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Probiotics: Insights and New Opportunities for *Clostridioides difficile* Intervention

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Abstract

Clostridioides difficile infection (CDI) is a life-threatening disease caused by the Gram-positive, opportunistic intestinal pathogen *C. difficile*. Despite the availability of antimicrobial drugs to treat CDI, such as vancomycin, metronidazole, and fidaxomicin, recurrence of infection remains a significant clinical challenge. The use of live commensal microorganisms, or probiotics, is one of the most investigated non-antibiotic therapeutic options to balance gastrointestinal (GI) microbiota and subsequently tackle dysbiosis. In this review, we will discuss major commensal probiotic strains that have the potential to prevent and/or treat CDI and its recurrence, reassess the efficacy of probiotics supplementation as a CDI intervention, delve into lessons learned from probiotic modulation of the immune system, explore avenues like genome-scale metabolic network reconstructions, genome sequencing, and multi-omics to identify novel strains and understand their functionality, and discuss the current regulatory framework, challenges, and future directions.

Keywords

Probiotics; *C. difficile* ; inhibition; virulence; immunomodulatory; genomic exploration; discovery informatics; metabolomics; multi-omics; regulatory requirements

Introduction:

Clostridioides difficile (*C. difficile*), also known as *Clostridium difficile*, is the most common infectious agent that leads to healthcare-associated diarrhea [1, 2]. Although first identified in 1935 [3], it was not until recently that the bacterium was implicated as the causative agent of pseudomembranous colitis with the severity and rate of *C. difficile*-associated disease (CDAD) increasing since the emergence of hypervirulent strains [4, 5]. As per recent estimates provided by the U.S. Centers for Disease Control and Prevention (CDC), 223,900 people were hospitalized with *C. difficile* infection (CDI) in 2017, which resulted in 12,800 fatalities in the U.S. alone [6] .

CDI has been primarily associated with the use of antibiotics to treat other bacterial infections and is highly prevalent in healthcare settings [7, 8] . Broad-spectrum antibiotics modulate the gut microflora, which facilitates *C. difficile* colonization in the large intestine [9–11]. Indeed, antibiotic treatment induces class-specific dysbiosis in the microbiota and a recent study noted remarkably low abundance of *Ruminococcus*, *Blautia*, *Porphyromonas*, *Bifidobacteria*, *Ezakiella* and *Ozoribacter* spp. along with a higher abundance of *Enterococcus* in the baseline samples collected from CDI patients [12]. Furthermore, antibiotics might promote *C. difficile* growth by altering bile acid metabolism. Specific bile acid signals are the prerequisite for the germination of *C. difficile* spores into vegetative cells [13]. Antibiotic use and abuse induce intestinal microbiome dysbiosis which results in population decline for gut microorganisms that possess 7-α dehydroxylase activity, which is involved in bile metabolism. This, in turn, brings about a reduction in secondary bile acids like deoxycholate. The decrease in deoxycholate levels is accompanied by an increase in taurocholate, a primary bile acid. Taurocholate stimulates the germination of *C. difficile* spores to metabolically active and toxin-producing cells [10, 14, 15].

Despite the pitfalls associated with antibiotics, they remain the treatment of choice for CDI. Current therapeutic options for mild-to-moderate CDI cases include the glycopeptide vancomycin or the macrolide fidaxomicin [16, 17]. However, there is no effective countermeasure for CDI recurrence wherein 1 out of 5 patients experience recrudescence of infection [18]. Treatment of subsequent recurrent infections can be challenging as the patient fails to mount an effective immune response against the toxins secreted by vegetative *C. difficile* cells [19, 20].

One biotherapeutic approach that has garnered considerable attention in recent years is fecal microbiota transplantation (FMT) [21, 22]. FMT is based on infusion of liquid filtrate feces from a healthy donor into the gut of a recipient to treat/control disorders where there has been a disruption in the community structure of the gut microflora [23, 24]. FMT is effective in resolving ~80 % of recurrent infections [25, 26]. However, FMT administration can result in the transmission of enteropathogenic *Escherichia coli* (EPEC) and Shiga toxin-producing *E. coli* (STEC). In fact, investigational FMT administration led to the development of invasive antibiotic-resistant *E. coli* infections in two immunocompromised individuals, with one individual dying [27]. These fatalities have prompted the need for intensive clinical work to comply with all related regulatory agencies. For example, the U.S. Food and Drug Administration (FDA) has mandated additional requirements for FMT that include the screening of donors for risk and carriage of multidrug-resistant organisms [27]. A recent population evidence-based study noted that washed microbiota preparation via microfiltration significantly reduced FMT-related adverse events as compared to conventional FMT [28]. However, there is a lack of standardization of FMT protocols and different methods of delivery, such as colonoscopy, nasogastric tube, retention enema, or orally delivered encapsulated stool, have different levels of efficacy and associated risks [29, 30]. Thus, numerous questions regarding FMT remain to be addressed highlighting the need for therapeutics that can provide sustained resolution of clinical symptoms.

The dwindling number of antibiotics effective against CDI and CDI recurrence has led to an increase in probiotic testing and research. As defined by the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO) in 2002, probiotics are “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” [31–33]. Currently, the commercialization of probiotics has led to the development of a multibillion-dollar industry, and probiotics now constitute one of the most consumed supplements [34–37].

Emerging insights from probiotic research interventions provide mechanistic aspects for their putative health benefits [38–42]. However, clinical trials that investigated the effect of supplementing probiotics to antibiotics therapy in treating CDI revealed mixed results; some trials validated the benefits of probiotics while other trials found negative results. In this perspective, we will delve into studies conducted on different probiotic strains to evaluate their ability to inhibit *C. difficile in vitro* and *in vivo*, highlight the efficacy of probiotics from a clinical context, and focus on the immunomodulatory properties of probiotics. We will also consider avenues like genome-scale metabolic network reconstructions that can identify novel strains of probiotics, discuss the role of genome sequencing in understanding the functionality of probiotics, highlight the importance of a multi-omics approach in

understanding the *C. difficile*-lumen-intestine interaction as a measure to evaluate probiotic efficacy, and explore the current regulatory framework and possible future directions.

Probiotic efficacy in a preclinical context:

Corthier *et al*., in 1985, conducted one of the first studies that revealed the positive effect of probiotics in mice infected with *C. difficile*. In this study, gnotobiotic mice that were inoculated with *Bifidobacterium bifidum* or *E. coli* exhibited reduced cecal cytotoxin levels and survived *C. difficile* challenge without exhibiting diarrhea [43]. Since this study was published, numerous studies have been conducted to investigate the ability of different probiotic strains to attenuate virulence or directly inhibit growth of *C. difficile*. The following section highlights the notable probiotic strains that have been proposed as plausible interventions based on findings conducted mostly using *in vitro* cell culture studies and a few *in vivo* models.

Lactobacilli:

The intestinal commensal organisms *Lacticaseibacillus paracasei* and *Lactiplantibacillus plantarum* demonstrated antagonistic activity against *C. difficile* strains *in vitro*. The antagonistic activity of the *Lactobacillus* sp. was strain-dependent and correlated with their ability to produce lactic acid and hydrogen peroxide (H_2O_2) indicating the probable role of lactic acid and H_2O_2 behind the antagonistic interaction (Fig. 1) [44]. A different study concluded that the cell-free supernatant (CFS) of *L. plantarum*, *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus casei*, and *Ligilactobacillus salivarius* exhibits a direct inhibitory effect on *C. difficile in vitro* in a pH-dependent manner hinting towards the production of organic acids[45].

