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Jingpeng Yang & Hong Yang

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REVIEW ARTICLE

Non-antibiotic therapy for *Clostridioides difficile* infection: A review

Jingpeng Yang and Hong Yang

State Key Laboratory of Microbial Metabolism, and School of Life Science & Biotechnology, Shanghai Jiao Tong University, Shanghai, China

ABSTRACT

Clostridioides difficile infection (CDI) is a common infectious disease that is mainly caused by antibiotics. Antibiotic therapy is still the dominant treatment for CDI, although it is accompanied by side effects. Probiotics, fecal microbiota transplantation (FMT), engineered microorganisms, bacteriophages, diet, natural active substances, nanoparticles and compounds are examples of emerging non-antibiotic therapies that have received a great amount of attention. In this review, we collected data about different non-antibiotic therapies for CDI and provided a comprehensive analysis and detailed comparison of these therapies. The mechanism of action, therapeutic efficacy, and the strengths and weaknesses of these non-antibiotic therapies have been investigated to provide a basis for the reasonable alternative of non-antibiotic therapies for CDI. In summary, probiotics and FMT are currently the best choice for non-antibiotic therapy for CDI.

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1. Introduction

Infectious diarrhea triggered by *Clostridioides* (previously known as *Clostridium*) *difficile* accounts for a large part of antibiotic-associated diarrhea (AAD), causing serious health hazards and considerable economic losses worldwide [1]. *C. difficile* is generally recognized as a conditional pathogen in the gut, and most strains cannot cause infection except in special cases, such as during dysbiosis in gut homeostasis induced by antibiotics. The ingestion of antibiotics kills most gut microbes that can defend against *C. difficile* and causes the destruction of the intestinal mucosa and immune system [2,3]. *C. difficile* survives during antibiotic intervention due to their spores. These recalcitrant spores can withstand multiple antibiotics; subsequently, they germinate and transform into vegetative cells again when suitable conditions manifest. Finally, without competitors, these regenerated *C. difficile* strains flourish in the gut, secrete toxins and cause infection [4]. Traditional antibiotics, such as vancomycin, metronidazole and fidaxomicin, are still the first choice in the treatment of *C. difficile* infection (CDI) or recurrent CDI (rCDI); however, these antibiotics have side effects, such as rash, multidrug-resistant strains and an imbalance in the gut microflora [2,5,6]. Therefore, finding novel alternative therapies to address these issues is an urgent need. In recent years, probiotics and fecal microbiota transplantation (FMT) have become the dominant non-antibiotic therapies for CDI [7,8]. In addition, other emerging treatments, such as engineered bacteria [9], diet [10], bacteriophages [11], natural active substances [12], nanoparticles [13] and compounds [14], have also exerted excellent antibacterial activity against *C. difficile*. Probiotics and FMT are the two main non-antibiotic therapies in the treatment of CDI. This review will give a more detailed discussion regarding these two mainstays.

2. *Clostridioides difficile* infection

2.1 Global trend and main route of infection

Infectious disease caused by *C. difficile* is one of the most common infectious diseases worldwide, resulting in serious harm to public health. In 2011, the US Centers for Disease Control and Prevention (CDC) evaluated ~453,000 cases related to CDI and found that this infection killed 29,300 patients that year, accompanied by a heavy burden of 5.4 billion dollars [15,16]. In Europe, a report from the European Center for Disease Prevention and Control (ECDC) indicated that there were 124,000 CDI cases and 14,000 deaths annually [6]. Meanwhile, in Asia, this trend has also attracted wide attention due to a surge in incidence [1], especially in China. Several reports have shown that CDI in China is a relatively mild disease, but it is on an upward trend

CONTACT Hong Yang hongyang@sjtu.edu.cn State Key Laboratory of Microbial Metabolism, and School of Life Science & Biotechnology, Shanghai Jiao Tong University, Shanghai, China

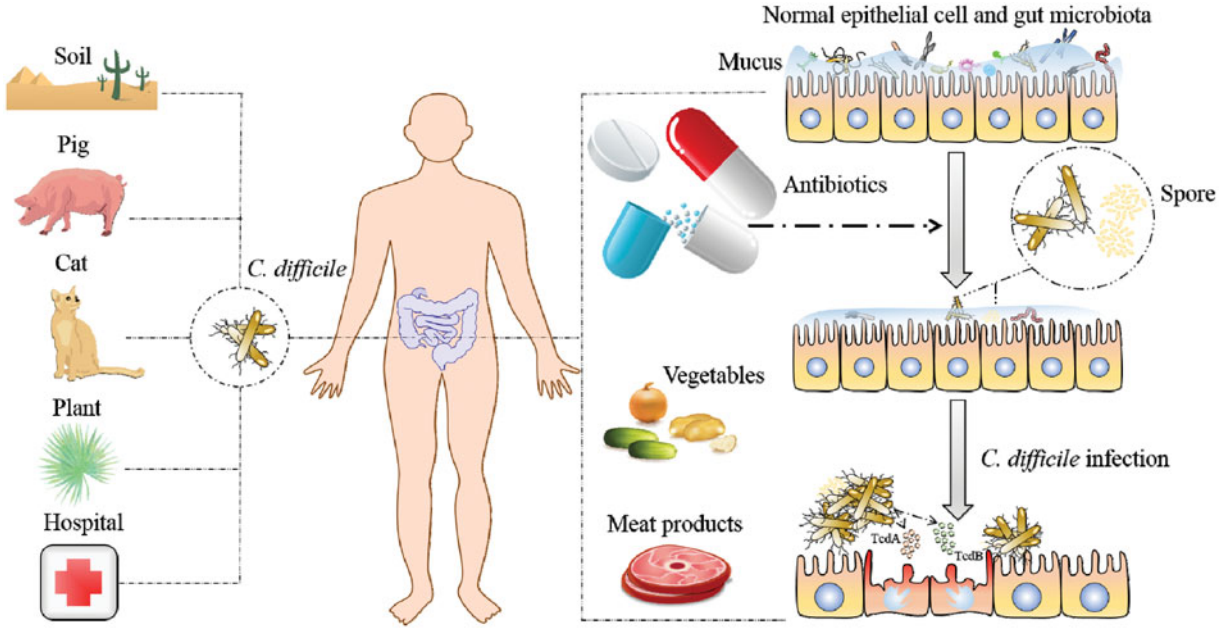

Figure 1. Routes of *Clostridioides difficile* infection and destruction of normal epithelial cells and gut microbiota. *C. difficile* exists in animals, plants and the human intestines. It spreads via many ways, such as vegetables, retail meat products and antibiotics. The use of antibiotics disrupt the normal epithelial cells and gut microbiota in humans, providing the chance for *C. difficile* to spread, grow and secrete toxin proteins (TcdA and TcdB). These toxin proteins further destroy epithelial cells and eventually cause inflammation and intestinal cell damage.

[17–19]. The main reason behind this trend of increased CDI incidence is likely due to the broader utilization of antibiotics, especially in developing countries [20]. The overuse of antibiotics is a severe public health issue. It has not only brought a huge medical cost but has allowed drug-resistant bacteria, including *C. difficile*, to flourish. Lack of specific medical guidance, inadequate health care systems and the shortage of public health funds contribute to the overuse of antibiotics, which is becoming widespread in most developing countries [21]. Multiple antibiotics are easy to obtain and are used in the treatment of several diseases, including ailments and serious illness, and these antibiotics accelerate CDI epidemics. For most developed countries, the use of antibiotics is under control, accompanied by an increasing development of specific drugs and medical guidance for CDI; however, emerging drug-resistant strains remain an unsolved problem [22]. Overall, many developed countries have a high CDI incidence, which causes huge economic losses; in addition, a growing trend of CDI has been observed in developing countries.

