Fecal Microbiota Transplantation: Current Challenges and Future Landscapes

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SUMMARY

Given the importance of gut microbial homeostasis in maintaining health, there has been considerable interest in developing innovative therapeutic strategies for restoring gut microbiota. One such approach, fecal microbiota transplantation (FMT), is the main "whole gut microbiome replacement" strategy and has been integrated into clinical practice guidelines for treating recurrent *Clostridioides difficile* infection (rCDI). Furthermore, the potential application of FMT in other indications such as inflammatory bowel disease, metabolic syndrome, and solid tumor malignancies is an area of intense interest and active research. However, the complex and variable nature of FMT makes it challenging to address its precise functionality and to assess clinical efficacy and safety in different disease contexts. In this review, we will outline clinical applications, efficacy, durability, and safety of FMT and will provide a comprehensive assessment of its procedural and administratiion aspects. The clinical applications of FMT in children and cancer immunotherapy are also described. We will focus on data from human studies in inflammatory bowel disease (IBD) in contrast with rCDI to delineate the putative mechanisms of this treatment in IBD as a model, including colonization resistance and functional restoration through bacterial engraftment, modulating effects of virome/phageome, gut metabolome and host interactions, and immunoregulatory actions of FMT. Furthermore, we will comprehensively review omics technologies, metagenomic approaches, and bioinformatics pipelines to characterize complex microbial communities and discuss their limitations. FMT regulatory challenges, ethical considerations, and pharmacomicrobiomics are also highlighted to shed light on future development of tailored microbiome-based therapeutics.

KEYWORDS fecal microbiota transplantation, human microbiome, *Clostridioides difficile* infection, microbial engraftment, donor screening

INTRODUCTION

The important role of the human gut microbiome in health and disease has been the subject of extensive research over the past decade. While the structure of a normal or healthy microbiome remains to be defined, alterations in gut microbiota composition and function—broadly termed intestinal dysbiosis or disturbed microbiota—are associated with many diseases. *Clostridioides difficile* (*C. difficile*) infection, caused by microbiota disturbance usually provoked by antibiotics, is the "poster child" of such, and the incredible success of fecal microbiota transplantation (FMT) in preventing *C. difficile* recurrence further confirms causality. This has spawned many clinical studies utilizing FMT as a research tool to modulate the intestinal microbiome for health benefits in other states with disturbed microbiota, including inflammatory bowel disease (IBD) and metabolic syndrome (MetS). The hypothetical axes of the gut with brain, lung, and liver resulted in an increased interest to develop microbiota interventions in many other diseases. These interventional trials, when conducted thoughtfully and with multidisciplinary engagement, have the potential to move beyond associative evidence and can inform potential microbial therapeutic targets in these chronic conditions. In this paper, we will first review the "healthy" gut microbiome, then we will discuss different states of disturbed microbiota and the dynamic interaction of microbiota with the human host. We will review indications where FMT has achieved variable levels of success, where FMT is recommended in clinical practice, and where promising preliminary results require further investigation. We will review evidence from clinical studies, with a focus on randomized trials and systematic reviews/meta-analyses when available. We will compare various aspects of FMT treatment regimens, outcome assessments, and potential mechanisms of action in these indications, followed by a review of FMT manufacturing practices including donor screening and selection as well as formulations and storage. A brief overview of microbiome analytical tools relevant to the study of FMT will follow. Finally,

we will summarize challenges and offer insight into knowledge gaps and potential future research directions.

GUT MICROBIOTA

Introduction to the Human Gut Microbiome

Recent advances in high-throughput sequencing techniques have improved our capacity to survey the breadth of the human gastrointestinal (GI) microbiota. Our current view of the gut microbial community has mainly been informed by the MetaHit and the Human Microbiome Project (1, 2). In health, a homeostatic gut microbiota is predominantly composed of Firmicutes and Bacteroidetes, with Actinobacteria, Proteobacteria, Fusobacteria, Verrucomicrobia, and others as minor phyla (3). The predominant genera in the Firmicutes phylum are *Clostridium*, *Lactobacillus*, *Bacillus*, *Enterococcus*, and *Ruminicoccus*, whereas *Prevotella* and *Bacteroides* are the most common genera in the Bacteroidetes phylum (4). The delicate balance of the gut microbial community is associated with metabolome output and impacts host-microbial interactions.