Limosilactobacillus reuteri 17938 is one of the most studied probiotics in clinical trials for different applications including inhibiting growth of *C. difficile*. A study by Spinler *et al*., 2017 identified *L. reuteri* 17938 as a potential candidate for an adjunct probiotic therapy to be used alongside antibiotics to treat CDI [46]. *L. reuteri* converts glycerol into a potent broad-spectrum antimicrobial compound, reuterin, that inhibits the growth of both Gram-positive and Gram-negative bacteria. Using fecal mini-bioreactor arrays (MBRA), the investigators demonstrated a 5-$\log_{10}$ reduction in *C. difficile* count in glycerol-treated reactors containing *L. reuteri*. *Ex vivo* inhibition of *C. difficile* growth and germination was noted in the cecal contents procured from germ-free mice when treated with a combination of *L. reuteri* and glycerol. Moreover, supplementation of *L. reuteri* with glycerol did not show a drastic and statistically significant change in community structure in the MBRA samples [46]. A more recent study noted the ability of reuterin to inhibit the growth of metabolically active *C. difficile* by inducing reactive oxygen species (ROS) production resulting in a shift in carbon metabolism followed by reduced toxin synthesis . Reuterin could also inhibit *C. difficile* outgrowth from spores. Importantly, *L. reuteri* is resistant to the antibiotics used for CDI treatment, including vancomycin, fidaxomicin, and metronidazole; furthermore, the susceptibility of the vegetative pathogen to these antibiotics was enhanced by *L. reuteri*, which makes it a promising strain for adjunct therapy [46, 47].

Lactobacilli-derived molecules can also attenuate virulence (Fig. 1). Lactobacilli have been found to inhibit the expression of virulence factors of *C. difficile* both *in vitro* and *in vivo*. Banerjee *et al.*, in 2009, investigated the efficacy of six commercially available and conventional lactobacilli strains in mediating *C. difficile*-induced toxicity on a human enterocyte-like Caco-2 (human intestinal epithelial cell line) cell culture model. Indeed, CFS derived from *Lactobacillus delbrueckii* ssp. protected Caco-2 cells from the cytotoxic effects of *C. difficile* toxins [48]. When Caco-2 cells were exposed to the CFS from a co-culture of *L. delbrueckii* and *C. difficile*, similar results were generated, which hinted at the presence of one or more antitoxic components in *L. delbrueckii*-conditioned medium that possibly exerted a proteolytic effect on the *C. difficile* toxins. Furthermore, CFS from *L. delbrueckii* inhibited adhesion of *C. difficile* to the Caco-2 monolayer [49]. However, the mechanistic aspects that could decrease the adherence of *C. difficile* to Caco-2 cells and detoxification remain to be elucidated.

Another study investigated the ability of *Lactobacillus acidophilus* in modulating *C. difficile* virulence [50]. At a low pH, the probiotic inhibited growth of the pathogen, which was attributed to the production of lactic acid (Fig. 1). The cell extract of *L. acidophilus* GP1B had an inhibitory effect on the production of the quorum sensing regulator autoinducer-2 (AI-2) in *C. difficile* and resulted in downregulation of gene expression associated with the production of AI-2 and toxins A and B (*tcd*A, *tcd*B, and sigma factor *txe*R). *L. acidophilus* GP1B was further found to confer protection to *C. difficile*-infected mice, which was evident from the structural integrity of the intestinal epithelial cells in *L. acidophilus*-treated mice. [50]. Another recent study revealed the efficacy of CFS from *L. acidophilus* La-5 in reducing attachment of *C. difficile* to human epithelial cells and conferring protection to two human epithelial cell lines, HT-29 and Caco-2, from the cytopathic and cytotoxic effect of *C. difficile* CFS [51].

The surface layer proteins (S-layer) of *Lactobacillus kefir*, a member of the microbiota of kefir grains, antagonized the effect of both *C. difficile*-spent culture supernatant (SCS) and purified toxins on the African green monkey kidney epithelial (Vero) cells in an *in vitro* study. The inhibitory effect was higher for the S-layer proteins belonging to the aggregating *L. kefir* strains (strains harboring auto aggregation capacity) as compared to the non-aggregating strains, while whole cells of *L. kefir* failed to confer any protection [52, 53]. In a different study, *L. lactis* Centro de Investigación y Desarrollo en Criotecnología de Alimentos (CIDCA) 8221 and a mixture of kefir-isolated bacterial and yeast strains (*L. kefir, L. plantarum, L. lactis ssp. lactis, Saccharomyces cerevisiae, and Kluveromyces marxianus*) antagonized the cytotoxic effect of *C. difficile* toxins on Vero cells. The anti-toxin activity was attributed to the heat sensitive, protease-resistant components (greater than 10 kDa) present in the *L. lactis* CIDCA 8221 supernatant, which provided new insights into the anti-virulence activity of the kefir microbiome [54]. Additionally, the mixture of the kefir-isolated microorganisms conferred protection *in vivo* against CDI in a hamster model where hamsters treated with kefir-isolated microorganisms had a 100% survival rate as compared to the placebo group which had a survival rate of 28.5% [55].

Composed of three bacterial strains (*L. acidophilus* CL 1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2), Bio-K+ is a commercial probiotic used as a supplement to prevent CDI.

The probiotic formulation inhibited toxin A/B-producing strains of *C. difficile*. Furthermore, CFS from the three lactobacilli strains harbored anti-cytotoxic activity that protected Caco-2 cells from the cytotoxic effects of CFS obtained from *C. difficile* [56].

Saccharomyces boulardii: *S. boulardii*, a non-pathogenic yeast, confers protection against *C. difficile* via indirect inhibitory mechanisms like attenuation of *C. difficile* virulence factors and modulation of the host immune response (Fig. 1). *S. boulardii* inhibited *C. difficile* toxin A-mediated receptor binding and the associated enterotoxic effects like intestinal secretion and mannitol permeability in a rat ileal loop model [57]. The significant reduction of the histologic damages caused by toxin A was attributed to a *S. boulardii* serine protease that hydrolyzed the toxin and thus prevented it from binding to glycoproteins present on the brush borders of the host epithelium [58]. *S. boulardii*-conditioned media also inhibited interleukin-8 (IL-8) production, induced by *C. difficile* toxin A in human mucosal epithelial colonocyte cells, in a dose-dependent manner. *S. boulardii* modulated the host signaling pathways pivotal to the intestinal inflammatory response and involved inhibition of ERK1/2 activation. *S. boulardii* supernatant also normalized toxin A-mediated fluid secretion and prevented the associated histopathologic changes in toxin A-treated ileal mucosa of mice [59]. Moreover, *S. boulardii* protease possessed enzymatic activity against *C. difficile* TcdB and inhibited the cytotoxic effects of both TcdA and TcdB on native human colonic mucosa and on colonic epithelial cells *in vitro* [60].

***Bifidobacterium* spp.:** Bifidobacteria have been evaluated for their ability to reduce the cytotoxic effect of clostridial toxins (Fig. 1). *Bifidobacterium longum* and *Bifidobacterium breve* reduced the toxic effect of *C. difficile* supernatant when exposed to a human intestinal epithelial cell line (HT-29) [61]. Of the strains that were investigated, *B. longum* IPLA20022 demonstrated the strongest ability to counteract the cytotoxic effect of clostridial toxins. The neutralized cell free supernatant (NCFS) obtained from the *B. longum* IPLA20022 strain permitted the least damage to the HT-29 cells, even after 22 hours of incubation with *C. difficile* supernatant; furthermore, the NCFS significantly reduced TcdA levels (12% remnant TcdA). Additionally, HT-29 cells incubated with the NCFS from *B. longum* IPLA20022 displayed an intact F-actin cytoskeleton and retained the integrity of the epithelial monolayer, which could be correlated to the reduced amount of *C. difficile* toxins [61]. *B. bifidum* CIDCA 5310 and *L. plantarum* CIDCA 83114 also antagonized the biological activity of *C. difficile* in a separate study [62]. Detection of *C. difficile* toxin concentration in spent co-cultures of the pathogen and the probiotics revealed that *B. bifidum* CIDCA 5310 significantly reduced the concentration of TcdA in the supernatant. The integrity of the F-actin network in Vero cells was maintained when the pathogen was co-cultured with the *B. bifidum* strain. Additionally, co-culturing with both *B. bifidum* CIDCA 5310 and *L. plantarum* CIDCA 83114 successfully inhibited cell rounding and detachment that occurred in the presence of the *C. difficile* supernatant [62]. Another study by Wei *et al.,* 2018 demonstrated the efficacy of a commercial *B. longum* JDM301 strain in attenuating CDI *in vivo*. Mice infected with *C. difficile* and treated with *B. longum* had a survival rate of 75% compared to 43% for untreated mice [63].

