Title: Beneficial effects of fecal microbiota transplantation in recurrent *Clostridioides difficile* infection

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Abstract

Fecal microbiota transplantation (FMT) is highly effective in preventing recurrent *Clostridioides difficile* infection (rCDI). However, the mechanisms underpinning its clinical efficacy are incompletely understood. Herein, we provide an overview of rCDI pathogenesis, followed by a discussion of potential mechanisms of action focusing on the current understanding of trans-kingdom microbial, metabolic, immunological, and epigenetic mechanisms. We then outline the current research gaps and offer methodological recommendations for future studies to elevate the quality of research and advance knowledge translation. By combining interventional trials with multi-omics technology and host and environmental factors, analyzing longitudinally collected biospecimens will generate results that can be validated with animal and other models. Collectively, this will confirm causality and improve translation, ultimately to develop targeted therapies to replace FMT.

INTRODUCTION

Clostridioides difficile is a Gram-positive, spore-forming bacterium that causes the most common nosocomial intestinal infection. The pathology arises from dysbiosis of the intestinal microbiota, usually triggered by antibiotic use, allowing *C. difficile* to proliferate. The clinical spectrum of *C. difficile* infection (CDI) ranges from mild diarrhea to toxic megacolon and death.[1] The recommended therapy for the primary episode of CDI is either vancomycin, a broad-spectrum Gram-positive antimicrobial, or fidaxomicin, a narrow-spectrum but expensive antibiotic.[2] A major clinical challenge is recurrent CDI (rCDI), which occurs in approximately 20% of patients after the primary episode and in 60% of patients following the third episode, because no effective standard drug-based therapy exists.[3, 4]

Recently, fecal microbiota transplantation (FMT), also known as intestinal microbiota transfer, has been increasingly adopted into routine clinical practice to treat rCDI because it is the most clinically-and-cost-effective therapy. A successful treatment outcome is usually defined as a lack of CDI recurrence after a follow-up period of at least 8 weeks. Using this criterion, the success rate of FMT has been reported to range from 60% to over 90%, depending on the route of administration (retention enema, nasogastric tube, colonoscopy, or capsules) and study design (randomized placebo-controlled or open-label).[5-7] The success rates tend to be lower with randomized placebo-controlled studies[5] than in open-label studies,[5] and are also lower in studies using FMT by retention enema than with other routes of delivery.[5, 7, 8] Although highly effective, there are substantial drawbacks with FMT, including infectious risks and sparse long-term safety data.[9, 10]

While substantial progress has undoubtedly been made in unraveling the "how" of FMT, most human studies are largely associative or correlative, and solely analyze stool microbiome. Although results from these studies have led to the development of defined

microbiota likely to influence FMT efficacy for rCDI, the variable success rate observed across clinical trials demonstrates critical gaps in knowledge about how FMT works. In addition to well-designed clinical trials, a mechanistic understanding of both microbe-microbe and host-microbe interactions that define successful recovery from rCDI is necessary. These include studies using strain-level metagenomic analyses to identify the ecological effects of FMT on the recipient microbiota,[11-13] as well as testable hypotheses on mechanisms of the FMT action in human and rodent studies. As such, identifying the key components responsible for the beneficial effect of FMT and the underlying mechanisms should remain a research priority.

In this review, we present an updated overview of *C. difficile* host-microbe interactions that may additionally influence FMT efficacy for rCDI. We first summarize these concepts in the context of CDI pathogenesis and recurrence. We will then discuss the current understanding of the potential mechanistic actions of FMT for rCDI, focusing on microbial (trans-kingdom), metabolic, immunological, and epigenetic mechanisms potentially underpinning FMT efficacy. We will consider both human observational studies and animal models, as the latter are able to make causal inferences. We will discuss the current research gaps and offer methodological recommendations for future studies to elevate the quality of research and advance translation. We will further explore the challenges and potential mitigation strategies for determining causality in humans.

PATHOGENESIS

Clostridioides difficile infection

As a complete overview of the pathogenesis of CDI is beyond the scope of this review and has been discussed elsewhere,[14] we will highlight *C. difficile* host-microbial interactions that may influence therapeutic efficacy of FMT (see Figure 1).

Colonization and germination of *C. difficile* spores are critical in initiating this toxin-mediated infection, usually occurring in the context of antibiotic-induced dysbiosis. This process is facilitated by adherence to the mucus layer and microbe-microbe interactions with mucin-degrading bacteria. *C. difficile* uses intestinal mucin as a chemoattractant and energy source with the aid of other gut microbes including *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, and *Ruminococcus torque*.[15] *C. difficile* has been shown to form intestinal biofilms *in vivo* with substantial co-colonization with *Fusobacterium* species.[16] These processes result in an acidic intestinal mucus layer, which consists of a higher level of MUC1 and lower MUC2 production.[17]

Toxins are a major virulence factor, and many *C. difficile* ribotypes produce up to three distinct toxins: toxin A (TcdA), toxin B (TcdB), and *C. difficile* transferase or binary toxin (CDT). Almost all clinically significant *C. difficile* strains produce TcdB, and the epidemic BI/NAP1/027 strain can produce all three toxins. The translocation of *C. difficile* toxins through receptor-mediated endocytosis leads to pore formation in the endosomal membrane, resulting in actin cytoskeleton disruption and cell rounding, changes in cytokine secretion, impaired cell proliferation, and barrier integrity;[18] these manifest clinically as diarrhea. Moreover, the induction of cellular apoptosis, especially by TcdA, may contribute to the development of pseudomembranes, crypt damage, and necrotic lesions.[19] Activation of inflammatory transcription factors such as mitogen-activated protein kinase (MAPK) and nuclear factor kappa B (NF-κB) trigger colonic inflammation and the acute influx of host immune cells.[20] Also, CDT toxin can trigger the activation of MAPK and NF-κB downstream in a toll-like receptor 2 (TLR2)/TLR6–dependent manner.[21]