C. difficile widely exists in soil, plants, megafauna, some small mammals and the human intestines [4]. It can infect humans with its spores in many ways, especially in the hospital; in addition, animals, vegetables and retail meat products also provide the chance for *C. difficile* spores to spread (Figure 1). A report from Carmen et al. [23] indicated that *C. difficile* spores can enter the food chain via different pollution sources, including water, soil, sewage treatment plants, shellfish, horses, pigs, and traditional organic fertilizer. The process of evisceration in animal slaughterhouses can be contaminated by spores, followed by spores spreading through retail meat products (beef, pork, and poultry). A previous report on the visceral processing line in Europe showed that *C. difficile* has been detected in 28% of pork intestines, 9.9% of beef cattle and 5% of broilers. Furthermore, a certain percentage of pork and beef stored in refrigerators was also contaminated by *C. difficile* spores. *C. difficile* RT017, RT027, and RT078 are the common types found in food. In Europe, RT001 and RT014 are found in poultry, vegetables and shellfish, and RT027 and RT078 are found in North America. Tkaleca et al. [24] investigated 142 kinds of retail products and 12 kinds of vegetables from 2014 to 2017 in Slovenia. The *C. difficile* detection rate in vegetables reached 18.2%, and 10 types (RT 014/020) of 115 isolated strains produced toxins. Further analysis found that the *C. difficile* detection rate was 28% in potato, 9.4% in leafy vegetables, and 6.7% in ginger. However, there are few reports about food contamination-induced CDI, and this fact might be due to individuals (with a normal gut microbiome) having innate defense against invasion by *C. difficile*.

2.2 Physiological and pathological features

C. difficile is a gram-positive anaerobic bacteria that produces spores and produces an awful smell [4,25]. As a

common conditional pathogenic bacterium, *C. difficile* exists in the gut in humans of all ages; almost half of infants are asymptomatic carriers, and the number of *C. difficile* bacteria in their gut drops rapidly with age [26,27]. For adults, there is no large-scale CDI epidemiological investigation that can reveal the proportion of asymptomatic adults carrying *C. difficile*. A clinical practice guideline on CDI in adults from the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA) indicated that there are 3%–26% of adult inpatients in acute care hospitals and 5%–7% of elderly patients in long-term care facilities that are *C. difficile* carriers without symptoms [28]. There is no direct evidence that *C. difficile* asymptomatic carriers are more likely to develop an infection with age. However, clinical data indicated that the morbidity of CDI in older *C. difficile* carriers reaches a high level [29]. This result might be associated with many factors, such as the degeneration of the immune system and the fragile gut microflora in elderly individuals [28], which provide the opportunity for *C. difficile* to expand. Clinically, more than three loose stools in 24 h together with a positive result of *C. difficile* toxin in stools is defined as CDI [29]. Mild diarrhea is the initial symptom of the infection induced by *C. difficile*; afterwards, it develops into severe diarrhea, pseudomembranous colitis, toxic megacolon, or even death [30].

The first report about *C. difficile* appeared in 1935, with no other new findings on *C. difficile*, especially on its pathogenicity, over the next four decades until 1978 [31]. In 1978, *C. difficile* was isolated from the feces of pseudomembranous colitis patients who received clindamycin, and this organism was considered the main cause of the disease. Subsequently, this presumption was followed and supported by a series of reports with regard to pseudomembranous colitis, diarrhea, antibiotics, *C. difficile*, and toxin secretion [32]. All these studies revealed that *C. difficile*, as a gut pathogen, can cause severe human gastrointestinal diseases, especially in people undergoing antibiotic therapy. Finally, the strong link between *C. difficile* and antibiotic diarrhea was confirmed, and the emergence of pseudomembranous colitis aroused wide attention. Pseudomembranous colitis is the most severe symptom induced by *C. difficile*. After antibiotic therapy, *C. difficile* releases toxins (TcdA and TcdB) in the gut [4]. These toxins act on intestinal epithelial cells, exert a cytopathic effect on tissue, and cause acute shock inflammation of the intestinal mucosa; subsequently, a pseudomembrane forms on the necrotic mucosa [33–35]. Diarrhea is the initial symptom of CDI, and this kind of diarrhea is a type of AAD, called *C. difficile*-associated diarrhea (CDAD) [36]. The difference between AAD and CDAD is mainly reflected by their clinical features. CDAD symptoms include colitis, cramps, fever, and fecal leukocytes that are common and of longer duration, while AAD is usually moderate in severity, without colitis, and of transient duration [37].

The genetic material of *C. difficile* consists of an ~4.3 Mb circular chromosome [31], and most of *C. difficile* can secrete two kinds of synergistic toxin proteins, enterotoxin A (TcdA, 308 kDa) and cytotoxin B (TcdB, 270 kDa), under suitable conditions [38]. TcdA and TcdB can destroy intestinal epithelial cells and subsequently induce inflammatory and tissue damage [39,40]. These two toxins are encoded and controlled by *tcdA* and *tcdB*, which are located in the pathogenicity determinant locus PaLoc (19.6 kb). In addition, the other three genes (*tcdC*, *tcdD*, and *tcdE*) in this area are associated with the production of toxin proteins [40]. *tcdC* is a negative regulator of toxin expression, whereas *tcdD* is a positive regulator. The role of *tcdE* is to release toxin proteins from *C. difficile* cells. Recently, Cdt, a new type of binary toxin, has emerged, and this toxin is controlled by the *cdtA* and *cdtB* genes outside the PaLoc; only some high virulence strains, such as RT 027, can secrete it [31]. *C. difficile* has resistance to various harsh environments due to its spores, and these spores can form when *C. difficile* stays in undernourished or special conditions [41]. Spores have strong resistance against high temperatures, oxygen, and even high concentrations of ethanol and thus cannot be killed easily. These spores can germinate and become vegetative cells when they are in a suitable environment and subsequently form pathogenic strains, produce toxin proteins, and induce tissue damage and infection again [41]. Clinically, this symptom is defined as rCDI [42].