Bifidobacterium-mediated direct inhibition of *C. difficile* growth was observed for *Bifidobacterium animalis* ssp. *lactis* [45]. Another study assessed the antagonistic activity of SCS from *Bifidobacterium* strains isolated from newborn babies [64]. All the *Bifidobacterium* strains used in this study showed strain-dependent growth inhibition of *C. difficile*. The supernatant from *Bifidobacterium* CIDCA 5323 exhibited the most potent inhibitory effect with a 4-$\log_{10}$ decrease in viable *C. difficile* colony forming units (CFU) observed within 1-hour post-incubation. Additionally, some of the probiotic strains significantly reduced the adherence of *C. difficile* to colonic epithelial cells (Caco-2) [64].

Pediococcus pentosaceus: *P. pentosaceus,* a potential probiotic belonging to the family Lactobacillaceae, is a human gut-borne bacterium that is known to possess antimicrobial activity against enteropathogens [65]. The efficacy of *P. pentosaceus* LI05 in a mouse model of CDI was recently assessed [66]. Interestingly, a 100% survival rate and reduced histopathological symptoms were observed for mice who were challenged with *C. difficile* VPI10463 and were orally administered *P. pentosaceus* LI05. The *P. pentosaceus* LI05-treated mice showed substantial alleviation of inflammatory mediators in the colon, inflammatory cytokines and chemokines in the serum, and CDI-induced disruption of zonula occludens-1 (ZO-1), occludin, and claudin-1, which led to reduced histopathological symptoms (Fig. 1). Additionally, treatment with *P. pentosaceus* LI05 resulted in an increase in the abundance of beneficial microbiota like *Lactobacillus*, *Prevotella*, and *Paraprevotella* and curbed the proliferation of opportunistic pathogens like *Escherichia* and *Flavonifractor* (Fig. 1) [66].

Streptococcus thermophilus: *S. thermophilus*, a thermophilic lactic acid bacterium, is traditionally used in the food industry and has been clinically established as a probiotic formulation that intervenes with the progression of chronic kidney disease [67, 68]. *S. thermophilus* filtrates have been found to exhibit a dose-dependent growth inhibitory effect on *C. difficile* along with a significant decrease in *tcd*A expression (Fig. 1). In addition, *C. difficile*-infected mice treated with *S. thermophilus* exhibited significantly less diarrhea and attenuated pathological features in the colon and cecum when compared to the controls [69]. The high levels of lactate (used as a marker for lactic acid which exhibits a bactericidal effect on *C. difficile*) observed in the lumen of mice treated with *S. thermophilus* was attributed to the better experimental outcome observed in terms of significantly less weight loss (46%), diarrhea, toxin levels, and cecal pathology [69].

Non-pathogenic *Clostridium* spp.:

The endogenous intestinal microflora of humans is dominated by members of the Firmicutes and Bacteroidetes phyla. Of the inferred microorganisms in the Firmicutes phylum, most belong to known butyrate-producing bacteria and are members of the Clostridia class [70]. *Clostridium butyricum* is one such non-pathogenic butyric acid-producing *Clostridium* spp. found in the intestine of humans that has been used clinically to prevent human gastrointestinal diseases like antibiotic-associated diarrhea [71]. *C. butyricum* MIYAIRI 588 (CBM588), when co-cultured with *C. difficile*, resulted in a complete loss of *C. difficile* toxicity. Spores of CBM588 and *C. difficile,* when co-inoculated, exhibited a greater inhibitory effect which suggests that *C. butyricum* spores can probably inhibit *C. difficile*

spore germination in the gut [72]. Moreover, CBM588 prevented CDI in gnotobiotic mice (80% survival in mice pre-infected with CBM588 vs. 14.2% survival in untreated) [73]; furthermore, administration of CBM588 reduced the incidence of watery diarrhea and the fecal-free water content in rats infected with *C. difficile* [74].

Colonization resistance using non-toxigenic strains of *C. difficile* successfully prevented colonization of toxigenic *C. difficile* in a hamster model of CDI (Fig. 1) [75]. The hamsters were colonized with non-toxigenic *C. difficile* strains M3, M23, and T7 which conferred protection ranging between 87-97% against the toxigenic strains.

Clostridium scindens is a bile acid 7α-dehydroxylating intestinal bacterium that possesses potent anticlostridial activity. *C. scindens* expresses enzymes that can convert the primary bile acids cholate and chenodeoxycholate to two secondary bile acids, deoxycholate and lithocholate. Albeit the fact that all cholate conjugates act as germinants for *C. difficile* spores, the secondary bile acid deoxycholate inhibited the growth of vegetative *C. difficile* cells [13]. *C. scindens* strains, because of their ability to efficiently convert cholate to deoxycholate, inhibited *C. difficile* growth and pathogenesis *in vitro*, *ex vivo*, in gnotobiotic mice, and in a *C. difficile* murine model (Fig. 1) [76–78]. Additionally, *C. scindens* and *Clostridium sordelli* secrete the tryptophan-derived antibiotics turbomycin A and B that mediate growth inhibition of *C. difficile* by interfering with the process of cell division. The activity of turbomycin A and B was augmented in the presence of deoxycholic acid and lithocholic acid, which indicates a second mechanism by which *C. scindens* prevents CDI [79].

***Enterococcus* spp.:** A component of the human and the animal gut microflora, *Enterococcus* spp., particularly *Enterococcus faecium* and *Enterococcus faecalis*, have been investigated for their ability to inhibit *C. difficile* growth [80, 81]. A screening assay identified the CFS of three isolates from infant fecal samples, *E. faecalis* NM815, *E. faecalis* NM915, and *E. faecium* NM1015, that exhibited more than 50% inhibition of *C. difficile* growth *in vitro*. The inhibitory action was presumed to be due to antibacterial substances and organic acids secreted by the enterococcal strains. A co-culture of the three probiotic enterococcal strains with *C. difficile* resulted in a 5-$\log_{10}$ reduction in the *C. difficile* CFU count. Administration of a mixture of the three enterococcal strains to mice protected the epithelial cells of mice from dense inflammation and mucin depletion when compared to the untreated group [81].

***Bacillus* spp.:** *Bacillus* spores may be a potential probiotic option to prevent CDI. Oral delivery of *Bacillus subtilis* PXN21 spores, a component of a commercial probiotic product, improved the survival rate for mice infected with *C. difficile* in a study conducted by Chen *et al.*, 2008 [82]. Mice treated with *B. subtilis* spores, prior to being infected with *C. difficile*, exhibited a survival rate of 41.6%, whereas treatment of mice post-infection with *B. subtilis* spores resulted in a 66.6% survival rate. Furthermore, damage to cell structure and tissue integrity was reduced in mice treated with *B. subtilis* spores compared to the untreated group. Additionally, the live PXN21 spores upregulated the expression of the Toll-like receptor 2 (*TLR2*) gene, which led to the induction of two proinflammatory cytokines, IL-6 and TNF-α, thereby stimulating the innate immune response of the host [83].