The sheer length, the unique functions within each segment, and the different speeds at which the luminal contents move contribute to the unique microbial compositions along the digestive tract. The oral cavity mostly harbors *Streptococcus*, *Haemophilus*, *Rothia*, *Neisseria*, and *Veillonella* genera (5), while the gastric niche is dominated by *Propionibacterium*, *Lactobacillus*, *Streptococcus*, and *Staphylococcus* genera (6). The small intestine microbiota is enriched with bile-resistant microorganisms, predominantly Gram-negative bacteria of the order of Enterobacterales (of which the family Enterobacteriaceae is most common), and facultative anaerobes of the family Lactobacillaceae (7). The reduction in the concentration of bactericidal agents, together with longer transit time in the large intestinal tract, promote the growth of fermentative polysaccharide-degrading anaerobes, especially Clostridiaceae and Bacteroidaceae (8).

The description of the microbial landscape in the gut would be incomplete without considering communities of viruses, fungi, and archaea, but they are much less studied than the bacteriome. The gut virome composition is mostly (97.7%) dominated by bacteriophages, which profoundly contribute to bacterial death and lateral gene transfer (9). However, this field remains understudied and the impact of phages on gut microbiota structure and diseases etiology is still in its infancy (10). Metagenomic evaluation of the fecal virome has led to the identification of novel bacteriophages (81%–93%) that can neither be assigned a bacterial host nor a taxonomic position. These "known unknown" bacteriophages pose a knowledge gap in gut virome research (11). The remaining phage components belong to non-enveloped DNA phages of Caudovirales, Microviridae, and Inoviridae (12).

Saccharomyces, *Malassezia*, and *Candida* are yeasts and represent the most prevalent fungal genera in fecal samples of healthy individuals (13). Eukaryotic microbes (protists) are less diverse than viruses and more patchily distributed than bacteria in the human gut (14). Notwithstanding, the influence of the gut protists, especially *Blastocystis*, on the diversity and structure of the bacterial communities merit consideration of these eukaryotic communities as ecosystem engineers (15). The gut archaeome mostly consists of *Euryarchaeota* and *Crenarchaeota* phyla and the *Methanobrevibacter* and *Methanosphaera* genera (16). Unlike the gut bacterial community, not a single archaeal species has been deemed a primary pathogen thus far. Given a high degree of inter-individual variability in the gut microbiota composition in health, it is challenging to define a "normal" or "healthy" composition. Furthermore, other aspects of the gut microbiome, such as function, should be considered when defining a healthy microbiome.

Microbiome and the Host Immune System

Microbial colonization of the human mucosal surfaces is critically involved in the education and maturation of the host immune system, especially during infancy, as exemplified in

germ-free (GF) animal models (17). The immune system in GF animals is mostly characterized by the disturbance in the development of gut-associated lymphoid tissues (GALTs). Microbial depletion affects the formation of crypt patches and isolated lymphoid follicles and leads to a substantial reduction in the size of Peyer's patches and germinal centers (18). GF animals as well as newborns demonstrate significantly fewer key elements of mucosal immunity such as immunoglobulin A (IgA) antibodies, interleukin (IL)-$17^{+}CD4^{+}$ T (Th17) cells, and B cells, which all are rapidly restored upon microbial colonization (19). Regulation of cellular signaling pathways and microbial gene expressions can orchestrate the production and secretion of cytokines, chemokines, and immune receptors (20).

The gut microbiota is associated with the structural development of GALTs through the recognition of pathogen-associated molecular patterns (PAMPs) by the host pattern recognition receptors (PRRs) (21). PRR-PAMP recognition further contributes to immune homeostasis by stimulating Peyer's patches through toll-like receptors (TLRs) to provoke the secretion of antimicrobial peptides (AMPs) (22). As the main AMPs presented in the mucus layer, defensins induce pore formation in the bacterial membrane and trap bacteria in extracellular net-like structures termed neutrophil extracellular traps (NETs) (23). Cathelicidin is the main AMP presented in infancy regardless of microbial composition, which considerably affects the early configuration of the gut microbiota (24). Following early-life development of the gut microbiota, the induction of immune tolerance is essential to regulate the host immune response. Commensal microorganisms can be discriminated from pathogens by the absence of virulence factors and low invasiveness. The inaccessibility and differential affinity of TLRs to commensals may also prevent commensals from initiating cytokine storms (25).

