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Probiotics for Prevention of *Clostridium difficile* Infection

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Abstract

Purpose of review—Probiotics may prevent *C. difficile* infection (CDI), a leading healthcare-associated infection in the United States. However, prior studies were limited by heterogeneity in products and patient populations. Recent clinical evidence and new approaches to probiotic development are reviewed.

Recent findings—Probiotic use may reduce incident CDI in high risk populations by as much as 50%, though prior clinical trials have yielded conflicting results. Combining probiotics with prebiotics improves growth and engraftment in the host. *Bacillus clausii* and *Lactobacillus reuteri* secrete compounds that directly inhibit *C. difficile.* Organisms that produce secondary bile acids, such as *Clostridium scindens*, enhance *C. difficile* colonization resistance. Non-toxigenic *C. difficile*, which provides nutritional niche competition, may prevent CDI. Refinements to fecal microbiota transplantation (FMT) blur the line between probiotics and FMT. These include a quality-controlled stool product (RBX2660), purified Firmicutes spores (SER-109), and sterile fecal filtrate. Bacteriophages may treat CDI but have unknown safety and efficacy in humans.

Summary—There have been a number of advances in probiotics and our understanding of their role in prevention of CDI, but a number of important safety and efficacy questions remain. An improved understanding of the native microbiota structure and function will allow for continued development of rationally designed probiotic therapy to provide enhanced protection against CDI.

Keywords

Clostridium difficile infection; probiotics; prebiotics; fecal microbiota transplantation; bacteriophage therapy

Introduction

Clostridium difficile infection (CDI) is a scourge of the modern healthcare system, causing over 500,000 infections and 30,000 deaths, with annual costs totaling over $1.5 billion.[1, 2] The Centers for Disease Control and Prevention currently lists CDI as an “urgent threat,”

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Conflicts of Interest

VBY has served as a consultant to Finch Therapeutics and Vedanta Biosciences.

requiring a prioritization of public health resources to identify infection and limit transmission.[3]

An anaerobic, spore-forming, toxin-producing Gram-positive bacillus, *C. difficile* has been found to asymptomatically colonize 2–15% of patients, depending on underlying comorbidities and degree of prior healthcare exposure.[4, 5] *C. difficile* spores can persist in the environment and are resistant alcohol-based cleaning agents and quaternary ammonium compounds, resulting in high transmissibility.[6] The primary risk factor for development of CDI is exposure to antibiotics, which perturb the indigenous microbiota's structure and function, allowing for *C. difficile* acquisition and symptomatic disease.[7, 8] *C. difficile* acquisition can result in a wide range of outcomes from temporary asymptomatic colonization to fulminant pseudomembranous colitis. Current standard of care for CDI involves antibiotic treatment with metronidazole, vancomycin, or fidaxomicin.[9] Outcomes are suboptimal, with 12–64% recurrence risk (median 22%).[10] Thus, adjunctive therapies that can improve outcomes are actively being sought. In particular, given the important role that disturbances in the gut microbiota play in the pathogenesis of CDI, this microbial community represents a potentially novel therapeutic target.

Probiotics, a century-old concept, are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host."[11, 12] In addition to the genus and species of a probiotic, the particular strain should be known, as health benefits can be strain-specific. Probiotics used to potentially prevent or treat CDI function through multiple possible mechanisms. [FIGURE 1] In many cases, these mirror postulated mechanisms by which the indigenous microbiota mediate colonization resistance against *C. difficile*.[7, 13]

Clinicians are often confused by conflicting data on probiotics for the prevention of CDI. This is compounded by the heterogeneity in prior clinical trials, utilizing different probiotic agents on varied patient populations and assessing different primary outcomes. Shen et al performed a recent meta-analysis of probiotic use for CDI prevention specifically in hospitalized adults receiving antibiotics.[14] They included 19 randomized controlled trials with 6261 patients treated with *Saccharomyces boulardii*, *Lactobacillus* spp, *Bifidobacterium* spp, and *Streptococcus* spp, alone, or in combination. The relative risk (RR) of CDI in the probiotic treatment groups was 0.42 (95% CI 0.3–0.57) without significant heterogeneity between studies (I^2 0.0%, P =.56) and no evidence of publication bias. While rigorously conducted and focused on a specific high-risk population, the review was limited by significant variability in probiotic agents, case definitions, and placebo rates of AAD and CDI in the included studies. As such, significant questions remain: 1) what is the optimal probiotic strain or combination of strains, and 2) are they safe for use in immunocompromised hosts, who bear an outsized burden of CDI? Furthermore, current national guidelines for the treatment of CDI do not recommend routine probiotic use.[15, 16] As probiotic use in the context of CDI remains controversial, a summary highlighting active research in the field is warranted.