Bacillus clausii, has also been investigated for both direct inhibition of *C. difficile* and mitigation of its virulence factors. *B. clausii* CFS previously was shown to exhibit an inhibitory effect on growth of Gram-positive pathogens, including *C. difficile* [84]. Further studies revealed the presence of serine protease (M protease) in the supernatant of *B. clausii* that prevented damage induced by *C. difficile* toxins on Vero and Caco-2 cells. [85].

A spore-forming novel probiotic species, *Bacillus coagulans*, has recently gained attention in probiotic-associated industries, including food and pharmaceuticals, due to the bacterium's stability and resistance to stress. *B. coagulans* attenuated chemokine release and reduced neutrophil influx and COX-2 expression in *C. difficile*-induced colitis in mice [86, 87]. The strain *B. coagulans* BC30 improved *C. difficile*-induced colitis in mice, with 66.7% of treated mice demonstrating normal stools on day 12 compared to 13.0% in the control group [86]. *B. coagulans* GBI-30, 6086 (GanedenBC^{30}), also enhanced indices of *C. difficile*-induced colitis in mice and prevented recurrence after vancomycin withdrawal [86, 87].

Lachnospiraceae D4:

A component of the murine gut microflora and a member of the Lachnospiraceae family, Lachnospiraceae D4 was found to confer protection to mice challenged with *C. difficile*. Mice that were treated with an antibiotic cocktail of kanamycin, gentamicin, colistin, metronidazole, and vancomycin in water for three days followed by a single intra-peritoneal dose of clindamycin and subsequent inoculation with *C. difficile* either developed lethal CDI or exhibited mild forms of the infection. The microbial flora of mice that exhibited mild symptoms were found to be colonized with Lachnospiraceae [88]. To further verify this, Lachnospiraceae and *E. coli* were isolated from the ceca of mice, and their ability to mediate colonization resistance against *C. difficile* was evaluated. Interestingly, 80% of mice pre-colonized with Lachnospiraceae, but not *E. coli*, had lower levels of *C. difficile* colonization, less severe forms of CDI, exhibited minimal weight loss, and were clinically healthy two days post-infection [89]. In contrast, mice pre-colonized with murine *E. coli* strains and then subsequently infected with *C. difficile* succumbed to the infection. Efficient nutrient utilization by Lachnospiraceae was proposed as a plausible reason for the lower levels of *C. difficile* colonization observed (Fig. 1). Alternatively, the production of metabolites or a different mechanism altogether may have provided colonization resistance to *C. difficile* [89]. Although there are limited studies regarding the role of human Lachnospiraceae isolates in preventing *C. difficile* colonization, it is known that a depletion of Lachnospiraceae, along with Ruminococcaceae and other butyrate-producing bacteria, is common for individuals with CDI and non-infectious diarrhea [90]. Hence, members of the human Lachnospiraceae family need to be investigated as this might provide a lead for a clinically efficacious probiotic to limit CDI.

The role of Synbiotics in preventing *C. difficile* infection:

While a prebiotic can be defined as a selectable, fermented, non-digestible food ingredient that triggers beneficial changes in the composition of the gut microflora, a synbiotic can be defined as a "synergistic combination of prebiotics and probiotics" [33, 91, 92]. Synbiotics have been the subject of extensive research because of their beneficial effect on the host

by improving the survival of live commensal microorganisms administered as supplements [93].

Synbiotics, such as the prebiotic xylitol combined with the probiotic *L. plantarum* Inducia, have been found to inhibit the germination of *C. difficile* spores *in vitro* and to protect hamsters from CDI *in vivo* [94]. In another study, a synbiotic formulation consisting of the probiotics *L. plantarum* F44, *L. paracasei* F8, *B. breve* 46, *B. lactis* 8:8, and the prebiotics galacto-oligosaccharides, isomalto-oligosaccharides, and resistant starch conferred protection against *C. difficile* in a mouse model of CDI with significant toxin inhibition observed [95]. Additionally, *B. longum* and *B. breve*, in the presence of short chain fructo-oligosaccharides, inhibited the growth of toxigenic *C. difficile* [96]. Furthermore, *Faecalibacterium prausnitzii* along with potato starch protected against *C. difficile* colonization in an antibiotic-treated mouse model, likely through immunomodulation [97].

Breast milk supplies human milk oligosaccharides (HMOs), a family of structurally diverse glycans that support infant gut colonization and are active in innate immunity. HMOs are prebiotics, and they promote the growth of certain bifidobacteria, including *Bifidobacterium infantis* and *B. bifidum* [98]. Furthermore, the human breast milk microbiome is rich in *Staphylococcus*, *Streptococcus*, and *Propionibacterium*; beneficial lactobacilli and bifidobacterial species have also been identified [99]. Accordingly, human milk is a synbiotic. In fact, in neonates, the formation of the gut microbiota is supported by the mode of feeding, where breast-fed infants have a higher abundance of bifidobacteria than formula-fed infants. Additionally, formula-fed infants have higher levels of *C. difficile* [100–102].

Clinical efficacy:

Assessment of probiotics in clinical trials for preventing CDI:

Several meta-analyses have been conducted with the aim of deciphering the collective outcome of the use of probiotics in preventing CDI and its associated morbidity. A 2013 meta-analysis of 4,492 cases conducted by Goldenberg *et al.*, concluded that probiotics were effective in preventing CDAD with moderate certainty evidence (RR 0.36; 95% CI: 0.26, 0.51) [103]. A follow-up analysis of 8,672 cases concluded with moderate certainty that probiotics were effective in preventing CDAD. However, a post hoc subgroup analyses revealed that probiotics had no significant effect on preventing CDAD in trials that had a low-to-moderate baseline risk [104]. A recent meta-regression analysis demonstrated that timely administration of probiotics can lower the risk of CDI by >50% in hospitalized patients that were administered antibiotics [105]. Another meta-analysis of six randomized control trials concluded that only *S. boulardii* is effective in preventing CDI recurrence when administered in combination with a standard antibiotic [106]. A later meta-analyses related to CDAD prevention found that *S. boulardii* significantly reduced the risk of CDAD only in children [107], with a low quality of evidence [108]. Another recent study noted that a 3-strain probiotic preparation of *L. acidophilus* CL1285, *L. casei* LBC8OR, and *L. rhamnosus* CLR2, commercially known as Bio-K+ 50 Billion (50 billion CFU per capsule), can lower the incidence of healthcare associated-CDI (HA-CDI) for at least half of the patients on

multiple-antibiotic regimens. Similarly, patients on ≥1 high-risk antibiotic regimen had a lower incidence of infection when on Bio-K+ during the intervention [0.9%, odds ratio (OR), 0.49] [109]. Contrary to this is the finding from another study which do not support the use of the Bio-K+ probiotic formulation for prevention of primary CDI in hospitalized adults >50 years receiving antibiotics in a setting which has relatively low baseline CDI rates [110] The fact that the majority of these meta-analyses tested a variety of probiotic strains and the variable baseline risks of CDAD among cohorts may explain the difference in the outcomes between these studies.

