
Hamster	TcdA and TcdB	Mouse mAb PCG-4 specific to toxin A; G-2 specific to toxin B	Oral	Animals were pretreated with 1 M NaHCO ₃ and treated with MAb and toxin mixture and observed for 72 hrs	PCG-4 MAb neutralized the effects of toxin A	105
Hamster	TcdA and TcdB	Sheep (ovine) IgG specific against recombinant toxin A and toxin B polypeptide of <i>C. difficile</i> VPI 10,463	Oral	Ovine antibody (IgG) doses of 2.5 and 25 mg was administered on the days 0 (before challenge), then day 3 and 6 (post challenge)	90 and 40% of the animals survived in the high- and low-antibody-dose groups respectively and at 12 days post challenge all surviving animals were asymptomatic	106
Hamster	TcdA and TcdB	Humanized mAbs (IgG1)	Oral	Animals were pre-dosed by intraperitoneal (i.p.) inoculation with mAb mixtures once/day for 4 days (days -3, -2, -1 and 0) – two doses of mAb – 50 mg/kg (high dose) and 5 mg/kg (low dose) of each anti-TcdA and anti-TcdB	High dose mAbs showed 100% protection on day 11 and ~82% (9/11) survival rate until end of the study on day 28. Low dose mAbs showed 100% protective effect only until day 3 then slowly succumbed to infection	91
Hamster	TcdA and TcdB + whole bacterium	Immune whey protein concentrate (WPC-40, Mucomilk)	Oral	Before and after challenge, then every 8 hrs during 10 days	80–90% protection	107
CDI patients				Three times daily for 2 weeks after antibiotic treatment	Significant decrease of recurrences	108
Randomized double-blind study in CDI patients	Formalin inactivated <i>C. difficile</i> cells	Immune whey IgG concentrate (CDIW)	Oral	Three times daily, 14 days	As effective as metronidazole in the prevention of recurrences	109
Mouse model of infection and relapse	TcdB-C-ter, inactivated spores, exo-sporium, inactivated vegetative cells, SLP LMW- and HMW-SLPs	Hyper-immune bovine colostrum TcdB-HBC, mixture 1-HBC, mixture 2-HBC	Oral	Two days before challenge and throughout experiment	HBC-TcdB alone or in combination (Mix1 and Mix2-HBC) prevents and treats CDI in mice and reduces recurrences	110
Hamster		Rabbit hyper-immune serum	Oral	Seven hour before challenge, during challenge, then 6, 17 and 24 hrs after challenge	Prolonged survival after challenge but no protection against death	111
Mouse	FlIC	Mouse hyper-immune serum	Intra-peritoneal	Twenty four hour before challenge	Eighty percent of protection	112

Abbreviations: TcdA, toxin A; TcdB, toxin B; PCG-4, mouse monoclonal anti-*Clostridium difficile* toxin A antibody; G-2, immunoglobulin G; VPI 10,463, *Clostridium difficile* strain VPI 10,463; WPC-40, whey protein concentrate 40%; CDIW, Immune whey IgG concentrate; TcdB-HBC, toxin B-hyperimmune bovine colostrum; HBC, hyperimmune bovine colostrum; HMW, high-molecular-weight; LMW, low-molecular-weight; SLPs, surface layer proteins.

via colonoscopic or nasoduodenal tube and rectal enema.¹¹³ Fecal matter is a complex mixture of bacteria, fungi, viruses, human cells, metabolites and more.¹¹⁴ Recommendations state that if there are three or more recurrences of CDI following pulsed vancomycin therapy, FMT should be considered the next therapeutic option.¹¹⁵ In a landmark randomized, open-label, clinical trial (RCT), van Nood and colleagues compared vancomycin alone and vancomycin bowel lavage to vancomycin and bowel lavage with FMT.¹¹⁶ Overall a 94% cure rate was reported for the FMT group while the vancomycin-bowel lavage and vancomycin groups reported cure rates of 23% and 31%, respectively. Further FMT clinical trials have reported similar cure rates.^{117,118} Recently, Bang et al showed FMT to be a highly effective therapy for refractory and recurrent *Clostridium difficile*. FMT was performed in nine patients with refractory/recurrent CDI. Bowel movement was normalized within one week after FMT.¹¹⁹ In a randomized double-blind clinical trial, where subjects were treated for rCDI by heterologous FMT (h-FMT) or autologous FMT (a-FMT) as a “placebo”, revealed that, while h-FMT resulted in higher cure rates than a-FMT (90% versus 63%; $P=0.019$), autologous FMT was, in some cases, successful.¹²⁰