Overuse of antibiotics can directly lead to infection, and this antibiotic-induced route accounts for the vast majority of CDI cases. Antibiotics make a great contribution to human health and can treat a wide range of diseases, such as cough, fever and postoperative infection; however, their side effects have become worse. Most CDI cases can be addressed with antibiotics, but there is still a relatively high recurrence rate and multiple complications. One of the serious side effects of antibiotics is destruction of the normal gut microbiota [43]. In Palleja et al.'s study [44], 12 healthy individuals received a 4-day antibiotic (meropenem, gentamicin, and vancomycin) intervention and then stopped. After half a year, the disrupted gut microbiota of most subjects returned to normal; however, a few species in the gut disappeared permanently. This result indicated that using antibiotics, on the one hand, can cure diseases; on the

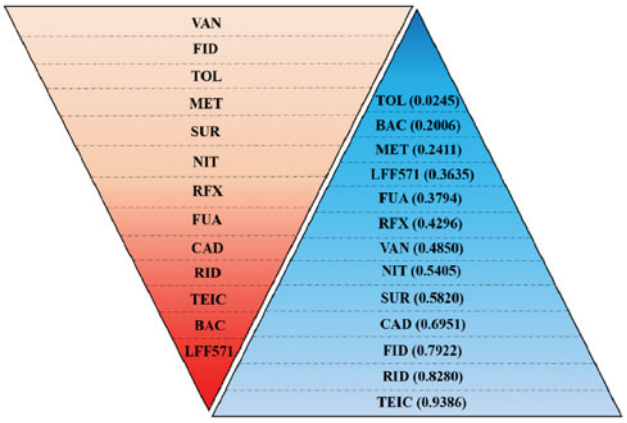

Figure 2. Rank of therapeutic drugs used in *Clostridioides difficile* infection (red) and their chance of being the best treatment (blue, P score) [46]. Red triangle shows therapeutic drugs ranked by the number of patients assigned to receive the therapeutic drug (the fewest used in the spire, the most used in the tower bottom). Blue triangle shows therapeutic drugs ranked by their chance of being the best treatment (the worst in the spire, the best in the tower bottom, P scores are used to rank the treatments). "Chance of being the best treatment", the sustained symptomatic cure, which was calculated as the number of patients with a primary cure (resolution of diarrhea, as defined by individual trial criteria) at the end of treatment, minus the number of patients with recurrence (recurrence of diarrhea or requirement for additional treatment) or who died during the follow-up period. BAC: bacitracin; CAD: cadazolid; FID: fidaxomicin; FUA: fusidic acid; MET: metronidazole; NIT: nitazoxanide; RFX: rifaximin; RID: ridinidazole; SUR: surotomycin; TEIC: teicoplanin; TOL: tolevamer; VAN: vancomycin.

other hand, it can cause permanent damage to some inherent microorganisms.

Generally, *C. difficile*, as a kind of resident bacteria in the gut, does not cause infection, and most *C. difficile* carriers have no symptoms due to the protection of the normal gut microbiota and intestinal immune system [4]. However, the infection only occurs under certain conditions, such as the destruction of certain intestinal microorganisms induced by antibiotics, chemotherapy, proton pump inhibitors, antacids or antimotility drugs [45]. In these specific contexts, the disturbed intestinal flora and immune system favor the colonization and expansion of *C. difficile* in the gut and a pathological change triggered in tissues. For example, some antibiotic treatments kill many beneficial microorganisms and stimulate the overgrowth of *C. difficile* [4]. These *C. difficile* strains flourish in the gut without competitors and secrete massive amounts of toxin, followed by intestinal infections and inflammation, which is called CDI. The process of CDI induced by antibiotics includes the following (Figure 1): (1) after intake of the antibiotics, most inherent bacteria and fungi are destroyed, and their corresponding niches are vacated; (2) vast numbers of spores are produced by *C. difficile* in the antibiotic-induced environment, and these spores can germinate into vegetative cells and strains again in suitable conditions; and (3) these pathogenic strains invade and occupy vacant niches and then indiscriminately grow and secrete toxin proteins (TcdA and TcdB), eventually causing inflammation and intestinal cell damage.

3. Antibiotic therapy

In terms of their cure rate and range of application, antibiotics are the first choice in the treatment of almost all bacterial diseases. Compared with the therapeutic effect of other drugs in bacterial- or fungal-induced gastrointestinal infections, antibiotics possess high-quality efficacy and a short treatment period. First-line antibiotics such as metronidazole, vancomycin and fidaxomicin are widely used in the clinical treatment of CDI [28]; these antibiotics have benefited hundreds of millions of people and promise to benefit many times more. Beinortas et al. [46] performed a systematic review and network meta-analysis to compare and rank the efficacy of different treatments for nonmultiple recurrent infections with *C. difficile* in adults (Figure 2), finding that fidaxomicin and teicoplanin were significantly better than vancomycin in their cure rate of sustained CDI; teicoplanin, ridinilazole, fidaxomicin, surotomycin, and vancomycin were better than metronidazole. Bacitracin was weaker than teicoplanin and fidaxomicin, and tolevamer was weaker than all drugs except for LFF571 and bacitracin. Overall, the frequency of fidaxomicin used in sustained CDI was the highest, followed by vancomycin and metronidazole. Fidaxomicin is an emerging antibiotic that exhibits effective and well-tolerated treatment for severe CDI and for patients with a high recurrence risk [47,48]. These data indicated that the status of metronidazole, vancomycin and fidaxomicin is still entrenched in CDI treatment over a short time.

3.1 Mechanism of first-line antibiotics

Metronidazole is a nitroimidazole antibiotic that can inhibit the synthesis of nucleic acids through interference with the activity of DNA molecules in bacterial cells [49]. Vancomycin, as a glycopeptide antibiotic, can bind the residues D-Ala-D-Ala of the UDP-MurNAc-pentapeptide, inhibiting the synthesis of the cell wall [50]. Fidaxomicin is a macrocyclic antibiotic that can bind to the DNA template-RNA polymerase complex and inhibit the initial separation of DNA strands, as well as prevent mRNA synthesis by interfering with the RNA polymerase

δ-subunit [51]. Maass et al. [49] used these antibiotics to study *C. difficile* 630Δ*erm* and found that specific proteomic responses (protein abundance and protein synthesis levels) of *C. difficile* corresponded to specific antibiotics. Vancomycin-induced signature proteins reflected various changes in cellular function in *C. difficile* cells and proteomic characteristics of metronidazole stress, including alterations in protein biosynthesis and degradation as well as in DNA replication, recombination, and repair; after fidaxomicin treatment, differences in protein expression were observed in mainly amino acid biosynthesis, transcription, cell motility, and cell envelope functions.

3.2 Disadvantages of antibiotics

Currently, antibiotics are the main therapy in the treatment of clinical CDI [52]. Several reports demonstrated that 75%–80% of primary CDI can be cured by antibiotics [53]. Ford et al. [54] used decision-analytic models to evaluate the therapeutic effects of metronidazole, vancomycin and fidaxomicin on initial episodes of mild-to-moderate CDI patients and found that the overall cure rates of patients reached 94.23%, 95.19%, and 96.53%, respectively. However, other reports indicated that the recurrence rate of CDI is relatively high (25%) after the initial treatment with antibiotics, and this rCDI is more difficult to treat [55]. Overall, the use of antibiotics leads to the emergence of resistant strains, body injury, dysbacteriosis and other complications.

Multidrug-resistant strains are a considerable threat to public health on a global scale, and most of them are difficult to address with conventional drugs, including multidrug-resistant *C. difficile* strains [56]. From 2007 to 2013, a report showed that the fluoroquinolone-resistant 027 (027FQR) strain accounted for 32% of all *C. difficile* isolates (3118) in southwestern Virginia, that the toxin levels of this resistant strain were significantly higher than those of the common 014/020 strain and that this strain induced more severe inflammation than other strains [57]. Similarly, Aptekorz et al. [6] found that a multidrug-resistant 027 strain (resistant to moxifloxacin, ciprofloxacin, imipenem and erythromycin) caused a higher CDI incidence than other strains in the Silesia region of Poland and that this type had a large proportion in all strains. Peng et al. [58] reported a new type, *C. difficile* LC693 (genotype was determined as ST201), that is associated with severe diarrhea in China; the genomes of this new type contained more than 40 antibiotic resistance genes, including 15 genes associated with vancomycin resistance. Increasing evidence has indicated that multidrug-resistant *C. difficile* strains are spreading worldwide.