In addition to secreting toxins, the vegetative cells express other intrinsic immunogenic factors. *C. difficile* cell wall peptidoglycan (PG) can stimulate CXC chemokine ligand 1 (CXCL1) production and neutrophil infiltration in a nucleotide-binding oligomerization

domain 1 (NOD1)–dependent manner. *C. difficile* surface layer proteins (SLPs) are also involved in the activation of the host innate and adaptive immune response through their effects on the maturation of dendritic cells (DCs).[22] *C. difficile* flagellin stimulation by TLR5 also results in the activation of NF-κB and p38 MAPK in the host epithelial cells. Additionally, TcdB pre-treatment *in vitro* can potentiate flagellin-induced inflammatory cytokine secretion.[23, 24]

The interaction of *C. difficile* with other enteric pathogens can also influence its fitness and virulence. Enterococci, enriched in *C. difficile*-infected gut, co-localizes with *C. difficile* in the lumen and in biofilms, markedly enhancing *C. difficile* colonization and survival.[25] Mutually beneficial, *C. difficile* toxin production is enhanced in the presence of *Enterococcus faecalis*, and *E. faecalis* growth is significantly increased in the presence of *C. difficile* toxin in mouse models.[25] Additionally, the core metabolism of *C. difficile* is significantly altered in the presence of enterococci through the arginine deiminase pathway: *E. faecalis* depletes arginine and exports high levels of extracellular ornithine, which in turn can be utilized by *C. difficile* for energy.[25]

C. difficile recurrence

Approximately 50% of rCDI cases result from reinfection by the original strain.[26] The recommended therapy for CDI, vancomycin, contributes to recurrence risk because it is a broad-spectrum antibiotic against Gram-positive bacteria and thus further perpetuates dysbiosis and a loss of colonization resistance. Other risk factors for rCDI include advanced age, concomitant antibiotic use, gastric acid suppression, gastrointestinal surgery, chemotherapy, hematopoietic stem cell transplant, cirrhosis, inflammatory bowel disease (IBD), prior CDI, and infection with a hypervirulent strain such as NAP1/B1/027.[27, 28] Additionally, recent evidence suggests that fibronectin-$\alpha_5\beta_1$ and vitronectin-$\alpha_v\beta_1$–dependent endocytosis of *C. difficile* spores into gut mucosa significantly contributes to spore persistence

and rCDI, because *bclA3* gene deletion or pharmacological inhibition of spore internalization reduces recurrence in a mouse model.[29] Adaptive host immune responses against TcdA and TcdB may offer some protection against rCDI, because high antibody titers are associated with reduced risk;[30] bezlotoxumab, a monoclonal antibody against TcdB, not TcdA, has been shown to reduce rCDI risk by 40%.[31]

Fecal microbiota transplantation

FMT is the process of transferring fecal matter from a carefully screened healthy donor into the gastrointestinal tract of a recipient in order to directly change the recipient's microbial composition and confer a health benefit.[32] Several practice guidelines, including those from the Infectious Diseases of America and the American College of Gastroenterology, have recommended FMT following the second recurrence, or third episode of CDI.[33, 34] FMT is regulated in the United States as a biological agent by the Food and Drug Administration (FDA). The use of FMT to treat rCDI is under FDA enforcement discretion,[35] and the source of FMT is largely supported by "stool banks" operated by clinical investigators or by OpenBiome. Recently, the FDA approved Rebyota, the first fecal microbiota product for the prevention of rCDI.[36] Simultaneous with this decision, the FDA also modified its previous guidance on FMT requiring stool banks who provide FMT products to comply with investigational new drug (IND) requirements.[35] Given the high success rate of FMT in preventing *C. difficile* recurrence,[37] there is intense interest in applying this treatment to other chronic conditions associated with intestinal dysbiosis, such as ulcerative colitis.[38, 39] Therefore, FMT for rCDI has become a paradigm for studying the consequences of host-microbial interactions in relation to pathology, and important aspects such as cause and effect relationships.[40] In the following sections, we will summarize mechanisms thought to contribute to the prevention of rCDI (see Figure 2).

Potential mechanisms contributing to FMT efficacy in rCDI

1. Restored microbial ecology

The term "microbiome" technically refers to communities composed of all microorganisms including bacteria, fungi, viruses, protozoa, and parasites, as well as their collective genomes and metabolites in the environment in which they reside. To date, most research has focused on bacterial diversity and community structure before and after FMT.[41] Other components of the microbiome including commensal fungi (the "mycobiome") and viruses (the "virome") coexist and interact in ways that may contribute to FMT efficacy,[42, 43] but these aspects remain understudied.

1.1 Effect of FMT on the gut bacteriome

Prior to FMT, the fecal bacterial community profile of rCDI patients has low diversity and richness with an over-abundance of potentially pathogenic and putatively inflammatory Proteobacteria, oral bacteria, and oxygen-tolerant bacteria.[11, 44-47] As early as 7 days after successful FMT, studies have shown a consistent microbial shift, with increased relative abundance of Bacteroidetes and Firmicutes and decreased relative abundance of Proteobacteria; this increased diversity and richness resemble the composition of the donor.[7, 11, 44, 46] Using shotgun metagenomics sequencing technology, Aggarwala and colleagues tracked bacterial strains of both donor and recipient and found that 70% of the donor strains, mainly from the orders of Bacteroidales and related Clostridiales, colonized the gut of 13 recipients and persisted up to 5 years post-FMT.[48] Bacteroidetes (especially *Prevotella* species) enrichment after FMT modulates Bacillis/Clostridia ratio in rCDI patients with concurrent IBD.[49] Higher levels of bacterial engraftment, as compared with FMT in other pathologies, were also detected for rCDI patients in three studies using strain-level resolution metagenomics.[11-13] Podlesny and colleagues showed that FMT not only resolved taxonomic (i.e., lower α-diversity, altered β-diversity) and functional (i.e., increased relative abundance of oral and oxygen-tolerant species) features of dysbiosis but also resulted in contributions of

60–90% of donor strains in the recipient microbiota.[50] Modeling using meta-analyses of metagenomics data from a wide array of pathologies established that strain engraftment is linked to antibiotic treatment, lower recipient α-diversity, and higher ratio of species abundance in the donors than in the recipients,[11, 12] suggesting that high engraftment in rCDI is facilitated by antibiotic-induced dysbiosis and reduced colonization resistance. Given the high success rates of FMT in rCDI, it is likely that engraftment of donor strains plays a key role in re-establishing colonization resistance and preventing CDI recurrence. However, because the recent metagenomics studies compared pre- and post-FMT only in cases with successful treatment outcomes, it is not possible to link engraftment to clinical outcomes. Additional studies are needed to confirm that bacterial engraftment is necessary and essential for FMT to work.