Several clinical trials have demonstrated the equality of fresh and frozen donor material to cure recurrent *C. difficile* infection (rCDI)¹²¹ More recently, Anand et al showed that the age of the sample donor does not affect the overall microbial diversity of the sample and the clinical efficacy of FMT in rCDI patients.¹²² All patients receiving FMT from their respective donors had resolution of rCDI symptoms and had a negative *C. difficile* toxin test 4–12 weeks after FMT. FMT has also been used to treat individuals infected with hypervirulent strains of *C. difficile*. In the case of a recent CDI outbreak in France, the treating physician adopted a new treatment algorithm by applying FMT in combination with antimicrobial therapy during the first infection episode, mortality of the patients dropped from 64% to 19% with early FMT treatment.¹²³ Tanaka et al also demonstrated that FMT is an effective treatment of new-onset CDI as well.¹²⁴

Increases in microbial alpha diversity are often reported in FMT recipients with improvements in *Bacteroidetes*, *Clostridium* clusters IV and XIVa numbers and a decrease in members of the *Enterobacteriaceae* family.¹²⁵ Higher diversity of gut microbiota has been observed in lean individuals when compared to obese individuals, yet diversity is a complex parameter as some recent microbiota studies

have shown higher diversity in disease states, such as colon cancer, coeliac disease and Alzheimer’s disease.¹²⁶ Thus, rather than counting the number of bacterial species, a comprehensive analysis (Source Tracker software program and Bayesian algorithm) of enriched and depleted microbial taxa must be performed and diversity alterations defined for each disease.¹²⁷ This point was eloquently demonstrated in a recent paper by Staley et al in which a partial engraftment was shown to be sufficient if functionally critical taxa were still present in the subjects following antibiotic therapy.¹²⁸ Notably subjects cured by a-FMT typically had greater abundances of the *Clostridium* XIVa clade and *Holdemania* bacteria prior to treatment, and the relative abundances of these groups increased significantly after FMT compared to heterologous FMT and pre-FMT samples. Provided *Clostridium* XIVa and IV can be identified in the feces prior aFMT it may be possible to further accelerate the reconstitution of the host flora by supplementing the slurry with phytochemicals (aryl hydrocarbon receptor ligands) thereby boosting colonization resistance.¹²⁹ Moreover, given aFMTs ability to rapidly improve the post-antibiotic reconstitution of the indigenous fecal microbiome and gut transcriptome in individuals,^{130,131} it may be prudent to offer the therapy as adjuvant to MET and VAN treatments, as both of these antibiotics are associated with the emergence of potentially pathogenic fungal operational taxonomic units, with predicted bacterial functions enriched for xenobiotic metabolism that could perpetuate the dysbiosis driving CDI (see Figure 3).

CDI is also a common comorbidity of irritable bowel disease (IBD). A recent study by Khortus et al compared the use of FMT in patients with CDI and IBD to those without IBD and found a lower efficacy in clearing the infection in those with IBD after one FMT (74.4% vs 92.1%).^{132,133} Anderson et al reviewed several case studies in which FMT was used in the treatment of rCDI and refractory CDI infection in IBD.¹³⁴ A resolution of CDI was found in 11 of the 12 patients and improved response to IBD medication in 6 of 7 patients. In addition, there have been many published case studies showing the positive effects of FMT in IBD of particular note is the work by Moayyedi et al in which the efficacy of FMT in active ulcerative colitis was investigated, remission of IBD was achieved in 24% of the patients.¹³⁵ A summary of recent FMT trials is shown in Table 2.