Drug reactions are another side effect during antibiotic treatment. Metronidazole can cause central nervous system poisoning in some patients [59]. Vancomycin can cause a maculopapular rash, urticarial, red man syndrome, dermatitis bullosa, kidney failure, and severe colitis [5]. Fidaxomicin, as a rising star in the area of antibiotics, demonstrated excellent efficacy against CDI or rCDI [54]. Considering current known clinical data, it seems that fidaxomicin has no apparent side effects in the treatment of CDI, and it should be the best choice among all three antibiotics [47].

Destruction of the normal gut microbiota is a serious consequence induced by antibiotics. The richness and diversity of the gut microbiota decrease after taking antibiotics, especially for some beneficial microorganisms, such as *Lactobacillus rhamnosus* [60]. Their populations sharply drop, followed by the vacation of niches; subsequently, several resistant and opportunistic pathogens, such as *C. difficile*, invade and occupy these niches and flourish in the gut, causing further disorder in the microbiota, affecting the immune and metabolic functions of the body, and eventually causing diseases [61]. *C. difficile* strains are resistant to many antibiotics due to their spores, and these spores can germinate and develop into *C. difficile* strains again under suitable conditions. Destruction of the gut microbiota, especially the damage to beneficial bacteria induced by antibiotics, involves the following two aspects: one is the direct inhibitory or bactericidal effects of antibiotics themselves against gut bacteria, and the other is the production of some special substances derived from *C. difficile* that can be activated or enhanced by antibiotics. These special substances are conducive to the survival and expansion of *C. difficile*.

Antibiotics can destroy beneficial microorganisms in the gut, such as *Bacteroides thetaiotaomicron* and *Bifidobacterium breve* [62]. These two bacteria induce the expression of C-type lectin, followed by regeneration of islet-derived protein III γ (REGIII γ). REGIII γ targets gram-positive bacteria and inhibits their growth [63]. Similarly, gut bacteria such as *Clostridium scindens* and *Clostridium sordellii* secrete tryptophan-derived antibiotics, which inhibit the division and proliferation of *C. difficile* [64]. These results suggest that some gut bacteria that antagonize *C. difficile* can be killed easily in an antibiotic-induced environment; subsequently, the levels of their antibacterial secretions decrease. In addition, antibiotics can also affect *C. difficile* itself. Kang et al. [64] found that *C. difficile* ATCC 9689 secreted a large amount of proline-based cyclic dipeptides under antibiotic-induced conditions, which improved the colonization ability of *C. difficile* and inhibited the growth of other bacteria and

fungi. Passmore et al. [65] found that *C. difficile* 630Δ*erm* produced *p*-cresol by fermenting tyrosine and that this substance competitively inhibited other bacteria (mainly beneficial microorganisms). In addition, a murine model showed that *p*-cresol was conducive to intestinal colonization, which enhanced the survival rate and pathogenicity of *C. difficile*. Subsequently, this process was accompanied by a rapid change in the intestinal microflora and metabolites. Many additional studies have demonstrated that specific species in the gut microbiota are closely associated with the functions of enterocytes and the immune system; deficiency in these species can seriously weaken human defense function and grant *C. difficile* a chance to invade [66–69].

In terms of pathogenicity, some antibiotics induce enhancement of toxin production and virulence gene expression in *C. difficile*. Zarandi et al. [70] found that vancomycin combined with clindamycin significantly reduced the toxin production of *C. difficile* but, in contrast, toxin production surged when ceftazidime was added. Aldape et al. [71,72] used ciprofloxacin to treat *C. difficile* and found that the expression level of *tcdA/B* was increased, and a similar trend was also observed under teicoplanin treatment. Our previous findings suggested that different combinations of antibiotics (metronidazole, vancomycin, clindamycin, ceftazidime, and ampicillin) had different effects on *C. difficile* [73], such as when the combination of vancomycin and ampicillin stimulated toxin production (2897.47 ± 7.24 ng/mL), which reached a higher level than that in the control group (2628.74 ± 3.62 ng/mL). For gene expression, ceftazidime induced the upregulation of *tcdA* and *tcdB* expression up to 20 and 14 times, respectively. These results indicated that some antibiotics might worsen the situation of CDI.

Overall, antibiotics weaken the diversity of the gut microbiota and create favorable conditions to promote *C. difficile* growth. Subsequently, *C. difficile* produces many toxins, and these toxins induce intestinal inflammation. Eventually, the situation develops into CDI or rCDI.

4. Non-antibiotic therapy

Where can we find better therapies to assist in the efficacy of antibiotics, or even to replace antibiotics in the treatment of CDI? New therapies can effectively treat CDI without affecting normal physiological function. Recently, a variety of emerging non-antibiotic treatments have attracted wide attention. Specifically, FMT, probiotics, engineered microorganisms, bacteriophages, diet, natural active substances, nanoparticles, and compounds are examples of non-antibiotic therapies.

4.1 Fecal microbiota transplantation

Currently, FMT has a high cure rate in the treatment of various diseases, especially for CDI and rCDI [74]. A previous report indicated that the cure rate of rCDI with FMT reached 85%–90% [75]. The main aim of FMT in the treatment of rCDI is to relieve inflammation induced by *C. difficile*, restore normal gut microbiota and metabolites, and eradicate *C. difficile* and its spores [75,76]. First-line antibiotics cured most primary CDI patients, but they still do not work on some individuals, even causing more serious infectious complications. Several reports demonstrated that the therapeutic effect of FMT on rCDI adult patients was prominent, with the exception of a few infant cases [77]. FMT seems to cause some side effects (nausea, sore throat, and abdominal pain) in infant patients [78]. A series of clinical research findings from Hota et al. revealed that FMT exerted a truly outstanding effectiveness in the treatment of patients with rCDI (a 80–96% cure rate), which was superior to that of antibiotics (a 56%–60% cure rate for vancomycin) [79–82]. In addition, the delivery modes of FMT, such as enema, colonoscopy, nasoduodenal tube, and even capsules, might directly determine the real therapeutic effects clinically [74,83].

The main therapeutic mechanism of FMT involves the extraction of stool that is donated by healthy individuals, which is transferred into the gut of patients (CDI or rCDI), and then restores and promotes the gut microbiota toward the normal structure of healthy individuals, accompanied by a disappearance of inflammation [84,85]. Gut microbiota from healthy individuals can rapidly replenish deficient species in the patient's gut, simultaneously stimulate the production of essential metabolites and motivate immune indexes to return to normal (Figure 3) [86,87]. Specifically, there are two indexes that change dramatically before and after FMT treatment: the level of metabolites and the structure of the microbial community. Julie et al. [87] explored the change in metabolites and found almost no valerate-producing species detected in rCDI patients' stools, but this situation changed after FMT; under treatment, the increased valerate inhibited the growth and spores of *C. difficile*, but by contrast, the concentration of the precursor of valerate decreased. This result indicated that FMT rapidly replenished valerate-producing species in the gut of rCDI patients, enhanced the production of valerate and finally achieved homeostasis. Lee et al. [88] analyzed changes in the metabolites and microbial community of rCDI patients treated with FMT and found that the proportions of *Bacteroides*, *Ruminococcus*, and *Blautia* increased and those of *Enterococcus*, *Escherichia*, and *Klebsiella* decreased; in addition, the levels of short-