In addition to the direct interaction of the gut microbiota with immunoreceptors, microbial by-products further influence host immunity. As the major microbial metabolites, short-chain

fatty acids (SCFAs), mainly acetate (C2), propionate (C3), and butyrate (C4), play a critical role in preserving the integrity of the gut barrier and regulating the host inflammatory response (26). Butyrate promotes the production of tight junction proteins probably through the stimulation of the AMP-activated protein kinase signaling pathway or the suppression of claudin 2 (CLDN2) expression (27). Acetate and butyrate further enhance the gut barrier with mucin secretion (28). The effect of SCFAs on TLRs, free fatty acid receptors (FFARs), G protein-coupled receptors, and histone deacetylase regulate the activation of mitogen-activated protein kinase, c-Jun N-terminal kinase, and nuclear factor kappa B to modulate the secretion of inflammatory and oxidative agents such as IL-8, IL-6, tumor necrosis factor, monocyte chemoattractant protein-1, and inducible nitric oxide synthase (29).

GUT MICROBIOME DISRUPTION

Despite temporal fluctuations by changes in diet, acute illness, or medications, gut microbiota composition is relatively stable during adulthood (Fig. 1). Diversity measurement is important for understanding community structure and dynamics, and historically relies on bacterial species as the fundamental unit of analysis (30). Diversity within a given community (alpha diversity) is characterized by the total number of species (species richness), the relative abundance of the species (species evenness), or indices that combine these dimensions. Beta diversity, in contrast, is a measure of dissimilarity between two microbial communities (30). Although defining a healthy microbiota is not currently possible, a “disturbed microbiota” is characterized by shifts in the gut microbial composition and reduced alpha diversity with purported functional alterations in the microbial transcriptome, proteome, or metabolome. The exemplar disease with definitive causality between disturbed microbiota and illness is *Clostridioides difficile* infection (CDI). In many other chronic conditions, most with complex pathophysiology, the relationship remains associative. One common feature in many of these conditions is intestinal barrier dysfunction, which can lead

to increased oxygen tension within the gut lumen resulting in mucin degradation and alterations in redox potential and microbial community structure (31). This disturbed microbial state is purported to facilitate intestinal inflammation. Collateral damage of the host inflammatory response includes epithelial necrosis, which leads to an increased presence of phospholipids that can be utilized as carbon and/or nitrogen source by certain microbes (32). Enteric infections and gut inflammation promote elevated mucin secretion to accelerate pathogen expulsion and preserve mucosal integrity. Furthermore, disruption of the resident microbiota by antibiotics and subsequent changes in availability of the mucosal carbohydrates within an inflammatory milieu in the gut lumen can be exploited by enteric pathogens such as *Salmonella typhimurium* and *C. difficile* to expand and induce host inflammation (33, 34). Mucosal hypoxia is another attribute of the mucus layer during gut inflammation, as highlighted by the respiratory flexibility of Enterobacterales to colonize the gut in low oxygen tension by utilizing nitrate, nitrite, trimethylamine-N-oxide (TMAO), and fumarate (35). Host inflammatory response triggers the production of reactive nitrogen species by epithelial cells and neutrophils and favors *Escherichia coli* nitrate respiration (36). However, the higher oxygen concentration of the lamina propria as a result of increased blood flow in the inflamed tissue favors colonization of facultative anaerobes, preventing the proliferation of obligate anaerobes such as butyrate-producing *Clostridia* (37). Depletion of obligate anaerobes from the Firmicutes and Bacteroidetes phyla results in disrupted gut microbiota with overgrowth of low abundance taxa or potentially pathogenic bacteria, and also facilitates the transfer of virulence factors and antibiotic-resistance genes (38, 39).

C. difficile is an obligately anaerobic Gram-positive, spore-forming rod-shaped bacterium. It spreads among humans and animals through the fecal-oral route and the environment and can cause CDI by production of toxins (40, 41). CDI is considered an iconic model of intestinal microbiota disruption (Fig. 2). Generally, the exposure to *C. difficile* spores alone is not

sufficient for the clinical onset of CDI and requires the coexistence of an altered microbiome, often due to antibiotic use (42, 43). The disturbed microbiome supports spore germination, promotes growth and stimulates toxin production of *C. difficile*, and alters primary and secondary bile acids ratio (44, 45).

Although many other diseases, such as IBD or obesity, have also been associated with gut microbiota disruption, it is difficult to identify specific "microbial taxonomic signatures" because of the significant heterogeneity of various studies; however, there can be some generalization. For example, the intestinal microbiota of patients with IBD is generally characterized by reduced alpha diversity and lower temporal stability (46). Multiple studies have linked microbial taxa to IBD, including enriched proinflammatory taxa or decreased beneficial bacteria, discussed further below in the IBD section (47-49). In obesity, an increased Firmicutes-to-Bacteroidetes ratio has been reported in many studies and can facilitate a positive energy gain (50). Similarly, microbial disruption has been associated with irritable bowel syndrome (IBS), chronic liver disease, and autism; however, characterizing these microbial signatures has proven to be challenging.