A few companies have initiated clinical trials for microbiome-based therapeutics. VE303 is the most advanced drug candidate, composed of an eight cryopreserved bacterial strain mixture, which successfully met its primary efficacy endpoint of preventing CDI recurrence in a Phase II clinical trial [111]. Two newer candidates added to the field of clinical probiotics are the investigational oral microbiome therapeutics CP101 and SER109. CP101 restored colonization resistance in a randomized, multicenter, placebo-controlled trial and has been granted fast track designation and breakthrough therapy designation by the FDA for preventing recurrent CDI [112]. SER109, on the other hand, constitutes the first targeted microbiome drug candidate that successfully met its Phase III primary endpoint that resulted in a statistically significant decrease in the proportion of patients experiencing recurrence as compared to the placebo (Clinical Trials.gov identifier: NCT03183128). There are two additional microbiota-based therapies for recurrent CDI, RBX2660 and RBX7455. RBX2660 is a microbiota suspension delivered via enema that is currently in Phase III clinical trials (Clinical Trials.gov identifier: NCT03931941). RBX7455 is a room temperature stable, orally administered probiotic that was recently found to be safe and effective at preventing recurrent CDI in a Phase I clinical trial (Clinical Trials.gov identifier: NCT02981316).

Probiotics for preventing CDI recurrence in the pediatric population:

The clinical outcomes of CDI in children are not sufficiently explored. A common clinical assumption is that symptomatic CDI does not occur in young infants due to the hypothesis that they lack *C. difficile* toxin receptors. As a result, testing for *C. difficile* is not typically performed in infants up to 2 years of age who present with diarrhea. However, there have been multiple cases of young infants presenting with recurrent diarrhea who tested positive for *C. difficile* [157]. Additionally, several studies have shown a rise in CDI in pediatric populations, which warrants further investigation to understand the clinical significance and optimal treatment for CDI in children. Case reports have found that probiotics have been successfully used to prevent CDAD and treat recurrent CDI in children [113, 114]. One study showed effective symptom management and reduced CDI recurrence when the probiotic *S. boulardii* was administered to a child with surgically treated Hirschsprung disease [115]. Further research is necessary to compare the efficacy of various probiotic agents in the treatment and prevention of CDI and its recurrence.

Advancing the understanding of probiotic mechanisms in the context of treating CDI:

Probiotic modulation of the immune system:

An improvement in the application of probiotics as a medical intervention requires us to develop a comprehensive understanding of the probiotic molecules and the response they can elicit in the host at the molecular and physiological levels. Primary studies have identified different probiotic strains that functionally modulate the host cells' response via strengthening the epithelial barrier, protecting against cytokine-chemical stress, and enteric pathogen induced disruption of the epithelial barrier (Fig. 1) [116–118].

L. plantarum strains produce plantaricin, which is a bacteriocin that modulates the level of the anti-inflammatory cytokine IL-10 [119, 120]. The secreted protein p40 of *L. rhamnosus* GG (LGG) activates the epidermal growth factor receptor (EGFR), which could reduce cytokine-induced epithelial cell apoptosis and thus protect against experimental colitis [121]. LGG can also adhere to macrophages; this interaction has been found to induce IL-10 mRNA and reduce IL-6 mRNA in murine macrophages, thereby promoting an anti-inflammatory effect [122]. LGG, in an *ex vivo* and *in vivo* trial with ulcerative colitis (UC) patients, demonstrated anti-inflammatory effects via a significant reduction of pro-inflammatory cytokines like TNF-α and IL-17 [123] The protease lactocepin secreted by *L. paracasei* degrades an array of pro-inflammatory chemokines and protected mice against colitis [124]. *L. salivarius* Ls33 has been found to harbor anti-inflammatory properties; it can confer protection to mice from chemically induced colitis via a nucleotide-binding oligomerization domain- containing protein 2 (NOD2)-IL-10-dependent response [125]. Interestingly, candidate probiotics (*L. acidophilus*, *L. casei*, *L. reuteri*, *B. bifidum*, and *S. thermophilus*) or a mixture of them can specifically upregulate CD4+Foxp3+ regulatory T cells (Tregs), thus harboring potent anti-inflammatory effects [126]. Similarly, *L.casei* DN-114 001 can alleviate disease severity in dinitrobenzene sulfonic acid (DNBS)-induced colitis through induction and expansion of colonic CD4+Foxp3+ Treg cells and reduce cytokine production and T cell apoptosis *in vitro* in intestinal tissue specimens from Crohn's disease patients [127–129]. Additionally, both *F. prausnitzii* and *Streptococcus salivarius* JIM8772 has been found to elicit strong anti-inflammatory responses in murine colitis models [42, 130–132].

In addition to its anti-toxin and anti-spore activity against *C. difficile,* CBM588 harbors immunomodulatory properties like inducing IL-17A-producing $\gamma\delta$ T cells and IL-17A-producing CD4 cells, which can enhance gut epithelial barrier function. CBM588 modulates the composition of the gut microbiome with an increased abundance of *Bifidobacterium*, *Lactobacillus*, and *Lactococcus* spp. CBM588 also upregulates anti-inflammatory lipid metabolites (palmitoleic acid, 15d prostaglandin J2, and protectin D1), enhances TGFβ1 expression, and reduces inflammatory cytokines [133].

Probiotics with anti-inflammatory properties might harbor therapeutic potential and should be investigated for their role in ameliorating diseases associated with inflammation, like CDI. The choice of which probiotic strains to use has mostly been arbitrary. It should

be noted that probiotics can have pro-inflammatory or anti-inflammatory properties. In addition, the therapeutic effect of probiotics also depends on its ability to provide colonization resistance against the pathogen, produce organic acids and antimicrobial substances, restoration of microbial community structure, and regulation of enzyme activity related to bile salt metabolism (Fig 1.) [134] . Hence, the choice of strains to use in clinical trials plays a crucial role in coming up with a probiotic that may be clinically efficacious.

It is also challenging to predict the effect of probiotics in humans based on *in vitro* and *in vivo* immunomodulation measurement designs. To determine the potential clinical impact of the probiotic strains, human mucosal responses should be investigated. Investigating the effect of probiotic strains in human volunteers at the transcriptome and proteomic levels can advance the knowledge base required to decipher a strain that might be clinically relevant [135, 136].

Genomic exploration of probiotic mechanisms:

In recent years, genome sequencing has been used to shed light on the presence of numerous genetic markers attributed to genuine probiotic features, such as bile and acid tolerance, epithelial cell adhesion, and the production of bacteriocins [137–139]. Defining core and pan-genomes in probiotic strains will undoubtedly provide a new baseline to examine the evolution and functionality of probiotics. Sun *et al.*, in 2015, completed a comparative analysis of bifidobacterial genomes (45 strains) and identified 402 core-genes and > 20,000 pan-genes that could be essential for colonization and survival in the human gastrointestinal tract [140]. Further functional analysis of these genes will provide insights into how probiotics exert a protective effect against *C. difficile*. Mining of the *B. breve* UCC2003.31 probiotic genome sequences supports the notion that genetic adaptation to the colonization and persistence of *B. breve* in the human gut is mediated by fimbria-like structures encoded by the *tad* gene cluster [141]. Genome mining of *B. animalis* ssp. *lactis* AD011, a probiotic strain derived from an infant fecal sample, revealed the presence of several glycosylases and the *fos* gene cluster which are involved in processing of fructo-oligosaccharides [142, 143]. In another study, prolonged culture of *B. longum* DJO10A, an intestinal isolate, loses its functionality by gene loss identified by genome sequencing. The mutant strain showed a significantly decreased competitive ability against *C. difficile* and *E. coli*; this suggests that genomic deletions in probiotics, which is often mediated by genetic mobile elements, confers a loss of competitive abilities in the human gut [144, 145].