The greatest impediment to the broad dissemination of FMT for the treatment of rCDI and primary cases as well is the uncertainty surrounding its regulation. Regulation of

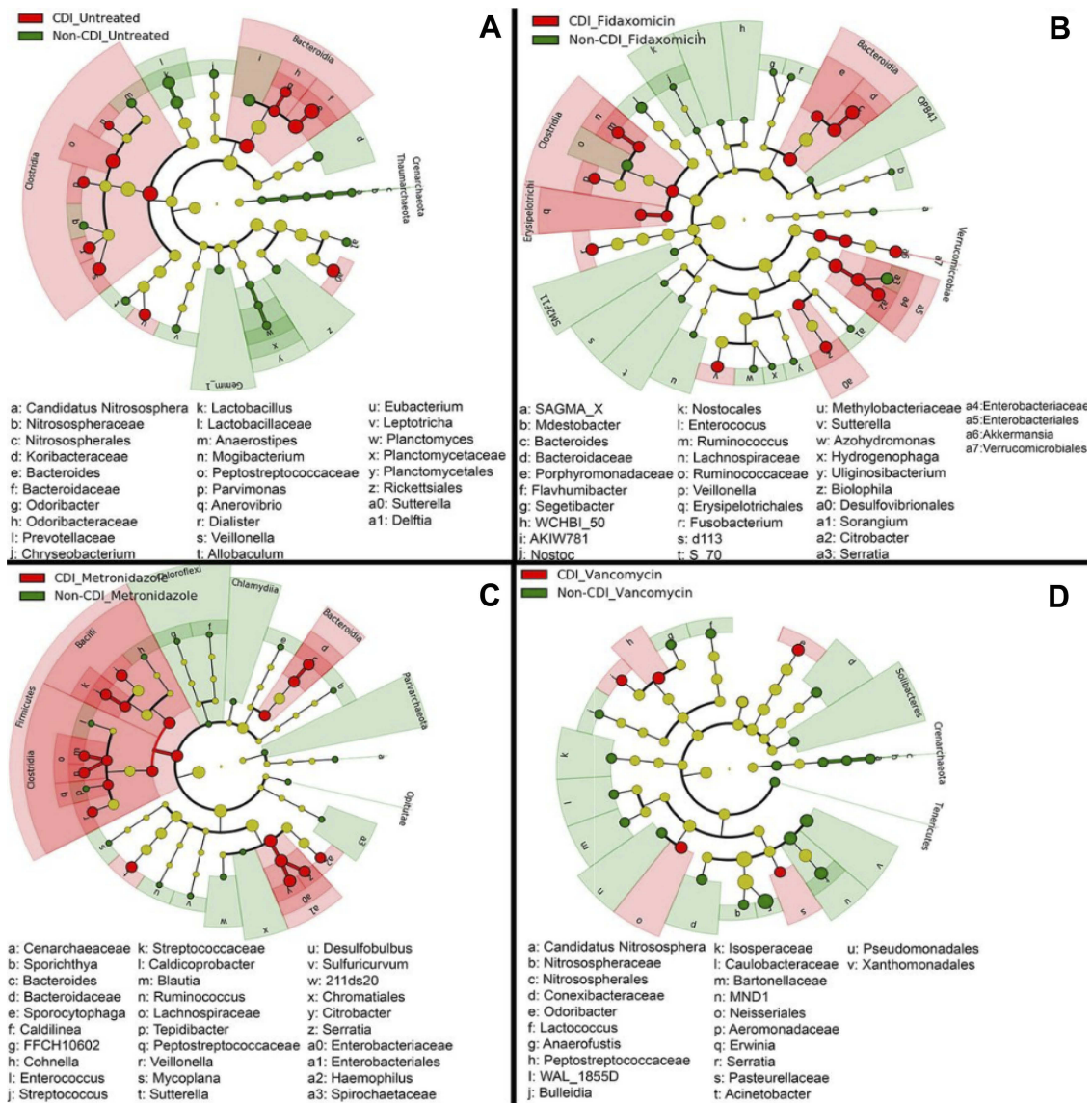


Figure 3 Cladogram plots were generated in Galaxy to visualize significantly enriched fungal taxa identified in *Clostridium difficile* infection (CDI) and non-CDI samples considering each treatment cohort separately (A, untreated; B, fidaxomicin; C, metronidazole; D, vancomycin).¹³¹

Table 2 Characteristics of some recent studies concerning fecal microbiota transplantation in *C. difficile* treatment

Patients (n)	Study type	Mode of delivery	Success rate (%)	Ref no.	Infection type
16	Randomized	Nasoduodenal tube	81%, 1st infusion	116	Recurrent
1	Case	Colonoscopy	100	117	Severe
46	Randomized	Colonoscopy	90.2(h), 62.5 (a)	120	Recurrent
16	Case Series	Nasogastric route	80	115	CD027 relapse
9	Case Series	Colonoscopy	100	119	Recurrent
272	Case	Colonoscopy	92, 75 & IBD	132	Recurrent
28	Prospective	Colonoscopy	100	122	Recurrent
24	Randomized	Colonoscopy	90.2(h), 43 (a)	128	Recurrent

Abbreviations: (h), heterologous fecal microbiota transplantation; (a), autologous fecal microbiota transplantation; IBD, inflammatory bowel disease; CD027, *Clostridium difficile* ribotype 027.