Although little is known about intestinal virome disruption, emerging evidence suggests that disease-specific alterations in enteric virome composition, which do not appear to be a consequence of changes in bacterial populations, may contribute to bacterial disturbance (51). For example, an increase in viral richness, specifically Caudovirales bacteriophages, has been found in patients with IBD when compared with healthy controls (51). Furthermore, patients with ulcerative colitis (UC) show an expansion of mucosal viruses, especially Caudovirales phages and phages that prey on Enterobacteria, and this correlates with intestinal mucosal inflammation (52). The role of yeasts and fungi in the intestinal microbiome is understudied and will not be reviewed here.

FECAL MICROBIOTA TRANSPLANTATION: INDICATION WITH DEMONSTRATED EFFICACY

The following section will summarize the available evidence in rCDI where efficacy of FMT is established (Table 1).

Prevention of Recurrent *Clostridioides difficile* Infection

The incidence of CDI has increased in the past two decades (53), and has become a considerable burden for healthcare systems, especially with rCDI. Depending on host immune response and *C. difficile* ribotype, 20%–40% of patients with CDI can recur after the initial episode, and nearly 65% of these patients will experience multiple recurrences (54). The treatment of patients with rCDI is a major clinical challenge because conventional antimicrobials are largely ineffective to obtain a global cure without recurrences, although the availability of fidaxomicin and bezlotoxumab has significantly decreased recurrence rates (55, 56). FMT has been recommended by several practice guidelines to prevent further CDI in patients with at least two recurrences (57-59), with efficacy of 80%–90% (60-62). To accommodate patients who may be at risk of significant morbidity or high mortality with subsequent recurrence, the most recent American Gastroenterological Association (AGA) guidelines refrain from specifying two recurrences (63), as there is emerging evidence supporting clinical benefits even after the first recurrence (64).

Many randomized controlled trials (RCTs), systematic reviews, and meta-analyses have built evidence for this indication, examining 1) FMT relative to a comparator (placebo, autologous FMT, no intervention, or antibiotics with activity against *C. difficile*), 2) FMT by different routes of administration (enteral tube, oral capsules, colonoscopy, or retention enema), or 3) FMT by different formulations (fresh, frozen, lyophilized). A recent Cochrane review and meta-analysis of six RCTs with 320 participants assessed the efficacy of donor-based FMT for rCDI and found that FMT is highly effective at preventing CDI recurrence compared with

the control [risk ratio (RR) 1.92, 95% confidence interval (CI) 1.36–2.71; *P*=0.02; numbers needed to treat (NNT) for an additional beneficial outcome (NNT=3)] (42). Of interest, one study with 290 patients found that FMT, compared with vancomycin alone, was associated with a significant decrease in bloodstream infections within 90 days and resulted in an increase in overall survival in hospitalized rCDI patients (65). Although FMT is seen as generally safe, there is no conclusive evidence regarding the safety of FMT for the treatment of rCDI because the number of events was small for serious adverse events and all-cause mortality (42).

The clinical efficacy of FMT for patients with rCDI appears comparable with various modes of delivery (nasoduodenal tube, capsules, gastroscopy, colonoscopy, and enemas) (66-69) or formulation (fresh, frozen, and lyophilized) (68, 70, 71). Examining differences in delivery routes, for example, a systematic review including 305 participants from 14 studies found that FMT delivered via the lower GI route was more effective than via the upper route, with the risk of treatment failure of 8.5% compared with 17.9% at 90 days after FMT (72). Another study including 7 RCTs and 30 case series found the success rate to be higher with the lower GI route of delivery of 95% compared with 88% with the upper route; however, this difference was no longer significant at 81% and 87%, respectively, following a single infusion (73). Two recent studies found success rates to be superior with colonoscopy delivery compared with enema or nasogastric tube delivery but comparable to capsule-delivered treatment (74, 75). Considering different formulations, lyophilized FMT can improve the logistics of product storage and shelf life and has demonstrated clinical efficacy in an open-label cohort with as few as 2–3 capsules (total dose ≈2.1–2.5 × 10^{11} cells) (70, 76). Another small RCT compared colonoscopically delivered 50 g of donor stool in fresh, frozen, or lyophilized formulations and found cure rates of 100%, 83%, and 78%, respectively, with no statistically significant difference between the frozen and lyophilized

FMT treatments (71). Although no study has directly compared different doses, a fecal amount of <50 g is associated with lower efficacy (61). It is also not known if bowel preparation is essential prior to FMT, provided sufficient time has elapsed from vancomycin treatment, because vancomycin can persist in the colon for up to 7 days. It should be noted that these observations only apply to patients with rCDI.