We take a mechanistic approach here to review the latest developments in probiotic research, focusing on their role in the prevention of primary and recurrent CDI. Methods to

manipulate the indigenous microbiota span the spectrum from traditional single agent probiotics to FMT. We discuss products that include undefined microbial consortia, phage viruses, and bacterial-derived small molecule therapy, that fall outside the traditional probiotic definition. While we do discuss new FMT developments, we do not review FMT in depth, as has previously been done [17, 18].

Trials of traditional single and combination agents

In the past year there has only been a single large-scale clinical trial involving traditional single-agent probiotics. This was a multicenter, double-blind, randomized controlled trial assessing effectiveness of *Saccharomyces boulardii* for the primary prevention of antibiotic-associated diarrhea (AAD) in hospitalized patients receiving antibiotics.[19]

This study of 477 patients, in which *C. difficile* infection was assessed as a secondary end-point, failed to find a benefit of *S. boulardii* in the prevention of AAD or CDI. However, the study was underpowered to detect a difference due to under-enrollment and fewer than anticipated primary events.

A recent phase 2 study was the first to assess the ability of a probiotic to reduce duration of diarrhea for the initial episode of mild to moderate CDI. Use of a daily multi-strain capsule (*Lactobacillus acidophilus* NCFM, ATCC 700396; *Lactobacillus paracasei* Lpc-37, ATCC SD5275; *Bifidobacterium lactis* Bi-07, ATCC SC5220; *B. lactis* Bl-04, ATCC SD5219) versus placebo for four weeks resulted in shorter duration of diarrhea (1 vs 0 days; P =.039). This exploratory pilot study of 33 patients will need to be replicated in a larger population to establish efficacy of probiotics as an adjunctive therapy for initial occurrence of CDI.[20]

Synbiotics

Prebiotics are non-digestible polysaccharides and oligosaccharides that promote the growth of specific genera of beneficial microorganisms by acting as a substrate for fermentation.[21, 22] When a prebiotic is administered with a specific probiotic to enhance the engraftment and growth of that microbe, the combination is termed a “synbiotic.” A recent study assessed the ability of *Lactobacillus plantarum* Inducia® and the prebiotic xylitol to inhibit the germination of *C. difficile* spores.[23] Pre-incubation with *L. plantarum* and xylitol fully inhibited *in vitro* germination of *C. difficile* spores. CDI was attenuated in mice fed xylitol and *L. plantarum* 5–6 days prior to ampicillin and *C. difficile* administration, with a reduction in mortality from 44% to 22%. No reduction in mortality occurred in mice who began synbiotic treatment after the *C. difficile* challenge. The generalizability of the findings is limited as it is a small *in vitro* and animal study. However, it does support further study on the enhancement of beneficial probiotic effects by dietary substrates and the relative temporal interaction of probiotic and antibiotic administration.

Another study assessed the ability of four different *Bifidobacterium* strains to inhibit *in vitro C. difficile* growth when co-cultured with various prebiotics.[24] Reduction in toxicity was observed when *B. longum* and *B. breve* were cultured in a cell line exposed to *C. difficile* cell-free supernatant using oligo-fructosaccharides as a carbon source. No beneficial effects were noted with use of inulin. Results suggest the optimal prebiotic substrate is strain specific. Further studies are needed to determine the optimal prebiotic substrate for each

probiotic strain and to measure effects of prebiotic administration on the larger intestinal microbiota.