FMT is complicated by the multifarious nature of fecal samples. Ideally, an FMT replacement modality would be safer to use, easy to apply and less expensive than current treatments.

Emerging biotherapies

Phages

Phage therapy entails the isolation and inoculation of phages that target and eliminate specific bacteria.¹³⁶ To date, phage treatments have been successfully developed for *Escherichia coli*,¹³⁷ *Pseudomonas*, *Proteus*,^{138,139} *Staphylococcus* and *Streptococcus* infections.¹⁴⁰ The lack of phage treatments for CDI reflects the technical difficulties (culturing) of working with sporulating anaerobes. The first reported isolation of *C. difficile* phages was in 1983, since then several phages (mainly temperate) have been described in the literature.¹⁴¹ All known *C. difficile* phage genomes are double-stranded DNA and belong to the Caudovirales (the order of the tailed phages). CD phages are characterized by their size and morphological type which includes the small myovirus (SMV) Φ MMP02, medium myovirus (MMs) ϕ CD119 and ϕ CDHMI1, long-tailed myoviruses (LTMs) ϕ CD27 and Φ MMP04 and two morphologically distinct siphoviruses (SVs) ϕ CD6356 and ϕ CD38-2.¹⁴² In 2016, two novel myoviruses CDKM15 and CDKM9 were isolated and selected for detailed sequence analysis on the basis of their broad host range.¹⁴³ CDKM15 infected 20/80 strains from 9/20 CD ribotypes, whilst CDKM9 infected 25/80 strains from 12/20 ribotypes. Both phages infected the clinically relevant ribotypes R027 and R001. Genome sequencing analysis of these phages identified new signals for horizontal gene transfer (HGT). The mechanism of DNA packaging for each myovirus could not be classified. Three *C. difficile* hosts, namely CD105HE1 (Ribotype 076, equine isolate), CD105LC1 (ribotype 027, human isolate) and CD105HS (ribotype 012, environmental isolate) were recently used by Clokie et al to propagate seven phages (6 ϕ CDHMI1-6 and ϕ CDHS1) producing phase titers ranging from 109 to 1010 PFU/mL.^{144,145} With the exception of ϕ CDHS1 the remaining phases were manufactured on a common host (CD105LC1), ensuring any lytic activity was attributable to the specific phage and not due to differences conferred by the host bacterial strain. Using a hamster model, the oral delivery of optimized phage combinations resulted in reduced *C. difficile* colonization at 36-hr post-infection.

The evolution of bacterial resistance to phages is of genuine concern as recent work suggests CD phages can mediate the horizontal transfer of genetic material via

transduction (antibiotic resistant and toxin genes). In a study by Goh et al, the ϕ C2 phage was shown to transduce the antibiotic marker *ermB* carried on a 13 kbp transposon.¹⁴⁶ Moreover, genome sequencing has revealed the presence of defense mechanisms including a clustered regularly interspaced short palindromic repeat (CRISPR)/CAS system^{147,148} and active type I and type II restriction modification system.

Although hamster CDI models demonstrate various clinical symptoms consistent with those seen in humans, the animals rapidly succumb to the disease. This has resulted in many groups employing artificial gut models, which have revealed many facets of enteric pathogens.^{149–151} In 2010 and 2013, Meader et al studied *C. difficile* phage-host interactions using two ex-situ model systems. The first involved studying their dynamics in a batch model and the second in a multi-vessel model (artificial gut model).^{152,153} Remedial and prophylactic treatments were tested using ϕ CD27, both models exhibited significant reductions in the levels of TcdA and B compared with the controls. The colon model illustrated the potential of phage therapy in treating CDI as well as other factors that could impact treatment.¹⁵³ Studies by Govind et al showed the single phage ϕ CD119 could lysogenize under the conditions in the mammalian gut and suppress toxin production.¹⁵⁴ On the other-hand, Sekulovic et al and Goh et al demonstrated toxin levels are most likely influenced by strain-phage specific interplay and that considerable variation in the physiological response to phage infection does occur.^{155,156}