Comparative genome analysis of probiotic genomes would provide insights into unique probiotic attributes that are representative of the organisms' niche. For example, *in silico* analysis revealed that intestinal lactobacilli showed enrichment of genes that encoded for mucus-binding proteins, bile salt hydrolase, and other enzymes that are involved in bile acid deconjugation and breakdown of carbohydrates; in contrast, milk-adapted lactobacilli showed enrichment of genes specific to metabolism of milk-derived sugars and other carbohydrates [146–149] . Huang *et al.*, in 2021, used metagenome shotgun sequencing and whole genome sequencing approaches and identified that *L. plantarum* can utilize a highly convergent adaptive evolution strategy in a diverse array of host environments (human,

mouse, and zebra fish); this confers fitness advantages to the strain and permits positive interactions between gut microbes and host cells [150]

In silico analysis of probiotic genomes could reveal a genetic repertoire that is essential for probiotic survival during transit through the harsh environment in the gastrointestinal tract. For example, analysis of the *B. coagulans* HS243 genome identified genes in the arginine deiminase (ADI) pathway, bacteriocin-producing genes, multi-subunit ATPases, adhesion proteins, and chologlycine hydrolase, which are required for survival and colonization of *B. coagulans* HS243 in the gastrointestinal tract [151]. Undoubtedly, analysis of the probiotic genome is the most valuable tool for safety assessments of probiotic products, which could alleviate the rising level of concerns over the risks of using probiotic products. Recently, Wang *et al.*, in 2021, conducted a whole-genome analysis of commercially available probiotics and identified genes related to virulence factors, toxins, and resistance to antibiotics, which poses a potentially serious health issue [152].

Genomic exploration and genetic manipulation of probiotic microorganisms are crucial for defining their functional roles and also facilitates the rapid discovery of essential genes or genetic loci that can be exploited for bio-therapeutics and precision medicine strategy [153]. In the field of probiogenomics, it is noteworthy that CRISPR-Cas tools can be used to precisely tweak the probiotic genomes based on their known genetic sequences to generate next-generation probiotic strains that are more effective and capable of achieving more desirable traits including their improved adherence to host GIT, bile salt tolerance, enhanced antimicrobial activities, fine-tuning immunomodulatory properties through surface protein expression/suppression, and increased shelf life. [154–156]. CRISPR-engineered probiotics for therapeutic benefits are still in infancy stage, however, the massive expansion of probiotic genome datasets and advancement in genome-editing tools gives us advantages to create next-generation probiotics. This can eliminate specific pathogenic bacterial strains through targeted delivery of CRISPR-Cas system or lytic bacteriophages, which will open new avenues for the development of novel therapies against CDI. SNIPR Biome, a preclinical start-up based out of Denmark, focuses on targeting endogenous microbiome using CRISPR based vectors to selectively kill pathogenic strains without targeting other species in the microbiome. In addition, Van Pijkeren Laboratory at the University of Wisconsin-Madison has been developing a probiotic bacterium containing bacteriophage, which can carry a customized CRISPR message (knock down genetic message) to tackle antibiotic resistance in pathogens. When the engineered probiotic bacterium (with bacteriophage) reaches the intestinal tract, the bacteriophage within the probiotic bacterium would burst and infect any nearby *C. difficile*, causing them to degrade their own genetic material (commit suicide) [157]. Besides these, Novome Biotechnologies has been developing Genetically Engineered Microbial Medicines (GEMMs) that uses genetically engineered Bacteroides (native gut bacteria) carrying a gene cassette to metabolize porphyran and create new ecological niche. It has been shown that Bacteroides GEMMs can compete with the resident microbes to durably colonize the GIT and has potential to deliver therapies in wide range of disease [158]. Neil et al., in 2021 generated CRISPR-cas9 delivery vehicle that eliminated > 99.9% of targeted antibiotic-resistant *E. coli* in the mouse gut microbiota. The same research group has also applied this strategy to a *Citrobacter rodentium* infection model and achieved complete elimination of *C. rodentium*

within 4 days of treatment [159]. Similar approach can also be used to eliminate *C. difficile* from the microbiota by targeting its essential genes or genetic loci of defined functional roles using CRISPR-Cas conjugative delivery vehicle, which could aid in development of preventive strategies for CDI. The development of genetic disruption techniques such as CRISPR-interference (CRISPRi) and CRISPR-activation (CRISPRa) to repress or activate genes has immense potential to quickly screen probiotics for phenotypes of both essential and non-essential genes. Applications of CRISPRi and CRISPRa have been successfully demonstrated in diverse probiotic bacteria, which provides deep insight into biology and the physiology of the probiotic species necessary for further strain improvement and exploitation [160]. Thus, the combination of probiogenomics and CRISPR-Cas tools enables selection of potential probiotic candidates and further enhancing their functional properties creating engineered probiotic strains with improved applicability in preventing CDI and its recurrence.

Emerging approaches to advance knowledge on probiotic use for the management of CDI:

Discovery informatics to identify novel probiotic strains:

Recently, several groups have deployed informatics-driven probiotic interventions to suppress *C. difficile* infection. Steinway *et al.* constructed a Boolean dynamic model utilizing time-series metagenomic data to capture therapeutic targets of probiotics. Genome-scale metabolic network reconstructions predicted the interaction between *C. difficile* and *Barnesiella*; this approach can be used to identify new probiotic strains to use as therapeutics [161]. A network meta-analysis was used to compare and rank the relative efficacy and tolerability of probiotic agents in antibiotic-associated diarrhea. *L. casei* exhibited better efficacy and medium tolerance in reducing *C. difficile* infection [162, 163]. Johnson *et al.*, performed a meta-analysis of randomized controlled trials that supports the efficacy of *L. casei, L. rhamnosus* and *S. boulardii* formulations in demonstrating protective effects. In one study, 30,000 isolates from a human fecal sample were screened for anti-clostridial activity and one isolate was identified that produced a strong narrow-spectrum bacteriocin that can be used for treatment of CDI [164]. Koenigsknecht *et al.*, performed time-course metagenomic sequencing and found that *Lactobacillaceae* and *C. difficile* dominated the small intestine and the large intestine, respectively. Furthermore, metabolomic analysis revealed antibiotic-induced alteration of the secondary bile acid compositions that inhibit vegetative *C. difficile* cells [165].

Metabolomics to identify colonization resistance metabolites:

Metabolomics offers an assessment of microbial activity through analysis of microbial metabolites. Furthermore, microbial impact on host metabolism can be investigated through host primary and secondary metabolites. The use of metabolomics in probiotic discovery identified a different response in fecal bile acids and SCFA upon probiotic administration that decreased colonization of *C. difficile* [166]. Additionally, metabolomics could be used to predict metabolic derivatives from diet or prebiotics, including bioactive microbial metabolites that affect microbiota composition and function [167].

Exploring the tripartite *C. difficile*-lumen-intestine interaction holistically via multi-omics to evaluate clinical efficacy of probiotics:

C. difficile is a pathobiont that lives primarily in peace with its host. Given an advantage, however, strains of *C. difficile* can exploit this advantage to potentially thrive and damage the host. The lack of microbial competitors, modifications in the chemical environment, and impedance of the intestinal host defenses can tip the balance towards *C. difficile* pathogenicity similar to other opportunistic pathogens; for example, *Pseudomonas aeruginosa* pathogenicity is dependent on the specific strain, chemical environment, and host defenses [168, 169]. A holistic personalized assessment of the intestinal environment should support evaluating the effectiveness of probiotics in a clinical setting. In the personalized assessment, the following questions could be asked to better understand person-to-person and patient-to-patient variation: What is the source of *C. difficile* strain-to-strain variation in virulence beyond the mere presence or absence of a virulence effector? For example, the metabolic capacity of the strain might be important to its virulence [168]. Also, what is the role of diet and the chemical environment before and after antibiotic treatment and concomitant CDI? Measuring the chemical output of a specific diet in the intestinal lumen may provide critical information that is relevant to treatment [169].