Bacterial secreted compounds that inhibit the activity of *C. difficile* toxins

A study by Ripert et al investigated the *in vitro* ability of *Bacillus clausii* O/C to neutralize *C. difficile* toxin, the major virulence factor of the pathogen.[25] Incubation of toxin-containing culture supernatants of *C. difficile* with supernatant from *B. clausii* protected mammalian cell lines from cytotoxic effects.[26] This was due to the production of a serine protease, M-protease, by *B. clausii*. Purified M-protease was able to protect Vero cells from the cytotoxic effects of *C. difficile* culture supernatants. Widespread screening of potential probiotic agents for enzymatic activities that can destroy *C. difficile* toxins may identify additional strains that can be combined for maximal inhibitory capacity.

Direct inhibition of *C. difficile*

Certain bacteria produce antibacterial compounds, that could prevent or treat CDI. This can include molecules such as the bacteriocin produced by *Bacillus thuringiensis* DPC 6431. Other bacteria produce non-protein antimicrobial compounds. *Lactobacillus reuteri* ferments glycerol to produce reuterin, an antibacterial substance with activity against numerous enteric pathogens, including *C. difficile*. A recent study by Spinler and colleagues utilized *L. reuteri* strain 17938, which displays high-level resistance to vancomycin, metronidazole, and fidaxomicin, making it an attractive option in patients receiving concomitant anti-CDI therapy.[27] These investigators utilized a mini-bioreactor system that contained a human-derived microbial community to compare the ability of *L. reuteri* 17938 with or without the addition of glycerol to inhibit *C. difficile* growth in bioreactors pretreated with clindamycin. The combination of *L. reuteri* and glycerol resulted in a 5-log reduction in the growth of *C. difficile* in these bioreactors (P =.008).

Other investigators have examined the synergistic effects of microbe-derived antimicrobials against *C. difficile*. Durancin 61, a bacteriocin produced by *Enterococcus durans*, was purified and examined for *in vitro* activity against *C. difficile* strain ATCC 630 alone and in combination with the antibacterial compounds nisin and reuterin.[28] The combination of durancin 61 and reuterin exhibited the most potent inhibition and synergy. These results suggest that combinations of probiotic organisms may exhibit beneficial synergy. Given the fact that the normal microbiota is a complex community it is not surprising that the use of multiple probiotics can lead to synergy. As yet, we do not have information on which combinations of organisms may have the most beneficial effect.

Restoration of bile acid homeostasis

Bile acids play a key role in the physiology of *C. difficile*, with specific bile acids (generally primary, conjugated bile acids) serving as germinants for *C. difficile* spores and others having inhibitory activity on vegetative *C. difficile*.[29] Antibiotic treatment alters intestinal bile acid abundance and composition. Repopulation with 7α-dehydroxylating bacteria that convert primary to secondary bile acids could therefore provide improved *C. difficile* colonization resistance.

A study by Pamer et al assessed changes in diversity and microbial composition by 16S rRNA sequencing in mice treated with various antibiotics.[30] Variance in the microbial structure was used to identify specific taxa that provided resistance against CDI; organisms belonging to *Clostridium* cluster XIVa, most notably *Clostridium scindens*, a known producer of secondary bile acids, provided the greatest protection. A consortium of four bacterial species including *C. scindens* displayed attenuated CDI in antibiotic-treated, *C. difficile*-challenged mice. Sequencing data showed engraftment of *C. scindens* that was dose-dependently associated with protection from CDI, along with presence of 7α-dehydroxylase capability in treated mice. These data suggest bacteria that synthesize secondary bile acids may form a crucial component of an engineered microbiota with resistance to CDI. This could potentially be accomplished through live bacteria expressing 7α-dehydroxylase or through direct enteral administration of secondary bile acids. The latter approach has proved successful in curing a single case of recurrent ileal pouchitis due to refractory CDI with ursodeoxycholic acid.[31]

Competition for Resources

As competition for similar ecological niches is believed to be an important mechanism of effective probiotics, strains that compete with toxigenic *C. difficile* represent a promising therapeutic avenue. In particular, non-toxogenic *C. difficile* (NTCD), which presumably shares the closest nutritional requirements, has shown efficacy as a method of CDI prevention.