More recently, Nale et al utilized *Galleria mellonella* larvae as an alternative model to study the therapeutic potential of a 4-phage cocktail on CD ribotypes 014/020.¹⁵⁷ It was found that multiple phage doses significantly improved the larval remedial regimen with 60% of the larvae surviving until the end of the experiment. The phages were most effective when vancomycin was given prophylactically before bacterial infection resulting in as little as 10 colony forming units (CFU) per larva being recovered. The study demonstrated that multi-phage therapy remains one of the most effective ways of clearing *C. difficile* and preventing the appearance of resistant/lysogenic clones.

Isolating new CD phages from the terminal part of the gut that exhibits minimal or no temperate activity on the target strain remains challenging.^{158,159} However, the mouth is a great source of bacteriophages and susceptible bacteria such as *E. faecalis* which is known to play a role in prolonging dysbiosis. Therefore, it may be suggested that

future phage treatments target such species as well as CD, potentially reducing the risk of recurrence and relapse.¹⁶⁰ A summary of the challenges CD phage therapy currently face can be found in recent review by Fortier.¹⁶¹

Endolysins

Many bacteriophages isolated from the host environment are not efficient in the rapid eradication of pathogenic hosts, as is the case with ϕ CD27.¹⁶² One way to overcome this problem is to clone and express the recombinant version of the endolysin from its phage. Endolysins are produced by many double-stranded DNA bacteriophages to affect the release of new virions from an infected cell by degrading the bacterial cell wall. They have been used to target many well-known infectious bacteria including *Streptococcus*,¹⁶³ *Staphylococcus*,¹⁶⁴ *Listeria*,¹⁶⁵ *Bacillus*¹⁶⁶ and *Clostridium*.¹⁶⁷ Unlike CDI,^{168,169} most infections involve multiple strains requiring the use of broad-spectrum antibiotics. Lysins possess the potential to satisfy this role without the risk of bacterial resistance. Moreover, they have been shown to provide better protection against pathogenic organisms such as *C. difficile* or *E. faecalis* compared to their respective phages.^{170,171} Investigations using ϕ CD27 found that a truncated version of the N-terminus was able lyse all 32 strains of *C. difficile* tested, as well as less closely related species *B. subtilis*, *Listeria innocua* and *B. amyloliquefaciens*.¹⁷² How the wider activity of the truncated endolysin impacted the broader bacterial community within the GI tract was not reported. In the future, endolysins will undoubtedly play a strategic role in the treatment of systemic (sepsis) and antibiotic resistant infections.

Small molecule inhibitors

The mammalian gut contains hundreds of small molecules whose function is yet to be discovered. Finding molecules that selectively inhibit different stages of the *C. difficile* life cycle, while sparing the indigenous gut microbiota is important for the development of alternatives to standard antibiotic treatments. 2-aminoimidazole (2-AI) molecules have been shown to overcome the protective mechanisms of multi-drug resistant pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Recent work by the group of Theriot et al in which the inhibitory effects of eleven 2-AI molecules on the life cycle of seven strains of *C. difficile* and an eight-member commensal library of bacteria associated with host colonization resistance were tested. Four of them were found to inhibit toxin production without affecting the growth of both *C. difficile* strains and the commensal library.¹⁶⁹ In addition to 2-AI, there are

number of anti-virulence compounds such as Ebselen and benzodiazepinedione that inhibit the glucosyltransferase activity of TcdA and TcdB and have the potential to reduce disease symptoms.^{170,173} Furthermore, Ebselen has been used in phase II clinical trials, and was recently reported to ameliorate β -amyloid pathology, tau pathology and cognitive impairment in triple-transgenic Alzheimer's disease mice.¹⁷⁴ Whether *C. difficile* or its toxins should be included in the current "infectious theory" of Alzheimer's disease is beyond the scope of this review.