The recent strain-level metagenomic analyses provide an ecological framework for the effects of FMT.[11, 12] Although the ecological dynamics after FMT are complex (with several ecological processes at play), these studies support the importance of deterministic, niche-based processes for post-FMT microbiome assembly, specifically the competition between and exclusion of closely related recipient and donor strains.[11] The outcome of such competition is determined by the fitness of the strains and the relative fitness (adaptation to the gut environment) differences of the incoming and recipient strains. Priority effects, which favor early arriving strains at an ecological site,[51] generally support recipient strains in undisturbed communities,[11] and provide an explanation for the low levels of strain engraftment in patients with undisrupted microbiota.[11] In rCDI patients, depletion of the resident microbiota by antibiotics frees up ecological niches, resulting in increased donor strain engraftment, and effectively overcoming priority effects. In the absence of further perturbation such as repeated antibiotic exposure or underlying chronic conditions linked to dysbiosis such as IBD, the newly established microbial community appears to remain stable over time.[52]

1.2 Effect of FMT on the gut virome

A stable and individual-specific viral community exists in a healthy human gut, dominated by temperate bacteriophages, mostly members of crAss-like, *Caudovirales*, and *Microviridae* bacteriophages.[53-55] While phages act as important modulators of bacterial community structure and metabolism, and their metagenomic composition has been associated with specific diseases, much remains unknown about their actual behavior in the gut.[56] Recently, bacteriophages have been shown to modulate both taxonomic composition and functional capacity of the gut microbiome.[56-58] For example, Hsu and colleagues showed that bacteriophage transfer in a mouse model nearly altered all KEGG pathways, including amino acids, peptides, carbohydrates, lipids, nucleotides, cofactors, vitamins, and xenobiotics.[56] Campbell and colleagues showed that *Bacteroides* phage BV01 altered the genome-wide transcriptome profiles of bile acids *in vitro*.[58] However, few studies have examined the gut virome/phageome in the context of FMT for rCDI. They have observed different gut viral abundance and compositions between rCDI patients and stool donors, as well as changes in the recipient virome following FMT. However, our current understanding of the causal role of the virome/phageome on the effects of FMT in rCDI remains vastly incomplete because most studies remain correlative. For example, high levels of donor-derived *Caudovirales* bacteriophages in the recipients are associated with FMT efficacy in a preliminary study.[42] Successful FMT is also positively correlated with the relative abundance of temperate crAss phages, a phage thought to predate on members of the genus *Bacteroides*, decreased in rCDI patients.[59-61] Fujumoto *et al.* found increased proportion of *Microviridae* in association with decreased abundance of Proteobacteria in rCDI patients after FMT, suggesting a potential role of lytic *Microviridae* in modulating bacteriome.[41] Additional evidence supporting the role of the virome in FMT efficacy comes from a pilot study where five patients did not have further CDI recurrence after receiving sterile fecal filtrate. In that study, remarkably, the viral

composition of the recipient, consisting of mostly *Caudovirales*, resembled that of the donor after treatment, while the bacterial composition did not.[62] Further supporting evidence of how the virome may contribute to FMT efficacy stems from the use of *C. difficile* targeted phage therapy. For example, Nale and colleagues used a cocktail of four *C. difficile* myoviruses (CDHM1, 2, 5, and 6) to successfully inhibit *C. difficile* growth and toxin production in a batch fermentation model.[63] Meader *et al.* showed that specific bacteriophages substantially reduced *C. difficile* burden in a human colon model.[64] Furthermore, colonization of eukaryotic viruses following FMT may contribute to therapeutic efficacy since their presence is critical for gut homeostasis by modulating both the host immunity and the resident microbiome.[65-67]

The mutualistic and antagonistic interactions between bacteriophages/eukaryotic viruses, bacteria, and the human host will remain difficult to entangle. Other challenges that need to be overcome include technical limitations of viral enrichment in biological samples, extraction and sequencing library biases toward dsDNA viruses, removal of ssDNA and RNA viruses, the limited number of annotated viral genome sequences available in reference databases, and the need to refine and modify methods for viral phylogenetics and taxonomic classifications.

1.3 Effect of FMT on the gut mycobiome

Many species of fungi have been detected in the healthy human gut, and may play an important role in intestinal homeostasis and disease pathogenesis. Fungi capable of growing in and colonizing the gut are limited to a small number of species, mostly *Candida* yeast.[68] An increase in *Candida* spp. has been consistently observed to inversely correlate with bacterial diversity across many chronic diseases,[43, 69, 70] thus this can be seen as a marker of dysbiosis. Other commonly detected fungi have dietary or environmental sources (*Saccharomyces* and *Aspergillus*) and likely also contribute to microbial ecology.[68]