A phase 2 randomized, double-blind, placebo-controlled study was conducted on NTCD-M3 spores for the prevention of first recurrence of CDI [32] Patients were randomized to a 14-day course of three different doses of NTCD-M3 spores or placebo. Of 168 patients, CDI recurrence was 30% in the placebo group vs 11% in the combined NTCD-M3 groups (odds ratio 0.28; 95% CI 0.11–0.69; P =.006). CDI recurrence was lower in those who developed colonization with NTCD (31% vs. 2%). NTCD colonization declined substantially after completion of therapy, so CDI protection may be transient without prolonged or repeated courses. There is also a concern that NTCD may acquire toxin production capabilities through horizontal gene transfer, as has been shown *in vitro*.[33]

Refinements to Fecal Microbiota Transplantation: use of fecal derivatives for the treatment of CDI

Treatment with undefined consortia of fecal bacteria that are quality-controlled and semi-standardized is another avenue of therapy being explored. RBX2660 is a standardized stool-derived microbial suspension containing live bacteria in a cryopreservative derived from screened healthy donors. This microbial suspension can be stored frozen and then thawed and delivered to patients via retention enema.

A phase 2 single-arm study of 34 patients assessed safety and efficacy of RBX2660 in non-immunocompromised patients with ≥two recurrences of CDI.[34] Patients with persistent diarrhea after a first RBX2660 dose could receive a second dose within ten days. Of 31 patients evaluated for efficacy, 14 (45%) required a second treatment, and 27 of 31 (87.1%) had treatment success after one or two doses. This study shows the promise of a

standardized fecal microbial suspension that is easy to administer, though the patient sample was small and the first dose efficacy was suboptimal. However, a follow-up phase 2b trial of 150 subjects receiving two doses of RBX2660 versus placebo failed to meet its primary end point of absence of CDI at 56 days (61% vs 45.5%, P=0.152).[35] Larger randomized trials will be needed to further assess efficacy for recurrent CDI.

Other strategies are being developed to repopulate the gut microbiota by methods other than the administration of viable microbes. SER-109 consists of purified spores, generally from bacteria of the Firmicutes phylum, collected from the stool of healthy, pre-screened donors formulated as a capsule. A single-arm, open-label phase 1b study of SER-109 was performed in 30 patients with ≥ three prior episodes of CDI. [36] Therapy was well tolerated, and 26 of 30 patients met the primary endpoint of no CDI recurrence within eight weeks. Analysis of microbiota composition by 16S rRNA sequencing revealed rapid alterations, with expansion of Firmicutes and amplification of organisms not contained in SER-109, such as Bacteroidetes.

However, as SER-109 is still derived from human stool, it remains relatively undefined with inherent variability in the microbial composition from donor to donor. The efficacy of SER-109 failed to validate in the yet-to-be published phase 2 study's results.[37] The company is now conducting a randomized, double-blind, placebo-controlled, parallel group study of SER-109 as well as a phase 1b study on SER-262, a defined microbial preparation with 12 different types of bacteria in spore form, for prevention of recurrent CDI.[38, 39]

Another approach to administer bacteriotherapy without the transfer of viable microorganisms is through the use of microbe-free fecal filtrates. In the study by Ott et al, fecal supernatant was passed through multiple sequential filters, with a final pore size of 0.2 μm.[40] No bacterial growth was detected after cultivation of the resulting product. All five patients treated for recurrent CDI experienced rapid clinical cure with a single administration and remained symptom-free for the six-month follow-up period. 16s rRNA sequencing revealed rapid and complex shifts in gut microbial composition. Proteome analysis of the fecal filtrate demonstrated a wide array of predominantly human-derived proteins and bacteriophages. Combined with a trial that did not demonstrate FMT's efficacy over standard therapy for acutely recurrent CDI, this study underscores the importance of further research into the relative importance of the various stool components in CDI treatment.[41, 42]

Bacteriophage Therapy

Bacteriophage therapy is another intriguing avenue for effective treatment of multidrug resistant bacteria. It is a particularly attractive option for CDI because of its targeted mechanism of action and lack of significant impact on the microbiota. Research has been hampered by a lack of identified lytic phages specific for *C. difficile* and concerns of using temperate phages that could potentially integrate viral nucleic acid into host DNA. A study by Nale et al investigated the ability of seven different phages, alone and in combination, to reduce the bacterial load of 80 different strains of *C. difficile* representing 21 different ribotypes.[43] The optimal phage combination was strain specific, but multiple three and four phage combinations were able to completely lyse *in vitro C. difficile* cultures within 2–

5 hours. Mice treated with combination phage therapy survived longer and had a four-log reduction in colonic *C. difficile* bacterial and spore counts.