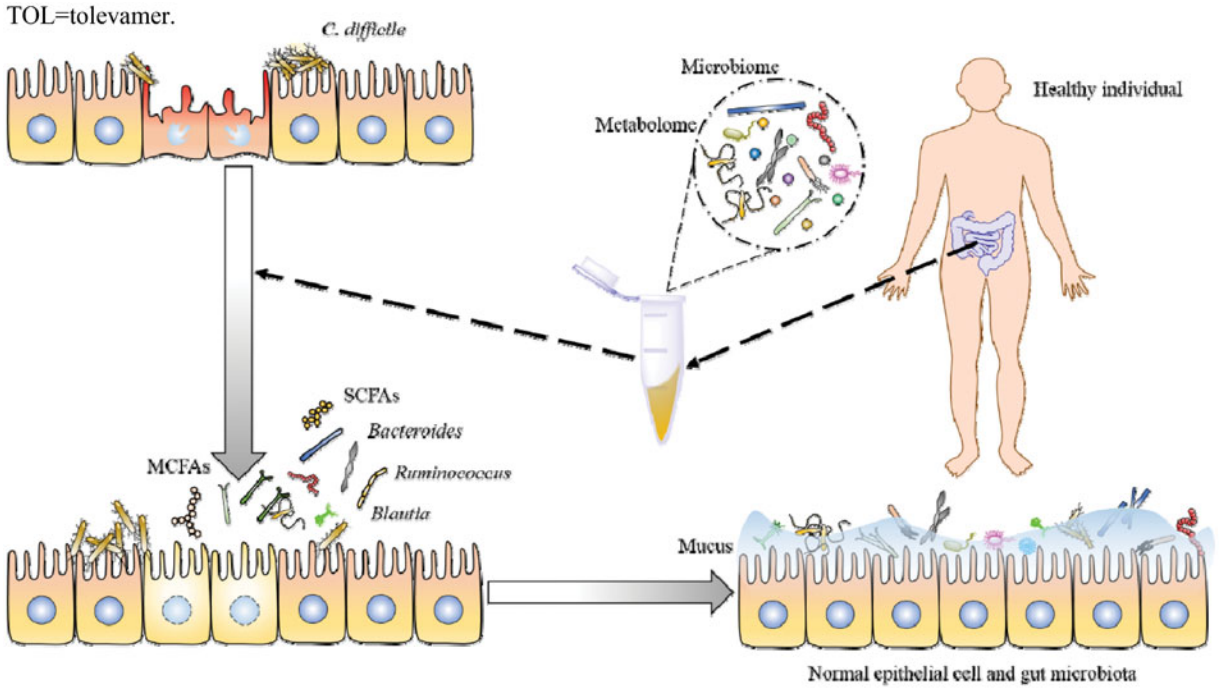

Figure 3. Fecal microbiota transplantation in the treatment of *Clostridioides difficile* infection. The extraction of stool is donated by healthy individuals and transferred into the gut of patients (CDI or rCDI). This restores and promotes the gut microbiota toward the normal structure of healthy individuals, including normal microbiome and metabolome, accompanied by a disappearance of inflammation. MCFAs: medium-chain fatty acids; SCFAs: short chain fatty acids.

chain fatty acids (SCFAs; butyric acid and acetic acid) were enhanced after FMT. Partial least squares regression analysis showed that there is a positive correlation between the production of butyric acid and the proportions of *Bacteroides*, *Ruminococcus*, and *Blautia*; conversely, a negative correlation was found between butyric acid and *Klebsiella* and *Enterococcus* proportions. These data demonstrated that the differences in gut metabolites and microbial communities between healthy individuals and rCDI patients can be eliminated by FMT [89]. After FMT treatment, the levels of metabolites and the structures of the microbial community in patients are closer to those in a healthy individual. In addition to the change in gut bacteria, a recent study reported that FMT also induces changes in gut fungi. Zuo et al. [90] found that the proportion of *Candida albicans* rose sharply during infection but this trend stopped after FMT. Simultaneously, the abundances of *Saccharomyces* and *Aspergillus* increased. Plenty of evidence suggests that FMT can help CDI or rCDI patients replenish and restore normal gut microbiota as well as their metabolites in a short time, eventually preventing recurrent *C. difficile* infection.

4.2 Probiotics

Probiotics are generally defined as a kind of living microorganism that reach the intestine in an active state when given in sufficient doses and thus exert positive health effects in humans, such as the modulation of the intestinal microflora and the activation of the immune system [91]. Lactic acid bacteria, *Bifidobacterium*, some bacillus and yeast are common representatives [92–94], especially the first two. *L. acidophilus*, *L. fermentum*, *L. plantarum*, *L. casei*, *L. paracasei*, *L. reuteri*, *L. rhamnosus*, *L. satsumensis*, *L. johnsonii*, *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Leuconostoc*, *B. adolescentis*, *B. animalis*, *B. bifidum*, *B. breve*, and *B. longum* are widely used in food and biomedical fields [95]. Most of them are generally recognized as safe and possess natural antibacterial activity [96,97]. Historically, they have flourished in the food field; however, increasing evidence suggests that they also play critical roles in the area of biomedicine, especially in the prevention and treatment of infectious diseases [98–101].

Clinically, probiotics are often used as an adjuvant, combined with antibiotics, to prevent, relieve and treat infectious diarrhea caused by CDI; however, they often exhibit beneficial and adverse effects in the treatment, and no substantive explanation has been provided for these effects. An investigation of multiple probiotics from Szajewska et al. [61] showed that *L. rhamnosus* GG used alone or combined with *B. breve*-12 and *L. acidophilus*-5 significantly reduced the morbidity of antibiotic-induced diarrhea in children; however, the

probiotic *Bacillus clausii* used alone or combined with *L. acidophilus* and *L. bulgaricus* had no effect on diarrhea. Zheng et al. used *B. longum* JDM 301 to treat a CDI mouse model and found that it exhibited excellent therapeutic effects, but this probiotic strain had no effect on the treatment of inflammatory bowel disease [102,103]. Similar gut inflammation reflected the different effects, meaning the efficacy of probiotics depends on the specific strain and target disease. Therefore, a vast majority of physicians are skeptical about the therapeutic effect of probiotics in the treatment of CDI. However, an authoritative survey report suggested that CDI patients treated with probiotics reached an infection rate of 1.6%, which was lower than that of the control group (5.5%); in addition, a combination of probiotics and antibiotics achieved a significant therapeutic effect with no side effects. Findings from this report support the fact that some special probiotics possess therapeutic effects in clinical CDI [7]. The main modes of probiotic use in CDI include single or combination probiotics, probiotics combined with prebiotics (synbiotics), and probiotics combined with antibiotics.

4.2.1. Single or a combination probiotics

These formulations are generally used for the prevention of clinical CDI. They can strengthen the human intrusion prevention system and protect against the invasion of *C. difficile*. Special strains, such as *Lactobacillus plantarum* 299v (LP299v), protected individuals efficiently from *C. difficile* infection [104]. CDI patients with antibiotics or immunosuppressive therapy received LP299v in the hospital, and the final data indicated that continuous supplementation with LP299v significantly prevented and reduced the infection rate of CDI, meaning LP299v is an effective probiotic strain for the prevention of CDI during hospitalization. Xu et al. [105] confirmed that oral *Pediococcus pentosaceus* LI05 enhanced the survival rates of CDI mice, relieved inflammation induced by *C. difficile*, reduced the levels of serum inflammatory and chemotactic factors, and decreased the damage to ZO-1 and claudin-1 induced by CDI. Further analysis found that LI05 enhanced the abundance of *Porphyromonadaceae* and *Rikenellaceae* and decreased the abundance of *Enterobacteriaceae*. These data suggest that LI05 reduces the inflammatory response and improves the diversity of the gut microbiota, subsequently preventing or curing CDI.