Few studies have examined the gut mycobiome in the context of FMT for rCDI. A higher relative abundance of *Saccharomyces* and *Aspergillus* has been reported in CDI recipients after successful FMT, whereas non-responders displayed a prominent presence of *Candida.*[43] In addition, the abundance of *Candida albicans* in donors and *Yarrowia* spp. in recipients prior to FMT are shown to be negatively correlated with FMT efficacy.[43, 71] Anti-fungal treatment such as nystatin was associated with the re-establishment of FMT efficacy in a mouse model[29] and simultaneously altered the gut mycobiome. However, understanding the effect of the mycobiome on FMT is in its infancy, and further studies are required to characterize the role of the mycobiome after controlling for dietary sources. It is crucial to improve methodological shortcomings in characterizing mycobiota. Specifically, the 18S rRNA gene sequence typically outperforms other markers [e.g., internal transcribed spacer 1 (ITS1), ITS2, and 28S rRNA] because of its ability to amplify and discriminate different species. However, the multicopy nature of rRNA regions in several filamentous fungi results in a strong bias toward those with more copies. In contrast, the ITS region is the standard marker for fungal DNA barcoding. However, primers amplifying ITS1 lead to preferential amplification of on-Dikarya while ITS2 is biased toward ascomycetes.[72] Lastly, the length of the ITS1 and ITS2 markers vary from 50 bp to several kb.[73] Incorrect mapping, and thus classification, leads to the inclusion of false positives or exclusion of valid taxa associated with FMT.

2. Changes in microbial-derived metabolites

Broadly speaking, the two classes of metabolites best described in FMT studies are bile acids and short-chain fatty acids (SCFAs); these are reviewed below.

2.1 Bile acids

Bile acids are steroids synthesized in the liver from cholesterol that facilitate absorption of fat and fat-soluble nutrients and also act as signaling molecules.[74] They regulate glucose and

energy metabolism as well as bile acid homeostasis through the Farnesoid X receptors (FXR)–fibroblast growth factor (FGF) axis. Primary bile acids produced by the host are exclusively transformed into secondary bile acids through bacteria upon secretion into the intestine, and both forms have been demonstrated to impact *C. difficile* pathogenesis in addition to host physiology.

Two key gut bacterial enzymes, absent in mammals, are known to facilitate bile acid transformation. First, taurine and glycine groups can be deconjugated by bile salt hydrolases (BSH), encoded by genes widely distributed in commensal bacteria including Bacteroidetes, Firmicutes, and Actinobacteria. Further transformation can occur via a second step, catalyzed by baiCD encoded 7α-dehydroxylase, that transforms primary bile acids [cholic acid (CA) and chenodeoxycholic acid (CDCA)] to their respective secondary bile acids: deoxycholic acid (DCA) and lithocholic acid (LCA). While only a small fraction of bacteria, mainly clusters XIVa and IV Clostridia (e.g. *Clostridium scindens*), contain 7α-dehydroxylase,[75, 76] recent studies suggest an underappreciated role for the microbiota in producing other secondary bile acids, including conjugation to other amino acids.[77]

A large body of work has established the important contribution of bile acids to CDI pathogenesis based on *in vivo* and correlative studies. *C. difficile* spores possess a soluble pseudoprotease receptor, CspC, which is stimulated by cholate-derived bile acids, promoting its germination.[78, 79] However, this process is competitively inhibited by CDCA.[80] *In vivo*, the inhibitory action of CDCA is probably limited by its low abundance after enterohepatic recirculation.[81] Collectively, members of the cholic acid family, including taurocholic acid (TCA) potently induce *in vitro C. difficile* spore germination, while members of the CDCA family, including LCA and ursodeoxycholic acid, inhibit spore germination and growth.[79, 80, 82]

Increased levels of primary bile acids (especially TCA) coupled with diminished secondary bile acids have been observed in rCDI patients prior to FMT. This altered bile acid composition is restored following successful FMT to resemble that of healthy donors,[83-85] which is associated with reversal of intestinal dysbiosis. Furthermore, increased levels of primary bile acids were also accompanied by reduced BSH levels in rCDI patients prior to FMT.[83] Successful FMT has been shown to enrich the gut for BSH-producing microorganisms and restore BSH functionality.[85] Similarly, the *bai*CD operon coding for 7α-dehydroxylase was also lacking in the pre-FMT samples.[85] In a landmark study, Buffie and colleagues demonstrated that colonization resistance against *C. difficile* could be established by the administration of a single bacterium, *C. scindens*, to antibiotic-treated mice, resulting in the recovery of microbial 7α-dehydroxylase activity that increased DCA levels.[86] Furthermore, *C. scindens* has an inhibitory effect on *C. difficile* through the secretion of tryptophan-derived antibiotics, 1-acetyl-β-carboline and turbomycin A, which is further augmented in the presence of DCA and LCA.[87]

While bile-acid transforming bacteria appear to be involved in *C. difficile* resistance, the host can also modulate circulation of primary bile acids depending on their presence. Bile acids vary in degrees of affinity for FXR receptors, with CDCA being the most potent endogenous agonist, and DCA and LCA being moderate agonists.[88] Following FMT, the increased levels of secondary bile acids LCA and DCA and reduced primary bile acids CDCA and CA are associated with upregulation of ileal FXR signaling and a rise in circulating FGF-19 in rCDI patients. The rise in secondary bile acids LCA and DCA and their moderate but collective activation of the FXR receptors may compensate for the decreased level of a more potent ligand CDCA,[88] resulting in reduced hepatic primary bile acid synthesis through a negative feedback response,[81] creating an unfavorable environment for *C. difficile* germination. Although these are interesting preliminary human data, it would be challenging to validate the

impact of FXR signaling on *C. difficile* in a mouse model since tauro-beta-muricholic acid, a naturally occurring FXR antagonist in mice, is not found in humans.[89]