The effect of secreted factors from *C. difficile* on the host epithelium, muscles, and immune cells can potentially be simulated in model organisms. For example, the expression of the catalytic subunit of the *C. difficile* toxin, CDTa, in the *Drosophila* midgut reduces body weight, fecal output, and overall survival. Mechanistically, the *Drosophila* enterocytes are informative to illustrate that CDTa induces F-actin network collapse, eliminates the intestinal brush border, and disrupts intercellular junctions by re-distributing Rab11 to the enterocytes' apical surface and the activation of the calmodulin/calcineurin pathway [170].

Thus, non-vertebrate or vertebrate model hosts can be used complementary to clinical development. *Drosophila* or mice exposed to with *C. difficile* secreted factors in multidimensional screens involving microbiota, prebiotics, and probiotics and the study of host epithelium response can teach us about the plasticity of *C. difficile* pathogenicity. Modulating host epithelium genetics or metabolism to alter the impact of CDI or virulence factors on the host is also a viable option, since genetic inhibition of Rab11 or chemical inhibition of the calmodulin/calcineurin pathway by cyclosporin A or FK506 reduces CDTa phenotypes [170].

Regulatory requirements and oversight for the approval of probiotics for *C. difficile*-associated diarrhea:

Most probiotic products are regulated as dietary supplements or foods for use by the general healthy population to improve their immunity or gastrointestinal flora. The increase in antibiotic-resistant infections, including the higher incidence of CDI, has resulted in probiotics being prescribed for the prevention and treatment of recurrent or refractory diseases in hospitalized patients [171]. The regulation of probiotics depends on multiple factors including the intended use, degree of health claims, warning statements on the label, dosage regimen, and geographical region. Based on the intended use, probiotics

can be subclassified into probiotic drugs (therapeutic use), probiotic foods (use as foods, food ingredients, and dietary supplements), probiotic feeds (for animal use), and designed probiotics (genetically modified probiotics) [172].

In Australia and New Zealand, probiotics are regulated as foods and covered by the appropriate Food Standards Code when used as foods with or without associated general level claims (enhance immunity and/or regulate gastrointestinal flora). Probiotic foods associated with a high level of health claims require approval from the Food Standards Australia New Zealand (FSANZ). Furthermore, probiotic drugs with a health claim, statements or instructions for use, and the dosage regimen are considered as therapeutic and hence listed on the Australian Register of Therapeutic Goods (ARTG) and are regulated as complementary medicine by the Therapeutic Goods Administration [173].

In the United States, probiotics that are intended for use as dietary supplements are regulated by the FDA's Center for Food Safety and Applied Nutrition. Unlike therapeutic drugs, the FDA does not need to approve dietary supplements; however, the regulatory body must be notified before a supplement can be marketed in the U.S., and the manufacturer should ensure product quality, efficiency, and safety [171].

The European Union (EU) possesses one of the strictest, if not the strictest, health claim regulations globally, has only authorized a small fraction of the health claims out of several claims submitted, and possesses no EU-wide legal framework that defines probiotic bacteria or probiotic foods. However, a list of bacteria at the species level, considered safe in foods and feeds, is kept and updated annually by the EU [174]. Health claim legislation in the EU revolves around consumer safety with an emphasis on controlled human intervention research and identification of the probiotic strains to the strain level by genotypic and phenotypic analyses, before approval [175].

According to the Joint Food and Agriculture Organization of the United Nations/World Health Organization Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics, the summary of the guidelines for evaluating probiotics that could lead to substantial health claims should include probiotic strain identification at the genus, species, and strain levels, *in vitro* testing to understand the mechanisms of action, clinical health benefits determined by human trials, and probiotic safety with respect to patterns of antimicrobial drug resistance, metabolic activities, side effects during clinical and post-clinical trials, toxicology studies, and investigation to determine a lack of infectivity in animal trials [171].

Challenges and future directions:

Several bacterial species confer protection against *C. difficile* and symptoms associated with CDI. Nevertheless, human studies using probiotics have generated contradictory results. Till date, no conventional probiotics have been clearly proven to be an effective prophylactic option for CDI prevention [176]. This may be in part due to disparity in study design, but it is also because of our limited understanding of the specificity and the mechanisms by which probiotics exert their action. Thus, a better understanding is needed regarding the gut

microflora secreted effector molecules, their bactericidal/bacteriostatic effect, involvement in the modulation of the host immune response, and modulation of the receptor signaling cascades. What should be noted is that most of the current mechanistic studies are based on *in vitro* cell culture models and will most certainly require obtaining better mechanistic insights into their effects in humans.

The probiotics currently on the market cater to healthy individuals and claim to reduce risk of diseases prophylactically without any validation of its prophylactic or therapeutic effects. What is currently lacking in the field is biologically coherent data on the ability of probiotics to prevent diseases and/or modulate immune responses in the host and thereby provide therapeutic benefits. Clinical studies to obtain such data must follow defined study parameters like population size, volume and duration of dose administration, control groups, and disease outcome. Well designed, scientifically sound clinical trials are necessary to minimize bias in assessing the effects of probiotic interventions [177].

Additionally, specific studies are required to validate probiotics as an adjunctive therapy to the existing treatment repertoire. This will require assessing the combination of probiotics with standard-of-care antibiotics used to treat CDI. Genetic manipulation of current probiotic strains (designer probiotics) can also offer intriguing prospects for circumventing the side effects associated with antibiotic therapy and preventing CDAD. Comparative analysis of probiotic genomes will provide insights into unique probiotic attributes and the genetic repertoire that is essential for probiotic survival in the intestine and confer a protective effect against *C. difficile*. Another limitation in the use of probiotics is the broad-spectrum effect of their secreted metabolites on gut microflora strains. This has been observed with reuterin, produced by *L. reuteri*, which exhibits broad-spectrum antimicrobial activity against intestinal flora [178]. Several antimicrobial peptide (AMP)-producing microflora strains are effective against *C. difficile* but addressing off-target effects associated with these strains remains a challenge. This issue might be addressed by genetically engineering the AMP to specifically target *C. difficile* without harming the beneficial microbiota [179]. Novel members of the host microbiota identified from recent advancements in microbiome research can also be investigated to determine their inhibitory effect on *C. difficile*.

One notable limitation of different probiotic formulations is the successful delivery of viable cells at a high enough burden to the gut. Thus, novel delivery systems that have been used to treat other enteric diseases should be investigated for probiotics [180].

Another important question regarding probiotic use is whether to use a single strain or multi-strain formulations to treat CDI. It is postulated that because of the complexity of the intestinal microbiota, the use of multi-strain probiotic formulations might confer an advantage over the use of single strains in restoring the gut microbiome [181]. Clinical studies of probiotic use for CDI prevention involve the use of both single and multi-strain probiotics. In fact, a systematic review and meta-analysis study by Johnston *et al.*, in 2012, concluded that clinical trials that used multiple species were more effective than clinical trials that used a single species [114]. However, as noted by the authors, the meta-analysis conducted was based on between-studies rather than within-studies comparison. Hence,

there is no conclusive evidence regarding the benefit of multi-strain species over a single strain. Therefore, affirmative clinical studies comparing the same strains administered alone versus in a mixture need to be conducted.

Overall, probiotics might harbor potential to be utilized for the prevention of CDAD and treating recurrent CDI alongside antibiotics. However, not all probiotics in the current repertoire can be expected to deliver promising results in human clinical trials. Probiotic is an umbrella term, and a careful selection of specific organisms using a mechanistic-based approach coupled with multicenter, randomized, controlled trials can address the potential of probiotics' supplementation as an option for treating or preventing CDI and its recurrence. In conclusion, a comprehensive understanding of the dynamic microbial community, deciphering the mechanistic relationship between the host microflora and *C. difficile*, and more evidence-based studies can help develop preemptive measures to better thwart the threat of CDI.