Some combinations of different probiotics exhibit enhanced therapeutic effects. A combination of *Bifidobacterium breve* Bb99, *Propionibacterium freudenreichii* subsp. *shermanii* JS, *Lactobacillus rhamnosus* Lc705, and *Lactobacillus rhamnosus* GG was given to infants who received antibiotic therapy, and this combination significantly promoted the alteration of the disordered gut microbiota toward homeostasis, as well as replenished the deficiency of some metabolic functions [106]. Golić et al. [107] found that *Lactobacillus helveticus* BGRA43 combined with *Lactobacillus fermentum* BGHI14 and *Streptococcus thermophilus* BGVLJ1-44 showed a strong suppression of the growth of *C. difficile* and *C. perfringens* and stimulated the activity of the immune system; further *in vivo* testing confirmed that this combination cured infected goats. *E. faecalis* NM815, *E. faecalis* NM915 and *E. faecium* NM1015 were isolated from infant feces and used in combination in the CDI mouse model; this combination inhibited *C. difficile* and protected the integrity of hepatocytes and enterocytes. In addition, other probiotics, such as *L. acidophilus* LA-5, *B. lactis* BB-12, probio 7, and symprove, *L. helveticus*, *L. rhamnosus*, *L. acidophilus* NCFM, *B. lactis*-04, *B. lactis*-07, and *L. paracasei*-37 used alone or in combination, exhibited significant antibacterial activity against *C. difficile* and showed excellent prevention, remission and treatment in CDI [108–111]. Several clinical reports have demonstrated that many combinations have outstanding effects on CDI in comparison to the effects of single strains, mainly dependent on a synergistic-action relationship among these strains. For example, *Bifidobacteria* fermented human milk oligosaccharides and produced trehalose, the latter of which promoted the growth of *L. rhamnosus* [112].

4.2.2 Probiotics combined with prebiotics

Prebiotics refer to indigestible polysaccharides or oligosaccharides that can be utilized by beneficial microorganisms and promote their growth and metabolism [113]. Probiotics combined with prebiotics are also called synbiotics. This synbiotic formulation has been adopted in the treatment of some diseases, such as neonatal septicemia, with a low cost [63]. However, little data are available about the clinical use of synbiotics in CDI. Some experimental reports demonstrated that synbiotics possess the ability to inhibit *C. difficile*. Xylitol combined with *L. plantarum* significantly inhibited the germination rate of *C. difficile* spores [114]. Inulin combined with special beneficial bacteria enhanced the production of SCFAs in the gut, with a decrease in *C. difficile* numbers, and reduced inflammation [10].

4.2.3 Probiotics combined with antibiotics

A clinical report from Goldenberg et al. [101] suggested that probiotics or probiotics combined with antibiotics reduced the morbidity of CDI; compared with a placebo

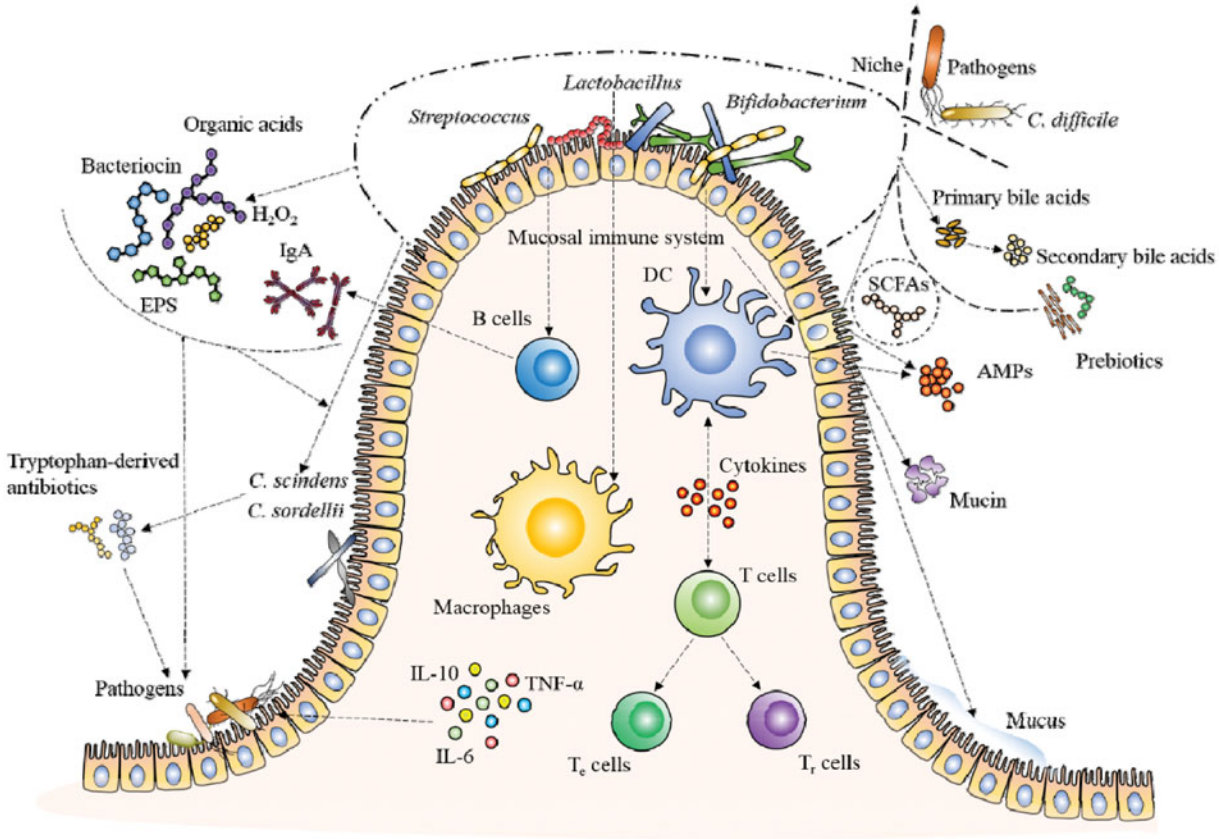

Figure 4. Mechanisms of probiotics in the treatment of *Clostridioides difficile* infection. Probiotics colonize the human gut, occupy niches, and secrete many antimicrobial substances (bacteriocin, organic acids) against pathogens (*C. difficile*). Simultaneously, these probiotics activate the body's immune defenses (mucosal immune system) to secrete mucin and antimicrobial substances and regulate the immune system to produce anti-inflammatory cytokines and growth factors to defeat the invasion of harmful bacteria and reduce susceptibility to infection. AMPs: antimicrobial peptides; DC: dendritic cell; EPS: exopolysaccharides; SCFAs: short chain fatty acids; Te cells: lymphocyte T effector cells; Tr cell: lymphocyte T regulatory cell.

and no treatment, this combination reduced side effects such as cramps and nausea. Furthermore, Shen et al. [7] found that probiotics were significantly more effective if given at approximately the same time as the first antibiotic dose; in addition, probiotics given within 2 days of antibiotic initiation were associated with an enhanced reduction of the risk for CDI. Taken together, these clinical data suggested that probiotics combined with antibiotics are an effective treatment for CDI, but the mechanism behind this phenomenon remains unclear. Our previous study indicated that *Bifidobacterium breve* YH68 combined with different antibiotics had different effects on *C. difficile* [73]. For some combinations, YH68 can enhance the antibacterial activity of some antibiotics against *C. difficile*, which was reflected mainly by the inhibition of growth, suppression of sporulation, and a significant drop in the production of A/B toxins; in addition, YH68 weakened the antagonism between some antibiotics. All these data from our study demonstrated that a combination of special probiotics and antibiotics might have great potential antibacterial activity against *C. difficile*.