The concept that restored bile acid metabolism plays a key role in establishing colonization and FMT efficacy was recently challenged by Aguirre and colleagues.[90] Using a germ-free mouse model with *Cyp8b1*$^{-/-}$ mutation (cholic acid deficiency), they observed no difference in disease susceptibility between *Cyp8b1*$^{-/-}$ and *Cyp8b1*$^{+/-}$ strains mono-associated with *C. scindens*, despite the absence of cholate-derived secondary bile acids in the *Cyp8b1*$^{-/-}$ mice. This suggests that 7α-dehydroxylation is dispensable for protection against CDI. The authors demonstrated the ability of *C. difficile* to use the Stickland pathway to metabolize amino acids to support its growth in the gut, as evidenced by lower proline and glycine (Stickland substrates) and increased 5-aminovalerate (a Stickland metabolic product) in mice mono-colonized with *C. scindens*, suggesting bile acid-independent mechanisms for *C. difficile* to overcome colonization. The importance of Stickland metabolism for *C. difficile* was also recently observed in another independent study, where mono-colonization of germ-free mice with another amino acid-fermenting bacterium, *Paraclostridium bifermentans*, could attenuate CDI.[91]

2.2 Short-chain fatty acids (SCFA)

SCFAs are produced by the gut microbiota during the anaerobic fermentation of carbohydrates and amino acids. Once produced, they are absorbed by intestinal epithelial cells (IECs). A multi-omics analysis first demonstrated markedly reduced SCFA concentrations in antibiotic-treated murine models.[92] The same study noted a higher concentration of SCFAs provided protection from *C. difficile* growth. An analysis of the human stool samples following FMT also showed a recovery of the major SCFAs: acetate, propionate, and butyrate.[93] Furthermore, this increase was positively correlated with the FMT-induced restoration of

unclassified families of Clostridiales and Firmicutes families such as *Lachnospiraceae* and *Ruminococcaceae*, known SCFA producers.[94]

The administration of butyrate in an acute CDI mouse model directly promoted the maintenance of the gut epithelial barrier via a hypoxia-inducible factor (HIF)-1 dependent mechanism.[95] Butyrate-activated stability in HIF-1 increases epithelial tight junctions of IECs and can potentially resist *C. difficile* toxin-mediated damage. Although this study found no influence of butyrate on toxin production or *C. difficile* colonization, it did identify direct protection of IEC integrity and a butyrate-mediated immune-modulatory effect by increased anti-inflammatory cytokine interleukin-10 (IL-10) and decreased pro-inflammatory cytokine IL-6 and chemokine ligand 1.[95] Additionally, butyrate attenuated intestinal inflammation by facilitating the extrathymic generation of T regulatory (Treg) cells, a population of T cells able to suppress inflammation that are discussed further in the adaptive immune section below.[96]

Another SCFA, valerate, may also play an important role in mediating FMT efficacy. In a chemostat model of CDI, the recovery of valerate was observed only after FMT, unlike other SCFAs that recovered upon antibiotic cessation.[97] While valerate inhibited vegetative growth of several *C. difficile* ribotypes in a dose-dependent manner, it had no effect on other commensals.[97] The 95% reduction of *C. difficile* total viable counts in mice after oral administration of valerate, and the sustained post-FMT recovery of donor-like valerate concentration in rCDI patients stool samples, further validate the importance of valerate both *in vivo* and *in vitro*.[98]

3. Immune-mediated mechanisms of FMT

Although CDI pathogenesis is largely due to the actions of TcdA and TcdB on the IECs,[99] most FMT studies have focused on clinical and microbiota-related changes, while immune-related changes remain poorly understood. This section will focus on what is known

about the immune effects of FMT in *C. difficile* patients and how the microbiome influences the immune system.

3.1 Effects on innate immunity

The innate immune system primarily responds to microbiota in a non-specific manner by cell surface pattern recognition receptors (PRRs) binding to microbe-associated molecular patterns (MAMPs). Activation of PRR signaling results in inflammation and recruitment of phagocytic cells, such as macrophages and neutrophils. Therefore, some FMT-induced inflammation may be protective in eliminating residual *C. difficile* via phagocytosis, while an ideal treatment would have minimal induction of pro-inflammatory cytokines, like IL-23 and IL-6, against novel commensal strains.

Eosinophils are an important innate immune cell at mucosal surfaces and may have a protective role in CDI, with undetectable eosinophil counts associated with increased in-hospital mortality and severe sepsis.[100] Similarly, a mouse study found virulence of the NAP1/027 *C. difficile* strain was enhanced by suppressing the eosinophilic response through binary toxin CDT.[101] Interestingly, restoration of the microbiota-regulated cytokine IL-25 drove colonic accumulation of eosinophils in mice and protected against CDI,[102] and a higher level of IL-25 was found in colon biopsies after FMT of CDI patients than in the pre-treatment biopsies.[103] Similarly, another microbiota-regulated cytokine, IL-33, increased following FMT in mice and could prevent CDI-associated mortality by activating group 2 innate lymphoid cells.[104] Understanding which commensals enhance secretion of these “protective” cytokines and attenuate “damaging” cytokines will help to determine the ideal FMT composition.

3.2 Effects on adaptive immunity

The key adaptive immune cells are T and B cells, which mediate long-lived cellular and humoral immunity, respectively. The concept that adaptive immunity may contribute to FMT efficacy is supported by a study showing that, in mice lacking T and B cells, CDI persisted

after FMT while immunocompetent mice fully recovered.[105] The two main types of T cells are cytotoxic $CD8^+$ T cells and helper $CD4^+$ T cells (Th), with Th cell subsets including type 1 (Th1), type 2 (Th2), type 17 (Th17), and regulatory (Treg) cells, which encompass subsets that express the transcription factor FOXP3 ($FOXP3^+$ Tregs) and those that are $FOXP3^{neg}$ but secrete high levels of IL-10 (Tr1 cells). Tregs can recognize both self and foreign antigens and play crucial roles in maintaining self-tolerance as well as preventing immunopathology through restraining inflammatory responses and mediating tissue protective and restorative effects.[106] Strikingly, a study of CDI in mice deficient in Tregs found they had increased inflammatory mediators, compromised engraftment of donor bacteria, and could not be cured by FMT.[105] Various metabolites, such as SCFAs and vitamins A and D, have been shown to increase Treg numbers and/or function,[107] and more recently, a role has emerged for secondary bile acids in promoting Treg development. Therefore, it is possible that FMT outcome may be influenced by a recipient's diet, since a diet rich in particular metabolites can activate Tregs that will establish and maintain tolerance to donor microbiota.