Major challenges with phage therapy include designing a phage that lacks integrase activity, to reduce risk of transmission of mobile genetic elements (i.e. drug resistance genes), and demonstrating human safety and efficacy.

Conclusion

Prior clinical trials involving traditional single and small combination probiotic agents have shown modest success in risk reduction of CDI in high-risk patients receiving systemic antibiotics. However, clinicians have been slow to adopt them into practice due to conflicting individual study results and a wide array of heterogeneous products. While incorporating them into treatment is appropriate based on the current body of data and has already been trialed by some hospitals, the search remains for an agent that is easy to administer and provides consistent, durable protection against CDI that is replicated in multiple clinical trials and real-world studies.[44]

The widespread availability of genomic and metabolomic analysis and recent developments in computational modeling result in a more precise understanding of the effects of targeted introduction of probiotics.[30, 45, 46] This will allow for more rational design of agents that specifically manipulate the microbiota to ameliorate dysbiotic changes. Since the native microbiome extends beyond bacteria and fungi and healthy stool includes many non-microbial components, further exploration of alternative treatments involving phage therapy and purified microbiota-derived small molecules is also warranted. An evolving understanding of the dynamics of the complex microbial community will allow for creation of better products to promote and restore homeostasis of the intestinal microbiota in the setting of antibiotic stress.

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Key points

- Probiotic use may reduce incident CDI in high risk populations by as much as 50%, though prior clinical trials have yielded conflicting results
- There are several promising new approaches to prevention of CDI including combining probiotics with prebiotics, use of strains that directly inhibit *C. difficile*, modulation of bile acid metabolism to improve colonization resistance, niche competition, and bacteriophage therapy.
- Refinements to FMT, including standardized stool-derived products, purified spores, and sterile fecal filtrate, blur the line between probiotics and FMT.
- Important safety and efficacy concerns still impede the widespread deployment and acceptance of probiotics.