Single-donor (related, or more commonly unrelated) instead of pooled multi-donor products are used in the treatment of rCDI (66-69). From a safety perspective, a single donor–derived product is safer and easier to track as there is always a 1:1 ratio between donor and recipient to mitigate potential risks of disease transmission. Moreover, a single donor also reduces the number of potential confounders, because each donor likely has a stable diet and lifestyle. Data from OpenBiome, the largest public stool bank in the US, have not indicated a donor effect in clinical efficacy of 1999 FMT-treated rCDI patients from 28 donors, with an overall cure rate of 84.4% (77). Moreover, because the success rate is high, there is no obvious advantage to consider pooled multiple-donor FMT products. Regulatory guidelines from the United States Food and Drug Administration (FDA) and Health Canada and several clinical practice guidelines also recommend that FMT products should be derived from a single donor (78, 79). On the basis of available evidence, it is difficult to conclude the ideal dosage, route of administration, or formulation of FMT for rCDI, and there is no consensus on the ideal dosage, route of administration, or formulation. FMT success may not critically depend on these variables, and how it is administered may be influenced by a clinician's evaluation of patient factors, provider expertise, healthcare infrastructure, and product availability.

350 **TABLE 1** FMT indication with demonstrated efficacy

Indication	Level of evidence	Clinical efficacy and durability	Dose/formulation/route and frequency of administration	Patient preparation and effect	Donor selection and effect	Serious adverse events	Clinical applications/comments	Potential strategies to enhance efficacy and safety	Potential mechanisms of action
Recurrent *C. difficile* infection (rCDI)	Multiple RCTs comparing FMT with a comparator or comparing different routes or formulations of administration (57, 64, 66-69, 71). Multiple systematic reviews and meta-analyses (42, 73-75).	Range from 60% to >90%. Studies including a control group tend to demonstrate lower clinical efficacy (80, 81). Treatment outcome assessed after >8 weeks post-FMT. Durable/sustained response observed (82).	Single dose, varying stool weights. Most studies used aerobically manufactured FMT (67, 68). Outcome generally not affected by formulations (fresh, frozen, lyophilization) (71) or routes of administration (enteral tube, endoscopy, enema, oral capsules) (83).	Patients on suppressive CDI directed antibiotic (e.g., vancomycin) until 24–72 hours prior to FMT (84). Vancomycin pretreatment to increase engraftment and eradicate *C. difficile*. Bowel preparation may not be necessary if suppressive antibiotic discontinued >24 hours prior to FMT, or FMT not delivered by colonoscopy (85).	Single donor. Little donor effect on clinical outcome (77). Donor may need to adjust diet if recipient has a food allergy.	Transmission of enteric aerobic Gram-negative organisms resulting in hospitalization and death due to inadequate donor screening (86). IBD flare in patients with underlying IBD receiving FMT for rCDI (69, 87, 88). Procedure-related complications such as aspiration following sedation for endoscopy and colonic perforation (89).	Recommended by multiple practice guidelines after two CDI recurrences (84, 90, 91).	Defined microbial consortia to improve safety (92, 93). Fiber supplementation following FMT to enhance efficacy. Addition of bezlotoxumab in high-risk patients.	Restored colonization resistance through a high degree of donor bacterial engraftment and/or modulation of non-bacterial components (66). Modulation of microbial ecology by virome/phageome and mycobiome (94). Inhibition of *C. difficile* growth and/or germination through bacterial-derived metabolites (95). Modulation of host immune responses (96). Modulation of host epigenetic responses (97).

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352 FMT, fecal microbiota transplantation; IBD, inflammatory bowel disease; *C. difficile, Clostridioides difficile*; RCTs, randomized controlled trials; UTI, urinary tract infection.

FECAL MICROBIOTA TRANSPLANTATION: INDICATIONS WITH PRELIMINARY DATA REQUIRING FURTHER CONFIRMATION

Modulating the gut microbiota for health benefit has been demonstrated by the remarkable efficacy of FMT in preventing recurrent CDI (rCDI), and there is a growing interest in applying FMT as a research tool across a multitude of indications beyond rCDI (Fig. 3 and 4). The following section will summarize the available evidence in a few key indications where preliminary data exists requiring further confirmation (Table 2).