Th17 cells, which secrete IL-17A, play an important role in gut homeostasis and anti-fungal responses, while IFNγ-secreting Th1 (and $CD8^+$) cells are involved in important responses to intracellular pathogens, and IL-4-producing Th2 cells are involved in responses to parasites. However, in a dysregulated gut both Th1 and Th17 cells can drive excessive inflammation.[108]

Recent work has identified robust $CD4^+$ T cell responses to TcdA and TcdB in CDI patients with these responses largely composed of Th17 cells. Importantly, rCDI patients had significantly reduced levels of circulating TcdB-specific Th17 cells[109] compared with healthy controls. In a follow-up study, it was identified that successful FMT results in a considerable increase in TcdB-specific Th17 cells in rCDI patients, with preliminary data showing that, post-FMT, these cells had increased secretion of IL-17A and IL-22 cytokines.[110]

A simultaneous increase in systemic anti-toxin IgA and IgG levels was also detected after successful FMT,[110] which has been previously associated with a reduced risk of CDI recurrence.[108] Consistent with the findings of Cook *et al.*, FMT-induced recovery of CDI in immunocompetent mice was associated with successful engraftment, increased Th17 cells, and increased levels of IL-17A and IL-22 in the large intestine lamina propria.[105] However, Th1 cell-deficient (*Tbet*$^{-/-}$), *IL-17A*$^{-/-}$, and *IL-22*$^{-/-}$ mice all recovered following FMT, suggesting that the FMT-mediated CDI cure in mice is not solely dependent on Th1 or Th17 cells.[105] It has also been proposed that immunosenescence (age-associated immune decline) may contribute to FMT failure,[111] as an observational study of four patients receiving sequential FMT for antibiotic refractory fulminant CDI found increased circulating immunosenescent cell populations in a non-responder compared with three FMT responders.[111] These data suggest that another mechanistic function of FMT is shaping the total and TcdB-specific CD4^{+} T cell repertoire, and potentially inducing an anti-aging effect. Taken together, these data suggest that an ideal FMT composition will need to activate a precise, and as yet undetermined, balance of Tregs and other Th cells to preserve intestinal homeostasis. Mechanistic insights gained from *in vivo* studies highlight the significance of colon-specific immune responses, which require further validation in clinical studies.

Although these human studies of FMT-treated CDI are largely preliminary, they suggest that the adaptive immune system is essential for FMT engraftment, and that anti-toxin antibodies, Treg and Th17 cell functions are associated with increased efficacy.[105, 109, 110] From studies of host-microbe interactions in the gut, we know how important the microbiome is in shaping immune development, tolerance, and long-lived immunity. Therefore, a big question that remains unexplored is how changing the microbiome through FMT may affect long-lived protective immunity, with one study showing reduced T cell responses to a childhood vaccine post-FMT.[110] It will be critical for larger studies to assess immune changes, ideally in both

peripheral blood and gut tissue, in parallel with microbiome/metabolome changes to understand the complete mechanisms underpinning the FMT efficacy.

4. Epigenetic-related mechanisms

In CDI, gut dysbiosis and reduced microbial diversity are likely to alter the levels of nutrients and metabolites, impacting epigenetic pathways and altering gene expression. Recently, FMT has emerged as a useful tool to explore the interrelation between microbiota composition and microRNA (miRNA) expression. To this effect, one study has reported the suppression of circulating miRNAs, small non-coding RNAs that post-transcriptionally regulate gene expression, in two independent cohorts of rCDI patients.[112] This effect was subsequently reversed following successful FMT and replicated in FMT-treated mouse models and *ex vivo* human colonoids. Analyses confirmed that TcdB mediated the suppressive effects of CDI on the miRNAs by dysregulating *Drosha* expression, an enzyme that plays a prominent role in miRNA biogenesis.[112]

Specific miRNAs that were upregulated in both rCDI patients and mouse models following successful FMT included miR-26b, miR-23a, miR-150, and miR-28-5p. Overexpression of these miRNAs in human blood resulted in reduced mRNA levels of *FGF-21*, *IL-12B*, *IL-18*, and *TNFRSF9* inflammatory gene targets, respectively.[112] In the same study, the investigators also determined that combined overexpression of miR-23a-3p and miR-150-5p could protect against TcdB-induced damage to the IEC (see Figure 3). There is still limited understanding of the impact of FMT on the human circulating, fecal, and tissue miRome and wider host epigenome, and further mechanistic studies are required to investigate the long-term epigenetic effects of FMT for rCDI and other disease states associated with gut dysbiosis. Future studies will necessitate comprehensively mapping epitranscriptomic changes associated with CDI, FMT, and dietary manipulation strategies. FMT-regulated miRNAs may represent

unique therapeutic targets, which alone or combined with live biotherapeutics, may augment therapeutic efficacy against *C. difficile* and help counteract drug resistance.[113]

Insights and future directions

As highlighted in this review, our mechanistic understanding of how FMT works in rCDI is still incomplete. Ecological, metabolic, immunological, and epigenetic mechanisms have all been studied at different depths (summarised in Table 1), but their individual contributions remain unclear, and the causal components (bacteria, viruses, fungi, specific metabolites) that contribute to clinical efficacy are still not fully elucidated. The recent meta-analyses[11-13] that combined metagenomics with strain-level resolution and predictive modeling clearly established antibiotic treatment, recipient factors (e.g., α-diversity and species distribution), and donor-recipient complementarity as important determinants of engraftment, but these studies were not sufficiently powered to determine if engraftment is necessary for a successful outcome. Nevertheless, the ecological and statistical approaches established provide frameworks that will inform future studies.