An alternative way of inactivating TcdB is by triggering its auto-proteolysis in the gut lumen prior to cell uptake using the allosteric activator inositol hexakisphosphate (IP6). Although IP6 can trigger the auto-processing of TcdB in vitro, the cleavage is abolished if performed in the presence of luminal concentrations (>10 mM) of calcium. In a recent study, Ivarsson et al attempted to address the problem of calcium chelation by synthesizing a series of IP6 analogs where the six phosphate groups were progressively replaced by sulfates culminating in inositol hexasulfate (IS6).¹⁷⁵ An optimal balance between allosteric activity and interference by calcium was reached using the phosphate-sulfate hybrid IP2S4. IP2S4 attenuated colitis in CDI mouse models after oral dosing; moreover, a thiol-phosphate form of the analog IT2S4 was shown to rescue mice in a fulminant CDI model. Figure 4 shows in-vivo IP2S4 and IP6 attenuating activities in mice.

Bacteriocins

Bacteriocins are a group of antimicrobial peptides ribosomally produced by Gram-negative and Gram-positive bacteria. A recent study, conducted by Egan et al, explored the role bacteriocins may have in the GIT. In a genome mining project, the authors retrieved 641 genomes (307 whole genomes and 334 draft genomes) from microorganisms in the human gut. The genomes represented 199 bacterial genera, including *Lactobacillus*, *Streptococcus*, *Clostridium* and *Bacillus*.¹⁷⁶

Nisin is a bacteriocin produced by a group of Gram-positive bacteria that belongs to *Lactococcus* and *Streptococcus* species. Nisin is classified as a Type A (I) lantibiotic that is synthesized from mRNA and has been used for many years as a food additive. Similar to vancomycin, lanthipeptides such as nisin also targets a cell wall component, in this case lipid II. Recent studies by Fliss et al¹⁷⁷ assessed the in vitro efficacy of nisin Z and A on *C. difficile* cells and spores. Nisin A and Z both inhibited the growth of twenty *C. difficile* isolates, and minimum inhibitory concentrations (MIC) were estimated at

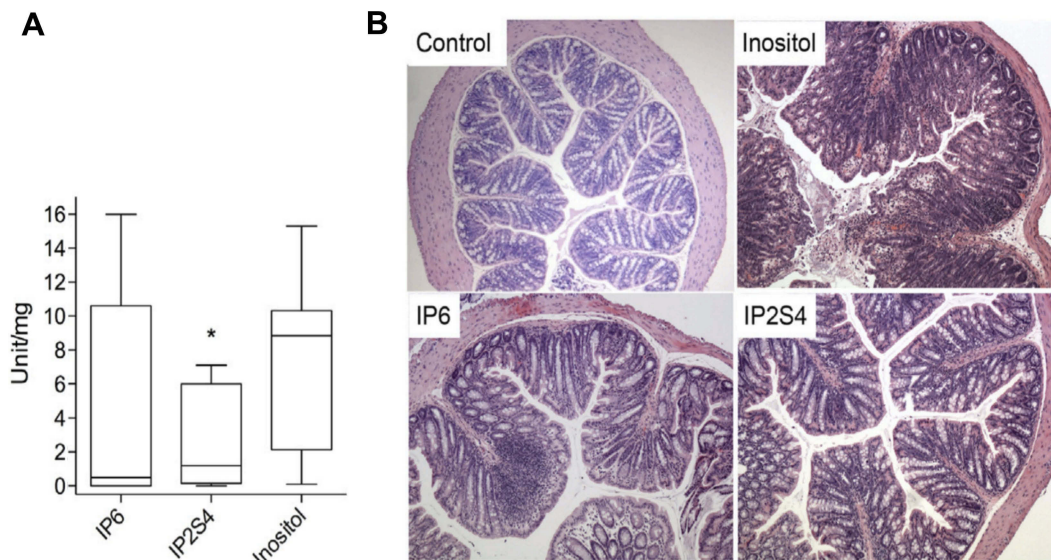


Figure 4 Swiss mice infected with fecal slur from a patient with recurrent *Clostridium difficile* infection. **(A)** Oral administration of IP2S4 but not IP6 significantly reduced the acute inflammatory component of colitis compared with administration of myo-inositol. **(B)** Histological sections of excised colons. Inositol-treated mice (negative control) displayed overt colonic structural changes characterized by mucosal ulceration and overlying exudate, marked acute and chronic inflammatory infiltrate and submucosal edema. IP2S4- and IP6-treated mice had decreased mucosal damage and inflammatory infiltrate. Copyright ©2018. Reproduced with permission from Elsevier. Ivarsson ME, Durantie E, Huberli C, et al. Small-molecule allosteric triggers of *Clostridium difficile* toxin B auto-proteolysis as a AQ3 therapeutic strategy. *Cell Chem Biol.* 2018;26(1):17–26.e13.¹⁷⁵