4.2.4 Mechanism of probiotic therapy

The therapeutic effect of probiotics on intestinal infection is reflected mainly by the secretion of antimicrobial substances and the activation of the body's immune defenses (Figure 4) [115], including the following: (a) exhibit competitive exclusion of pathogens, occupy niches, colonize the gut, restore the unbalanced microbial community structure and eventually achieve homeostasis [116,117]; (b) utilize prebiotics (such as oligosaccharides) and produce organic acids (such as SCFAs and branched-chain fatty acids) to reduce the environmental acidity, as well as secrete many antimicrobial substances (such as bacteriocin, hydrogen peroxide, and exopolysaccharides) to inhibit or destroy pathogenic microorganisms [118,119]; (c) regulate the activities of enzymes related to bile salt metabolism, enhance the conversion rate of secondary bile acids and inhibit the division and proliferation of pathogenic microorganisms [120,121]; (d) interact with other intestinal microbes and enhance the effectiveness of antibacterial substances [63]; (e) activate the mucosal

immune system, secrete mucin and antimicrobial peptides, and increase the production of target immunoglobulins, which can prevent the invasion of harmful bacteria and reduce susceptibility to infection [117,122]; and (f) regulate the immune system (mainly dendritic cells, macrophages, and T cells) and stimulate the production of anti-inflammatory cytokines and growth factors (i.e. interleukin [IL]-6, IL-10, and tumor necrosis factor-α) [123,124]. Overall, the mechanism of probiotic therapy in the treatment of CDI may include multiple of the interactions described above.

4.3 Engineered bacteria

Artificial modification of natural microorganisms by biological techniques to generate specific biological functions results in a kind of engineered microorganism that is regarded as engineered bacteria. The utilization of engineered bacteria against special pathogens in clinical therapy is an emerging treatment. Chang et al. [125] used synthetic biology to modify the genomic system (quorum sensing, killing and degradation parts) of *Escherichia coli*, and these engineered bacteria produced pyocin targeted at pathogenic *Pseudomonas aeruginosa*. Subsequently, another upgrade was performed in *E. coli* Nissle 1917 (anti-biofilm part), and this engineered strain exhibited excellent prevention and treatment in an infected animal model [126].

There are two types of engineered bacteria against *C. difficile*. One is an engineered microorganism that can target *C. difficile*, and the other is an engineered *C. difficile* that loses the ability to produce toxin or virulence gene expression. Engineered *Lactobacillus paracasei* BL23 expresses TcdB-neutralization antibodies (VHH-B2 and VHH-G3), and both can neutralize TcdB [127]. Similarly, engineered *Lactococcus lactis* ATCC 11454 expresses nontoxic Tcd-AC and Tcd-BC recombinant fragments [9]. Both engineered strains were inoculated into a CDI mouse model, which was followed by relief of inflammation and decreased mortality. For *C. difficile* itself, bioengineering modification is also effective. Passmore et al. [65] created a gene knockout in *C. difficile* 630, depriving it of its *p*-cresol producing ability, and reduced its colonizing ability in the gut. Senoh et al. [128] extracted the *C. difficile* membrane fraction (ntCDMF) from *C. difficile* JND13-023 and injected it into a CDI mouse model as an antigenic vaccine. The results indicated that mice treated with ntCDMF achieved a low mortality, which was accompanied by a significant decrease in *C. difficile* numbers. These data suggest that engineered bacteria target special pathogens with high precision and are expected to be a new generation of alternatives to antibiotics. However, all these engineered bacteria that target *C. difficile* infection still require further clinical validation, and their safety in the human body needs comprehensive assessment, such as their effects on the immune system and the gut microbiota.

4.4 Diet

Diet has a considerable effect on the composition of the gut microbiota [129]. A growing number of studies have suggested that there is a close relationship among diet, the gut microbiota and immune responses [130–132]. Feces from CDI patients were transferred into germ-free mice to analyze changes in the gut microbiota, resulting in an increase in amino acids in the mouse gut that induced infection susceptibility [43]; further, a wild-type *C. difficile* that cannot utilize proline had difficulty infecting germ-free mice. These data revealed that low concentrations of proline or low protein food relieve inflammation in CDI mice, indicating that amino acids play a critical role in CDI and that dietary intervention could be an effective preventative method. Hryckowian et al. [10] found that microbiota-accessible carbohydrates (MACs) can be utilized by several gut bacteria. Furthermore, a mouse model demonstrated that dietary MACs inhibited the growth of *C. difficile* and directly influenced the change in microbiota and metabolites related to inflammation. In addition, mice treated with MACs had increased gut microbiota diversity as well as a high level of SCFAs. The authors believe that dietary MACs can alleviate the damage induced by CDI and that this dietary pattern should be recommended for people in Western countries whose diets lack fiber.

4.5 Phagotherapy

Phages are a kind of bacteria-specific virus that can combat pathogenic bacteria associated with infectious disease; thus, this therapy is called phagotherapy [133]. Currently, phagotherapy has been used in the treatment of some infectious diseases induced by multidrug-resistant strains [134]. Some clinical evidence has shown that phagotherapy contributes greatly to the treatment of sepsis, urinary tract infection, postoperative infection, pancreatitis, otitis, diarrhea, systemic infection, gastrointestinal infection, pulmonary infection, and pyrosis [11]. However, another study found that a high abundance of phages aggravated ulcerative colitis symptoms through the regulation of TLR9 and interferon-γ [135]. These results suggest that the safety

of phagotherapy in some diseases remains unclear and requires further assessment. For CDI, there is no high-credibility clinical data that strongly supports the remarkable effect of phagotherapy for the treatment of CDI. Nevertheless, data from several *in vitro* and *in vivo* experiments showed that phagotherapy exerted an outstanding therapeutic effect. Bacteriophage ΦCD27 was used in a human colon model that was infected by *C. difficile*; subsequently, a significant decrease of *C. difficile* cells and toxin production was observed during the treatment with phage [136]. Ramesh et al. found that CD140 phages improved the survival rates of CDI hamsters, revealing the potential usefulness of phages for CDI [137]. It seems that phagotherapy can inhibit *C. difficile* and have no effect on the normal gut microbiota, but this therapy in the treatment of CDI still lacks clinical evidence and needs further investigation [138,139].