Even though FMT is highly effective in preventing rCDI, we still do not understand why a small portion of rCDI patients do not benefit from FMT. Additional ecological factors, such as diet and host genetics, have not been considered in studies of FMT in rCDI, and may potentially be the missing links to these "failed" FMT cases. Although diet has been proposed to be relevant because it affects the ecology of microbiome dynamics after FMT, the topic has received virtually no experimental validation. As the relative importance of host genetics on microbiome assembly is rather low (and explains <10% variation),[114] diet might influence FMT outcomes in several ways. First, the diet of the recipients would influence the diversity of substrates and resources (in the form of nutrients) that are available for the incoming microbes, therefore directly influencing the niches available for engraftment. As such, pairing donor-

recipient combinations based on their dietary patterns and preferences could further optimize efficacy, because the donor microbiota would be pre-adapted to the recipient's diet. Other knowledge gaps to be addressed include whether engraftment or live bacteria are necessary for efficacy, how other non-bacterial components modulate microbial ecology, what relative contributions of adaptive or innate immune response play in outcomes, or whether specific immunological factors such as immune senescence[115] or low IgA diversity may affect efficacy.[116]

Clinical and mechanistic insights provided by FMT in rCDI have extended the potential therapeutic value of FMT to other dysbiosis-associated chronic conditions. A recent search on clinicaltrials.gov yielded 429 studies utilizing FMT in a variety of conditions (accessed on Feb 7, 2023), highlighting the intense interest surrounding microbiome-based therapeutics. It is worth noting that the pathogenesis of many chronic conditions is complex and multifactorial, where dysbiosis is only a piece of the puzzle, and potentially not causal to the pathology.[117] For example, IBD is thought to be caused by immune dysregulation, gut dysbiosis, environmental triggers and genetic susceptibility. Thus, the magnitude of therapeutic benefits and the degree of engraftment following FMT in these chronic conditions would not be expected to be as high or as durable as in rCDI, where dysbiosis is the main pathogenic driver.[11, 12] The best example is in ulcerative colitis (UC), a form of IBD, the indication for which the strongest evidence from randomized, placebo-controlled trials exists. Irrespective of how FMT was delivered, how frequently FMT was given, or whether the FMT was from a single donor or from pooled multiple donors, the remission rate in mild to moderate UC was only 30-40%.[38, 39, 118, 119] Furthermore, all these studies are relatively short term, with primary outcomes assessed around 7-12 weeks after FMT. Additionally, many responders during the trials ended up with disease flares after they completed the trial.[38] FMT for irritable bowel syndrome, on the other hand, has generated conflicting results in randomized, placebo-controlled trials, with

some studies demonstrating modest efficacy,[120, 121] while others showed no benefits.[122-124] Promising preliminary results also came from FMT for other indications, such as metabolic syndrome,[125, 126] hepatic encephalopathy,[127] check point inhibitor induced colitis,[128] graft-versus-host disease,[129, 130] decolonization of multidrug-resistant organisms,[131] to name a few. However, much remains unknown, such as how to select patients most likely to respond favorably to FMT, how to design optimal dosing regimens, or how to improve durability of responses.

Although highly effective, FMT also has several disadvantages. First, there is a risk of transmitting an infectious agent because stool is sourced from a donor, and such risk is highlighted by a death due to extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* which prompted the FDA safety warning.[10, 132] Emerging pathogens detectable in stool, such as SARS-CoV-2 and monkeypox virus contribute to FMT safety concerns,[133, 134] making stringent donor testing protocols essential. Second, the composition of each FMT treatment is not known and even varies over time from the same donor. Stool is a complex mixture, and the current regulatory frameworks for drugs do not apply, making regulatory approvals challenging. Third, there is a lack of long-term safety data. As many chronic conditions are associated with intestinal dysbiosis, a donor phenotype could potentially be transferred to a recipient. Fourth, the precise mechanisms of action of FMT remain unknown. Better treatment options for rCDI that are targeted, safe, and donor independent are thus desired.

By recognizing the disadvantages of FMT, a reductionist approach has been taken with the development of more refined live biotherapeutic products. A mixture of 6 phylogenetically diverse intestinal bacteria can resolve relapsing CDI in mice.[135] Furthermore, SER-109,[136] a product containing only the spore-forming Firmicutes, and MET-2,[137] a product which contains 40 strains of rationally selected commensal microbes, have shown promise in treating rCDI

patients, with success rates, 88% and 79%, respectively, comparable to that seen with FMT. Interestingly though, a mixture of 12 strains of bacteria is not as effective as FMT for rCDI patients in a randomized clinical trial, showing efficacy of 52% when compared with 76% in FMT.[138] They highlight that full microbial spectrum in FMT is not required for clinical efficacy, at least for rCDI. Perhaps there are key strains that can provide "scaffolding" or early functional restoration, and they are essential and permissive for medium and late colonizers. The minimum number of microbes or which microbes required in a consortium to retain efficacy, and whether this is host-factor dependent remain unknown.

To deepen our understanding of FMT mechanisms and to establish causality, human intervention trials using not just stool, but stool derivatives with defined compositions and characteristics, or with defined consortium of bacterial, viral, and metabolic components alone and/or in combinations, will serve as an important experimental platform. These trials should use well-defined outcomes, and combine multi-omics (metagenomics, transcriptomics, proteomics, metabolomics), host based (immune phenotyping), and dietary or other environmental factors that analyse samples from both recipients and donors with predictive modeling (e.g., with machine learning or artificial intelligence) using an ecological framework to determine the relative importance of major determinants of clinical and ecological outcomes. The challenges of integrated multi-omics research not only lie with addressing the shortcomings of each "omic" technology, but also how to integrate different molecular data sets. Data libraries need to be further developed, particularly for virome and metabolome. Additional bioinformatics tools are required to standardize normalization and integration of multi-model experiments. Ideally, clinical assessment and sampling in the recipients should be longitudinal to allow statistical approaches (e.g., mediation analyses) that permit the identification of causal factors. Although animal models and other models (e.g., organoids, organs-on-chips) have limitations in their translatability, they remain important to establish

mechanisms, confirm causality, and identify causal components (see Figure 4). This work will provide information to refine FMT approaches (for example through donor-recipient pairings based on the microbiome and/or diet) while we await the development of refined and targeted biotherapeutics to replace FMT.