Adjunct Therapy in Fulminant CDI

Distinct from rCDI, fulminant CDI (fCDI) is clinically characterized by hypotension or shock, ileus, and toxic megacolon (90). The recommended therapies include 1) oral and/or rectal vancomycin and intravenous metronidazole with 2) consideration of adding intravenous tigecycline, and 3) surgery in medically refractory cases (59). Mortality rate can still approach 60% even with surgical intervention (98). In this context, FMT has been used to treat an active infection, in contrast to rCDI where FMT is used to prevent a recurrence. There is less evidence in fCDI than rCDI, and available evidence consists of small retrospective cohort studies and one RCT comparing single versus multiple FMTs (99). Studies varied in definition of fCDI, routes of delivery, FMT doses, frequency or number of treatments, duration of concomitant vancomycin, and follow-up period. The results have been summarized in two recent systematic reviews (100, 101). Because most studies included both severe and fCDI, it is difficult to estimate the success rate of FMT in fCDI; however, the pooled estimate for both populations is approximately 61.3% (95% CI 43.2–78.0) after a single FMT (100), increasing to 88% (95% CI 0.83–0.91) after multiple FMTs (101). The pooled all-cause mortality was 15.6% (95% CI 7.8–25) and the pooled colectomy rate was 8.2% (95% CI 0.1–23.7) after FMT. These results are promising, but future multi-center trials

with well-defined inclusion and exclusion criteria and thoughtful and pragmatic treatment protocols are required to validate these potential benefits.

Induction of Remission in Ulcerative Colitis

IBD includes UC and Crohn's disease (CD) and is characterized by chronic and relapsing inflammation of the intestinal mucosa. The pathogenesis of IBD is linked to several factors, including genetic susceptibility, immune dysregulation, environmental triggers, and alterations of the intestinal microbiome (102). Medical treatments consist of 5-aminosalicylates, immunomodulators, and biologics, with many patients requiring surgery at some point of their disease due to non-response or complications.

The disturbed microbiota in patients with IBD is characterized by both quantitative and qualitative changes: alpha diversity was reduced in both UC (103) and CD (104) patients compared with healthy controls. Additionally, a decrease in the abundance of bacterial species with anti-inflammatory properties, mainly SCFA production (such as *Roseburia hominis*, *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, and *Eubacterium rectale*) and an enrichment in proinflammatory species belonging to the Enterobacteriaceae family (such as *E. coli*) have been reported (104, 105). Patients with IBD also have an increased risk of becoming colonized with *C. difficile* and subsequently developing rCDI (106). Thus, therapies aimed at restoring gut microbiota using FMT in patients with both IBD and rCDI and patients with only IBD have received intense interest in recent years. A number of systematic reviews have found FMT to be effective at preventing CDI recurrence in patients with IBD, similar to those without IBD (107-109). However, serious adverse events (SAEs) may be higher in IBD patients than those without IBD, the most common being IBD flares and IBD-related hospitalization or surgery (107, 110).

Several RCTs have assessed the efficacy of FMT specifically at inducing UC remission (111-115). However, there is considerable variability in study designs, such as the use of single or

pooled stool donors, FMT dosage, frequency, routes of administration, and definition of remission. Most studies reported remission rates of approximately 30%–40% with FMT intervention (111-113, 116), much lower than seen for rCDI. A recent Cochrane systematic review including 12 RCTs with 550 participants showed that FMT for UC may increase rates of clinical and endoscopic remission relative to placebo with short follow-up duration of 6–12 weeks (clinical remission: RR 1.79, 95% CI 1.13–2.84; endoscopic remission RR 1.45, 95% CI 0.64–3.29) (117). The review also found uncertainty about the risk of SAEs given the low number of events in reported studies (RR 1.77, 95% CI 0.88–3.55), but hospitalization and surgery due to IBD flares have been reported (117). Another systematic review and meta-analysis including 10 randomized and 4 non-randomized studies found the use of a multi-donor strategy to be significantly more effective than single-donor FMT at inducing remission of IBD (118). However, another systematic review and meta-analysis including six high quality RCTs found no difference in outcomes with respect to single versus multiple donors, fresh versus frozen FMT, or routes of delivery (119). Rates of clinical improvement appeared to be higher with <275 g donor stool (120).

The current evidence of efficacy of FMT in inducing UC remission is promising but limited because of the significant heterogeneity in study design, small sample sizes, and short follow-up durations. UC flares following FMT have been reported, but it remains uncertain whether this was a result of FMT or a natural progression of the IBD itself. Further studies are needed not only to evaluate the efficacy and safety of FMT, but also to identify reliable predictors of response.