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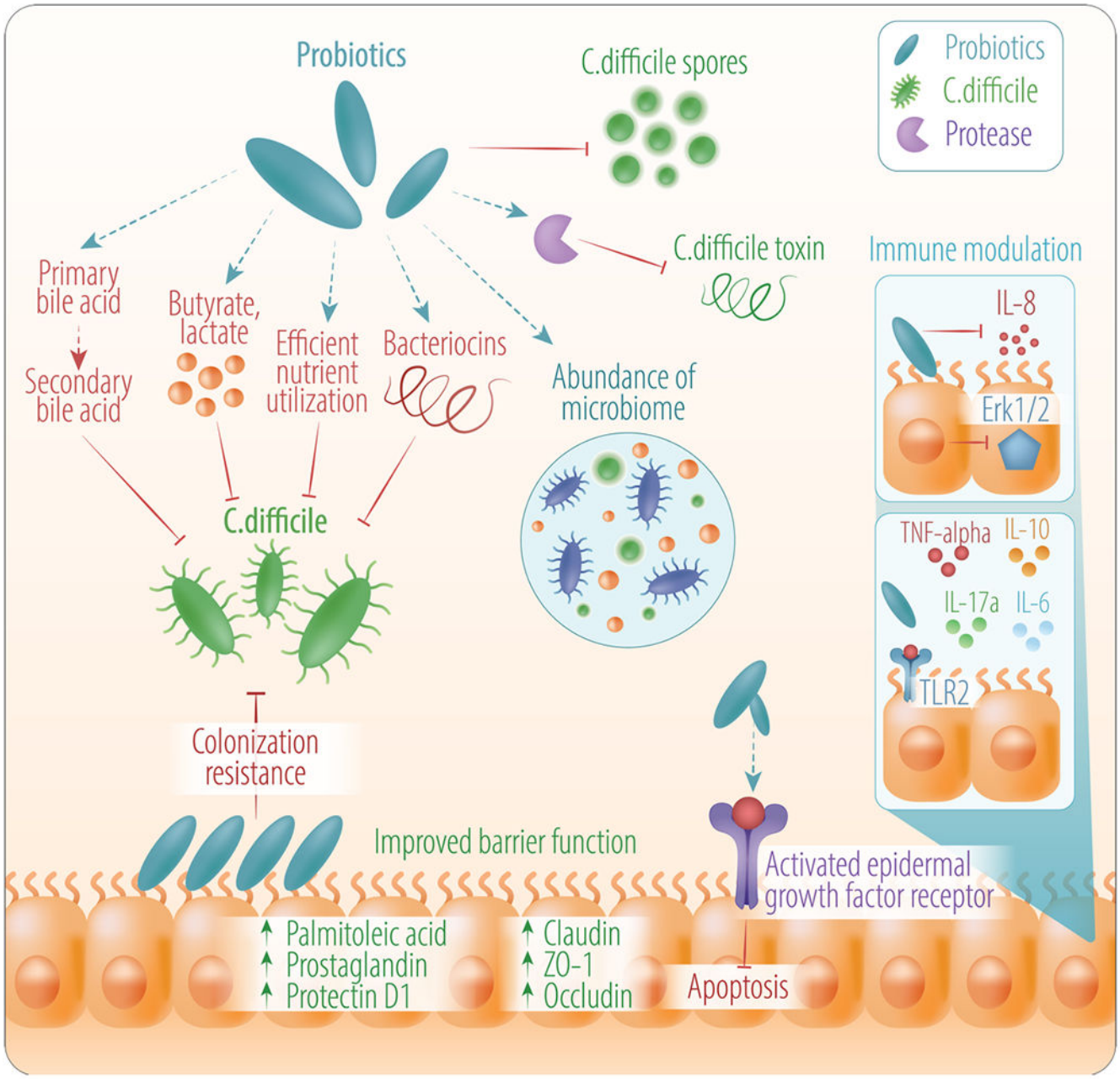

Fig.1:

Schematic depiction of the effect of probiotics in the context of treating/preventing CDI. Probiotics can have several effects that can be useful for treating/preventing *C. difficile* including competition for nutrients, resulting in inhibition of pathogen outgrowth through conversion of bile acids, improved barrier function, abundance of microbiome, and modulation of the immune system. Probiotics an also play a potential role in inhibition of *C. difficile* virulence factors (toxins and spores).

Table 1:

Summary of probiotic strains used in human clinical trials to treat CDAD.

Strain(s)	Clinical Trial Application	Outcome/Results	Reference(s)
Lactobacillus plantarum 299v	Reducing colonization of *C. difficile* in critically ill patients treated with antibiotics.	None of the patients who received the probiotic were colonized with *C. difficile* compared to 19% colonization was present in the control group.	[182]
Cocktail of 4 strains *L. acidophilus* NCFM, *L. paracasei* Lpc-37, *B. lactis* Bi-07 *and B. lactis* BI-04	Probiotic supplement for *C. difficile* infection in adults.	Probiotic adjunct therapy was associated with a significant improvement in diarrhea (rate and duration).	[183]
Cocktail of two strains*: L. acidophilus* CL1285 and *L. casei* LBC80R	Proprietary probiotic formulation for reducing CDAD in hospitalized patients on antibiotics.	Probiotic blend effective in lowering CDAD incidence in patients treated with probiotics vs. placebo.	[184, 185]
Probiotic formulation composed of *L. acidophilus* CL1285, *L. casei* LBC80R, and *L. rhamnosus* CLR2	Adult hospitalized patients were treated with the probiotic formulation from the initiation of antibiotic use to prevent nosocomial CDI.	Highly effective infection prevention strategy with the lowest rate of nosocomial CDI on average over the past 15 years.	[186]
Cocktail of *L. casei* DN114-001 (*L. casei immunitass*), *L. bulgaricus*, and *S. thermophilus*	Consumption of the probiotic cocktail by hospitalized patients taking antibiotics.	Patients on probiotics did not develop CDAD as compared to placebo (17%).	[187]
Lactobacillus GG	Patients with recurrent *C. difficile* were treated with *Lactobacillus* GG.	Treatment showed a high cure rate for recurrent CDI.	[113, 188]
Saccharomyces boulardii	Supplement to prevent antibiotic associated diarrhea including CDAD in children.	Patients had lower incidence of CDAD (3.4%) as compared to the placebo (17.3%).	[189, 190]
Saccharomyces boulardii	Probiotic used with antibiotics (vancomycin or metronidazole) in adult patients with active *C. difficile* disease (CDD) or one prior CDD episode.	Significant improvement in recurrence rate in patients treated with probiotic (34.6%) vs. placebo (64.7%); however, no significant outcome in adults with initial CDD.	[191]
Saccharomyces boulardii	Hospitalized patients exposed to antibiotics were administered this probiotic.	No significant evidence was observed in prevention of CDAD in a population of hospitalized patients on antibiotics.	[192]
Spores of non-toxigenic *C. difficile* strain M3 (NTCD M3)	Patients diagnosed with CDI were administered three different doses of NTCD M3 spores.	Significant reduction in CDI recurrence in the combined NTCD M3 groups (11%) vs placebo (30%). Recurrence was also significantly lower in patients who developed colonization to NTCD (31% vs. 2%).	[193]
Multi-strain preparation of *Lactobacilli* and *Bifidobacteria* (*L. acidophilus* CUL60, *B. bifidum* CUL20 and *B. lactis* CUL34)	Pragmatic efficacy trial of the probiotic preparation in elderly hospitalized patients (≥65 years) exposed to oral or parenteral antibiotics.	No significant evidence suggesting the efficacy of the multi-strain probiotic in preventing CDAD.	[194]