6.2 µg/mL for nisin Z and 0.8 µg/mL for nisin A. In addition, *C. difficile* spores were also susceptible to nisin A (25.6 µg/mL), reducing spore viability by 40–50%. The MIC value for nisin A was comparable to the MICs obtained for lacticin 3147. However, when used as standalone therapy resistance to nisin A frequently occurs.¹⁷⁸ A simple way to minimize resistance as well as improve therapeutic efficacy is to incorporate a germinator or potentiate the antibiotic/antibiotic peptide with a primary metabolite. The effectiveness of this approach was recently demonstrated by Se-Wook Oh et al in which the synergistic action of nisin and lysozyme (20 nmol/L nisin and 0.2 mmol/L lysozyme) resulted in no viable *C. difficile* spores being detected after 2 hrs of incubation.¹⁷⁹

Lacticin 3147 is another bacteriocin produced by strains of *L. lactis*¹⁸⁰ with potent anti-*C. difficile* activity with concentrations as low as 18 µg/mL capable of eliminating 10⁶ CFU/mL of *C. difficile* <30 mins, comparable in efficacy to metronidazole and vancomycin in a model fecal environment. An alternative to 3147 is the lantibiotic actagardine. When combined with ramoplanin or metronidazole it behaves in a partial synergistic/additive fashion against 61.5% and 54.4% of target *C. difficile* strains investigated.¹⁸¹ In addition, a recent study demonstrated that combinations of the class II bacteriocin, durancin 61A and the broad-spectrum antimicrobial reuterin yielded fractional inhibitory concentration index (FIC) indices of 0.2 against *C. difficile*, indicating highly synergistic

activity.¹⁸² But perhaps the bacteriocin with the most therapeutic potential was thuricin. Initial work revealed that thuricin was as effective as metronidazole and vancomycin against *C. difficile* in a distal colon human model. Moreover, further studies showed thuricin interacted in a partial synergistic manner when combined with ramoplanin against 31% of the target CD strains investigated.¹⁸³

Conclusions

With the recent emergence of hypervirulent strains in Europe, Australasia⁴⁵ and North America, there is an urgent need to develop alternative/adjunctive therapeutic options to metronidazole and vancomycin in order to minimize the ongoing problem of recurrence and prevent the spread of vancomycin-resistant enterococci in hospital environments.

The alternative therapies discussed each have their advantages, vaccination and monoclonal antibodies are probably the most cost effective in the long term.¹³¹ On the other hand, they do not reduce the bacterial load nor prevent *C. difficile* colonization or potential spore transmission. Moreover, challenges to vaccination strategy will arise from a patient's inability to generate a rapid, long-lasting and protective response. However, it is pleasing to note that many anti-toxin therapies are on the cusp of approval and when combined with other biotherapeutic options such as FMT or tailored spore formulations individual therapeutic solutions will become more available.

In addition to FMT and immunotherapies, multi strain-phage treatments are one of the most promising emerging therapies. However, numerous obstacles persist regarding the isolation and therapeutic application of *C. difficile* phages. Of particular concern is how different combinations or the same combination can affect toxin production in different hosts. Moreover, it is important know the exact phage and antibiotic resistance patterns of *C. difficile* strains in order to minimize the risk of recurrence. As of yet, no experimental models have investigated the use of multiple bacterial phages in the treatment of rCDI and dysbiosis.

Although the biotherapies discussed herein have the potential to improve patient outcomes, the most difficult step is translating these discoveries into therapeutics that are safe for humans. This review has not covered other treatment options, such as alternative antibiotics and antimicrobial agents.^{184–187} Future treatments will undoubtedly include a combination of these therapies with the aim of reducing rCDI, and the number of antibiotic resistant genes in *C. difficile* patients.¹⁸⁸

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Disclosure

The authors report no conflicts of interest in this work.

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