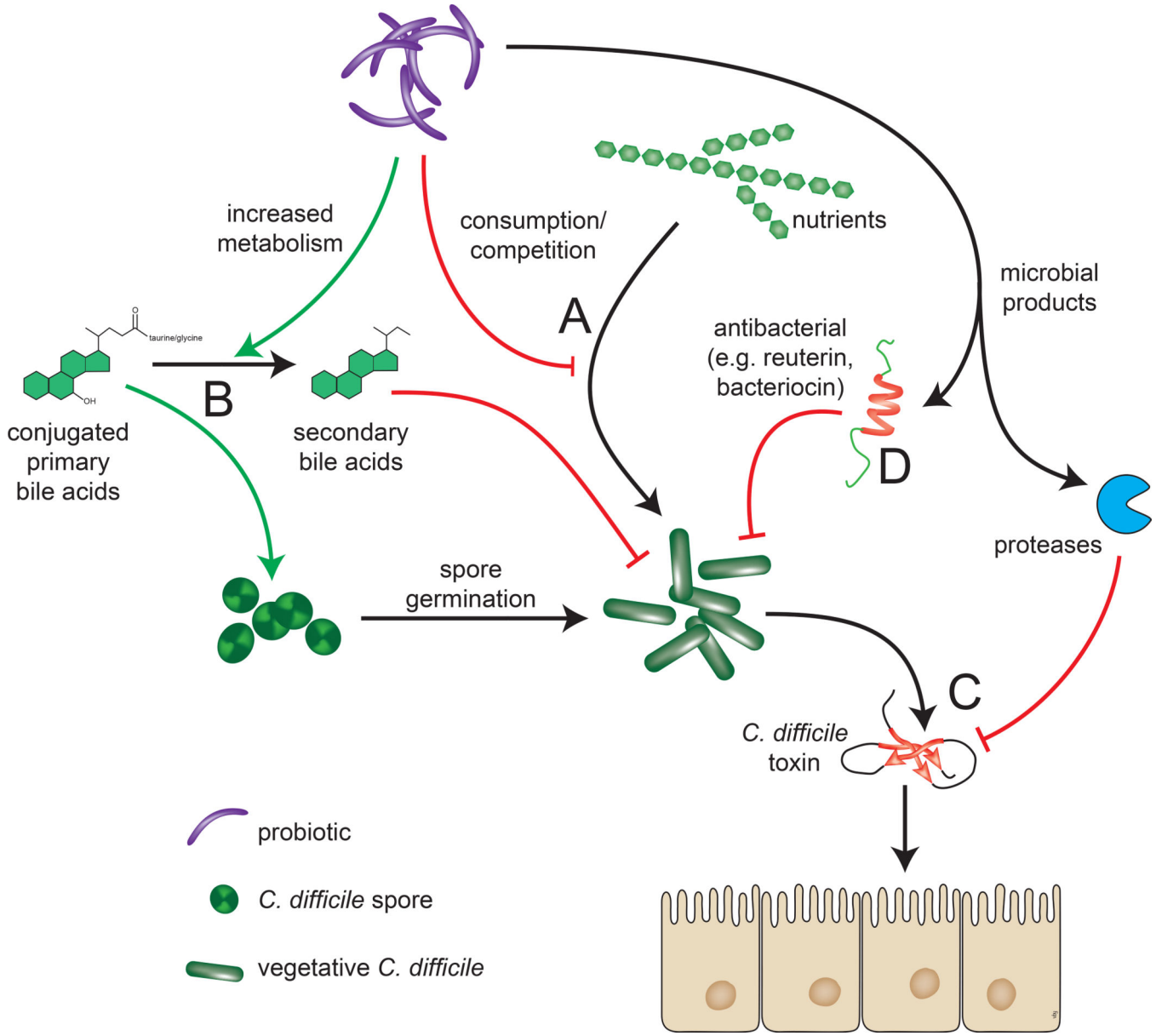

FIGURE 1: Mechanisms of probiotic protection against CDI.

There are multiple potential mechanisms by which probiotics could modulate the onset and course *C. difficile* infection. A) Competition for resources. For example, increased levels of nitrogen-containing amino acids, sialic acid, succinate, and host-derived glycans, and decreases in short chain fatty acids provide a favorable environment for growth of vegetative *C. difficile* and their consumption by probiotics could be inhibitory. B) Decreased metabolism of primary to secondary bile acids by 7α-dehydroxylase promotes germination of *C. difficile* spores, and this can be counteracted by administering bacteria that have such activity. C) The *C. difficile* toxins TcdA and TcdB are responsible for the symptoms of disease and these can be counteracted through secretion of inhibitory compounds. D) Bacteroicins, a class of antimicrobial peptides, can be directly secreted by probiotics. Non-

specific effects of probiotics that alter pH, increase mucosal IgA levels, or increase mucin production can also inhibit *C. difficile* (not depicted).[47–49]

TABLE 1:

Microorganism or microorganism-derived therapies that are actively in use or development for prevention of CDI.

	Advantages	**Disadvantages**	**References**
Single or small combination of bacterial/fungal strains	Extensive clinical trial and real-world experience Safety well-established	Modest efficacy Lack of standardization, heterogeneity among products	[11, 14, 50]
Non-toxogenic *C. difficile* (NTCD)	Safe and effective in phase II study Provides nutritional niche competition	Transient NTCD colonization provides short-lived protection	[32]
Fecal consortia (e.g. RBX2660)	Deliver complex native microbial communities (mimicking FMT) May promote clearance of other MDROs	Undefined, variable product Theoretical risk of transmitted infection	[34, 51]
Firmicute spores (e.g. SER-109)	Very low risk of transmitted infection	Undefined, variable product Limited clinical trial data	[36]
Sterile Fecal Filtrate	Very low risk of transmitted infection Unaffected by systemic antibiotics	Undefined, variable product Limited supportive data	[40]
Bacteriophages	Unaffected by systemic antibiotics Narrow spectrum of activity	Lack of supportive human data Risk of unanticipated gene exchange	[43]