4.6 Natural active substances

Natural active substances refer to some natural active molecules that are derived from a variety of natural products, such as plants or animals [140]. Several studies have shown that natural plants have the potential to protect against *C. difficile*. Roshan et al. [12] found that zingerone (0.3 mg/mL) protected the Vero and HT-29 cell lines from damage from *C. difficile* toxins. In addition, three kinds of manuka honey (4%, w/v), fresh onion extract (12.5%, v/v) and cinnamaldehyde (0.005%, v/v) reduced the toxin production and activity of *C. difficile in vitro*, although garlic powder (4.7 mg/mL) could only weaken the toxin activity. Lauric acid, one of the main components of coconut oil, has been used in the treatment of the CDI mouse model. Subsequently, the results suggested that this substance inhibited the growth of *C. difficile* and the production of proinflammatory cytokines, with this antibacterial activity depending on the reactive oxygen species derived from lauric acid [141]. In a study by Piotrowski et al., manuka honey also exhibited excellent antibacterial activity against four clinical isolates of *C. difficile* (RT017, RT023, RT027 and RT046) *in vitro*, which was mainly reflected by the inhibition of biofilm formation [142]. The aqueous solution of black seed (*Nigella sativa* seeds) and *Commiphora myrrha* (myrrh) inhibited the growth of *C. difficile in vitro*, and this antibacterial activity was not influenced by a change in pH (1.5–7.0) [143]. However, all these results indicated the effective antibacterial activities of these natural active substances *in vitro* or *in vivo*. There is no direct evidence that these natural active substances can work in humans. Therefore, the real effects of these natural active substances should be further tested in clinical studies. At present, these data suggest that some natural active substances have hypotoxicity and potential medicinal value in CDI and that they are likely to be new antibacterial agents in the future.

4.7 Nanoparticles and compounds

Nanoparticles (NPs) are particles with lengths that range from 1 to 100 nanometers and generally have two- or three-dimensional structures [144]. Most NPs are widely used in different fields, such as bioscience and medicine [145]. NPs possess the ability to suppress and kill a variety of pathogens [13,146]. Lee et al. [147] used synthetic $Fe_{3-\delta}O_4$ (octahedron iron oxide nanocrystals) NPs to target *C. difficile* and found that these NPs significantly inhibited the germination of spores. Studies using mouse models indicated that $Fe_{3-\delta}O_4$ reduced inflammation without affecting gut microbiota or cells, suggesting these NPs have a considerable effect on *C. difficile* and are safe at a certain concentration. Compounds such as niclosamide ethanolamine (NEN), which is the main component of niclosamide, inhibit the production of TcdA, TcdB and Cdt secreted by *C. difficile* and interfere with the toxin invasion of epithelial cells [14]. *In vivo*, compared with the effect of VAN, NEN (50 mg/kg) significantly reduced the primary and recurrent rate of CDI in a mouse model and increased the diversity of the gut microbiota, indicating that highly safe NEN has the potential to become a new drug in the treatment of primary and recurrent CDI.

5 Strengths and weaknesses of non-antibiotic therapies

The wide variety of non-antibiotic therapies have different modes of action and therapeutic effects, as summarize in Table 1. In the treatment of rCDI, FMT has an excellent effect within a short time; however, this therapy is mainly used in adults and not in infants or children due to the lack of definitive standards and safety assessments. Furthermore, FMT induced some side effects (stomachache, nausea) in a few individuals; in addition, the preparation and operational processes of FMT are complex and costly. Probiotic therapy has a large number of applications in the clinical treatment of CDI. Overall, mild or moderate CDI could be prevented or treated by probiotics without side effects. Probiotics and related products are not only accessible to everyone at a low cost but are also suitable for all ages;

Table 1. Advantages and disadvantages of different non-antibiotic therapies.

Non-antibiotic therapies	Advantages	Disadvantages	Main mechanism
Fecal microbiota transplantation (FMT)	High cure rate for rCDI, rapid curative effect, avoid recurrence	High cost, complicated operation, not suitable for infants, mild side effects	Restore the normal microbiome and metabolites, achieve homeostasis
Probiotics	Affordable, suitable for all ages, no side effects, easy operation, assist and enhance drug effects, dietary supplement	Sluggish curative effect, limited effect in rCDI	Multiple probiotic activities, antibacterial, activate immune system, assist the restoration of normal microbiome
Engineered microorganisms	Target *C. difficile* with high precision	Inadequate mechanism of action, high cost, complicated manufacturing process, lack of clinical validation	Direct genetic modification of microorganisms, act on targeted pathogens
Diet	Affordable and available, promote beneficial microorganism growth, alleviate symptoms	Inadequate mechanism of action, cannot cure CDI or rCDI alone	Some specific nutrients can be used by beneficial microbes and transformed into antibacterial substances
Bacteriophage	Against multiple resistant bacteria, remarkable curative effect	Inadequate mechanism of action, lack of safety assessment and clinical validation	Bacteria lysis
Natural active substances	Affordable and available, easy to obtain from large amount of natural plants	Inadequate mechanism of action, lack of clinical validation	Effective antibacterial constituents can inhibit *C. difficile*
Nanoparticles and compounds	Inhibit *C. difficile*, almost no damage to the body, therapeutic effects are remarkable *in vitro*	Lack of clinical validation	Directly on pathogen cells, induce cell damage, destroy pathogen without disrupt the normal microbiome

rCDI: recurrent *C. difficile* infection; CDI: *C. difficile* infection.

however, this therapy requires prolonged treatment for clinical CDI. For rCDI, probiotic treatment seems to have no significant effect. Currently, the main aim of probiotics in clinical CDI treatment is prevention. Engineered bacteria exhibit excellent therapeutic effects in the laboratory stage, and they can rapidly target specific pathogens without affecting other microorganisms. However, the manufacturing process of engineered bacteria is complex, costly, and still has a large gap between the laboratory stage and real mass production, as well as clinical use. Dietary supplementation can prevent or relieve symptoms induced by CDI, but its effectiveness varies with each person, and prolonged treatment is needed. Phagotherapy showed an outstanding therapeutic effect on some infectious diseases, but little clinical safety assessment data can support this therapy. In addition, phages have a dynamic interaction with the host and regulate human immunity; however, they also cause inflammation by increasing the permeability of epithelial cells [148]; therefore, it is necessary to have a comprehensive safety assessment and in-depth clinical investigation of this therapy prior to implementation in clinical practice. At the laboratory stage, natural active substances, such as manuka honey, showed excellent antibacterial activity against *C. difficile*; however, the specific active ingredients in these natural active substances remain to be determined. Similarly, NPs and compounds exerted outstanding therapeutic effects on CDI *in vivo* and *in vitro*, but they still lack comprehensive clinical assessment.

Overall, both FMT and probiotics in all these non-antibiotic therapies can achieve high expectations and meet the need for CDI treatment. Engineered bacteria and phage are largely in line with the goal of precision medicine in the future. Dietary supplementation can relieve symptoms induced by CDI. Natural active substances, nanoparticles and compounds have the potential to become new options in the treatment of CDI.

6. Conclusions

Currently, antibiotics are still the first-line treatment for CDI. However, the side effects of antibiotics are becoming increasingly prominent, and thus, it is necessary to find new drugs or treatment methods to address these issues. Probiotics and the combination of probiotics and antibiotics are the best choice for the treatment of mild and moderate CDI due to their outstanding clinical therapeutic effects, but the mode of use needs further optimization, with an investigation of strain specificity and individual variation. Personalized probiotics treatment is the trend for the future. For severe rCDI individuals, FMT is the most effective therapy. Future research on FMT should focus on standardization and personalization, especially in the treatment of infant and pediatric patients.

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ORCID

Jingpeng Yang http://orcid.org/0000-0002-0802-2891

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