Treatment for Irritable Bowel Syndrome

IBS is characterized by alterations in stool frequency and consistency and abdominal pain or discomfort. It can be further categorized into IBS-D (diarrhea predominant), IBS-C (constipation predominant), or IBS-M (mixed). The symptoms are chronic and bothersome,

resulting in reduced quality of life and productivity, with an estimated annual cost between $1.7 to $10 billion in direct medical costs and $20 billion for indirect costs in the United States (121). The pathophysiology is multifactorial, involving intestinal dysmotility, visceral hypersensitivity, and disordered gut-brain interactions. Traditional therapies such as laxatives, antispasmodics, and promotility agents are only partially effective, leaving many patients dissatisfied with their care (121). There is also evidence linking altered gut immune activation and gut microbiota disturbance to IBS. For example, studies have found differences in the composition of the gut microbiome within a subset of IBS patients compared with healthy individuals (122, 123), as well as reduced diversity, stability, and butyrate- and methane-producing microorganisms (124-126).

FMT has been utilized in nine randomized trials targeting the gut microbiome (127-134), and the results have been summarized in several systematic reviews (135-137). There is significant heterogeneity in inclusion criteria; FMT dose, frequency, duration, and route of administration; follow-up period; and outcome assessments in these studies. The most recent systematic review including 8 RCTs (484 participants) found that one single dose of FMT resulted in no significant benefit to IBS symptoms three months after treatment compared with placebo (RR 1.19. 95% CI 0.68–2.10). One positive RCT randomized 165 participants to placebo, 30 g FMT, or 60 g FMT by gastroscopy in a single dose; this study found a significantly higher proportion of patients in the FMT groups, compared with placebo, to have reduced IBS symptom scores by at least 50 points 3 months later (23.6%, 76.9% [*P* <0.0001] and 89.1% [*P* <0.0001], respectively), accompanied by a significant improvement in quality of life and fatigue. There was also increased relative abundance in *Eubacterium biforme*, *Lactobacillus* spp., and *Alistipes* spp. and reduced relative abundance in *Bacteroides* spp. in the responders following intervention in the FMT group, but not in the placebo group (132). This cohort was followed for 3 years, and the response rate remained high (27%,

64.9%, 71.8%) (138). It should be noted that participants were unblinded after the initial randomized trial, and they became aware of their treatment assignment during the 3-year follow up. Interestingly, this study used a single donor aged 36 who was reported to be very healthy; he was born by vaginal delivery, was breastfed, rarely used antibiotics, and had a very active lifestyle (132).

Most studies did not use antibiotic treatment prior to FMT, although antibiotic pretreatment may facilitate bacterial engraftment (139). One study found that antibiotic pretreatment prior to FMT with ciprofloxacin and metronidazole or rifaximin for 7 days reduced engraftment compared with FMT alone, although the response with the chosen antibiotics is not the same as data from vancomycin treatment (134). Although the overall quality of the evidence was low due to inconsistency, small number of participants, and imprecision (135), there likely is a subgroup of IBS patients who could benefit from FMT with a particular microbiota signature.

Amelioration of Metabolic Syndrome

Metabolic syndrome (MetS) with insulin resistance has become a global epidemic in recent decades with substantial morbidity leading to reduced life expectancy. Sustained weight loss is often possible only after bariatric surgery. Newer agents such as glucagon-like petide-1 (GLP-1) agonists have shown promise with substantial weight loss (140-144), although long-term efficacy and safety remain unknown. Many studies have found an association between MetS and intestinal microbiome disruption that not only has altered composition with reduced microbial diversity, but also has an increased functional capacity to harvest energy and produce cardiotoxic metabolites (145-147). Given limited therapeutic options and potential relevance of intestinal microbiota, FMT has been used to explore potential therapeutic benefits.

Several RCTs have evaluated FMT in MetS, comparing FMT from lean donors with controls (sham, saline, autologous FMT, or placebo). A single-dose FMT was used in most studies, while one study used weekly dosing for five weeks by oral capsules (148). Various endpoints have been included, such as changes in HbA1c, cholesterol, insulin sensitivity, body weight, gut microbiota, or intestinal permeability after FMT. Some studies also included other adjunct therapies, including metformin, diet, fiber supplementation, or exercise. Table S1 in the supplemental material summarizes 18 RCTs comparing FMT with control. Only two studies examined weight loss as a primary outcome, and neither found an effect with FMT. Changes in glycemic control and lipid profiles were examined in four studies with conflicting results. When examining insulin sensitivity as an outcome, five of nine studies have found a positive but transient effect favoring the FMT intervention at weeks 2–6. Most studies have investigated changes in the microbiome after FMT, showing non-consistent shifts in community structures (149). Repeated FMT +/- lifestyle modification led to a significant increase in the proportion of "lean" microbiota (>20% of the population) compared with sham + lifestyle modification alone after 24 weeks (100%, 88% and 22%, respectively) (150), while less intense FMT regimens reported less favorable changes in microbial community of recipients (148, 151). Intestinal barrier function, assessed by the presence of bacteria in the mesentery as a measure of bacterial translocation, did not improve following FMT in one study (152). Similarly, carnitine- or choline-to-TMAO conversion and markers of arterial wall inflammation did not improve after FMT from lean donors (153-156)