Declaration of Interests

D.K. has served on adjudication board for Finch Therapeutics and has received consulting fees and speaking honorarium from Rebiotix/Ferring Pharmaceuticals. The rest of the authors have nothing to declare. A.M.S. has received consultation fees from Finch Therapeutics and Rebiotix/Ferring Pharmaceuticals.

In Brief

Fecal microbiota transplantation (FMT) is highly effective in preventing recurrent Clostridioides difficile infection. In this review, we discuss its potential mechanisms of action from ecological, microbial mediated metabolic, immunological, and epigenetic perspectives. We also highlight limitations and propose future research directions.

Table 1. Hypothesized mechanisms of the action of FMT in recurrent *C. difficile* infection (rCDI)

Mechanisms	Evidence	Knowledge gaps
Restored colonization resistance through bacterial engraftment and/or modulation of non-bacterial components	Animal studies: • Defined bacterial consortium (6 strains) resolves relapsing CDI in mice[135] Human studies: • Clinical benefits associate with restored bacterial composition and diversity, and high level of durable bacterial engraftment[7, 11-13, 44-47, 50, 52] • Spore formulation (SER-109 with Firmicutes)[136] shows similar clinical efficacy to FMT in a randomized placebo-controlled trial • Defined bacterial consortium (MET-2 with 40 strains)[137] shows similar clinical efficacy to FMT in an open-label preliminary study	• Is engraftment necessary for efficacy? • What is the minimum number of bacterial strains required to retain efficacy? • How does diet influence engraftment or efficacy? • Are live bacteria necessary for efficacy?
Direct effect on *C. difficile* or through modulation of microbial ecology by virome/phageome	*In vitro* studies: • *Bacteroides* phage BV01 alter the genome-wide transcriptome profiles of bile acids[58] • Phage therapy inhibits *C. difficile* growth and toxin production[63] *Ex vivo* studies: • Bacteriophages reduce *C. difficile* burden[64] Animal studies: • Phage therapy effective in treating *C. difficile* infected mice[139]	• How do non-bacterial components, e.g. virome/phageome or mycobiome modulate microbial ecology and FMT efficacy? • Are phages alone sufficient for FMT efficacy?

	• Phage administration results in altered microbiome and metabolome[56] Human studies: • Correlation study shows FMT efficacy associates with higher levels of donor-derived *Caudovirales* in recipients[42] • Correlation study shows increased *Microviridae*, associated with decreased Proteobacteria in recipients[41] • Sterile fecal filtrate without any live bacteria is effective in preventing rCDI in an open-label preliminary study, with recipient viral composition resembling that of the donor, consisting of mostly *Caudovirales*[62]	
Inhibition of *C. difficile* growth and/or germination through bacteria-derived metabolites	*In vitro* studies: • Primary bile acids promote *C. difficile* germination and growth; secondary bile acids inhibit germination and growth[78-80, 82] Animal studies: • Mice treated with antibiotics (*C. difficile* susceptible) have increased primary bile acids and reduced secondary bile acids, as well as reduced SCFAs e.g. valerate[92] • *C. scindens* alone mediates bile acid-mediated *C. difficile* resistance[86]	• How do other microbial derived metabolites (e.g. tryptophan and indole) influence efficacy? • Is FMT efficacy bile acid and SCFA dependent?

	• Oral valerate supplement reduces *C. difficile* growth without negatively affecting gut commensals[98] Human studies: • Correlation studies show restored bile acid metabolism,[83-85, 93] and increased signaling in the bile acid-FXR-FGF pathway after successful FMT[88] • Correlation studies show increased SCFAs, including valerate in stool samples after successful FMT[93, 98]	
Modulation of host immune response	Animal studies: • Modulation of microbiota-regulated cytokine IL-33 secretion[104] • Establishment of immune tolerance to donor microbiota[105] Human studies: • Modulation of microbiota-regulated cytokine IL-25 secretion[102] • Restoration of *C. difficile* toxin-specific cellular immunity towards that seen in healthy controls[109, 110] • High level of systemic anti-toxin IgA and IgG after successful FMT[110] • Decrease in circulating immunosenescent signals in FMT responders[111]	• What are the relative contributions of innate and adaptive immune response in FMT efficacy? • How do human leukocyte antigen (HLA) polymorphisms affect engraftment or efficacy? • What is the characteristic profile of Tregs (e.g. tissue-specific adaptation and transcriptional dynamics) in FMT responders? • What is the precise role of immunosenescence in shaping FMT response? • Can we harness organ-on-chip models to determine real-time interactions between donor microbiota and host immune response? • What are the FMT effects on long-lived immune responses that were educated with original recipient microbiome?
Modulation of host epigenetic response	Human and animal mechanistic studies:[112]	• How do specific miRNAs influence the growth of specific bacterial strains?

	• Upregulation of circulating miRNAs (miR-26b, miR-23a, miR-150, and miR-28-5p) following FMT for rCDI in humans with corresponding decrease in expression of FGF-21, IL-12B, IL-18 and TNFRSF9 mRNAs, respectively) • FMT reversed the effects of *C. difficile* on circulating and cecal tissue miRNAs in a mouse model of rCDI • *C. difficile* TcdB mediates the suppressive effects of CDI on miRNAs in mouse colon and human colonoids	• How do microbial metabolites and by-products influence miRNA biosynthesis? • Are circulating miRNAs alone sufficient to account for FMT efficacy? Or can they be used as a treatment adjunct? Or as a predictor of treatment outcome? • How do rCDI and FMT impact on the fecal miRNA profile? • Which specific dietary components can influence the expression of (anti-inflammatory) miRNAs in the human host? • Can exogenous diet (e.g., plant)-derived miRNAs directly impact *C. difficile* growth?

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