A systematic review in 2020 including 6 RCTs with 154 participants found that 2–6 weeks after intervention, mean HbA1c was lower in the FMT group (MD=−1.69 mmol/L, 95% CI −2.88, −0.56, P=0.003) and mean HDL cholesterol was higher in the FMT group (MD=0.09 mmol/L, 95% CI 0.02, 0.15, P=0.008); however, there were no differences in other clinically important obesity parameters 6–12 weeks after intervention (157). Another systematic review

in 2023 including 9 RCTs with 303 participants reported statistically significant changes in the short term in the following parameters in the FMT relative to control groups: fasting blood glucose (MD=−0.12 mmol/L, 95% CI −0.23, −0.01, SD: ±0.04, I^2=7%), HbA1c (MD=−0.37 mmol/mol, 95% CI −0.73, −0.01, SD: ±0.13, I^2=46%), HDL cholesterol (MD=0.07 mmol/L, 95% CI 0.02, 0.11, SD: ±0.02, I^2=25%), and insulin levels (MD=−24.77 pmol/L, 95% CI −37.60, −11.94, SD: ±4.76, I^2=0%). Other parameters such as weight, body mass index (BMI), homeostatic model assessment for insulin resistance (HOMA-IR), and total cholesterol did not differ between groups (158). Given the complex pathophysiology and chronicity of MetS and heterogeneity of these studies, it is not surprising to see these mixed results. While the current evidence suggests a role of the microbiome in MetS, microbial manipulation alone is unlikely to be sufficient; rather, it may eventually be an integral part of a multi-faceted approach (i.e., pharmacotherapy and bariatric surgery) once definitive causality can be demonstrated.

Immune Checkpoint Inhibitor Modulation in Patients with Malignancy

Immune checkpoint inhibitors (ICI) have become the cornerstone of cancer immunotherapy and have dramatically improved survival in patients with melanoma, lung cancer, gastric cancer, and kidney cancer. Development of severe colitis is one of the most frequent immune-related adverse events (irAEs) in ICI-treated patients (159). Emerging evidence has demonstrated the critical roles of the gut and tumor microbiota in modulating tumor immunosurveillance and response to immunotherapy (160). The influence of the microbiota on the efficacy and irAE of immunotherapy has been observed in patients taking antibiotics (161, 162). As such, targeted modification of the gut microbiota represents an innovative strategy in cancer immunotherapy for treating severe intestinal complications and for enhancing ICI effect (163). For example, case reports and case series have found the efficacy of FMT in resolving ICI-associated colitis (164-167). The microbiota in the recipients had

increased alpha diversity and increased abundance in beneficial taxa (e.g., *Collinsella* and *Bifidobacterium),* which were depleted prior to FMT in one study. This provides evidence that modulating the gut microbiota may alleviate ICI-associated colitis. Microbiome-based interventions may also augment immune defense against malignant cells. Anti-PD-1 therapy, together with responder-derived FMT, has been shown to modify the tumor microenvironment and promote the response to anti-PD-1 in PD-1-refractory melanoma in two small case series (168, 169). One of these case series found the fecal microbiota of patients after FMT mostly resembled that of the donor, with significant enrichment in the proportion of favorable taxa Lachnospiraceae, Ruminococcaceae, Bifidobacteriaceae, and Coriobacteriaceae and a decreased abundance of *Bacteroides* species (168). However, an independent analysis of these two case series did not find a correlation between donor microbiota engraftment and anti-PD-1 response in recipients (168-170).

One potential strategy to enhance donor engraftment is to pretreat recipients with oral vancomycin and neomycin, but there is no consensus on approach. The selection of donors for FMT in studies to complement ICI therapy is also not clear, because the two studies used donors who were themselves treated with anti-PD-1 and had partial remission or complete remission (168, 169). A recent multi-center phase I trial combining healthy donor-derived FMT with anti-PD-1 in 20 patients with advanced melanoma showed four (20%) patients with a complete response and additional nine with a partial response. In this study, all responders had engrafted strains from their respective donors and the engrafted strains increased over time. Furthermore, responders had an enrichment of immunogenic bacteria and a loss of deleterious bacteria after FMT (171).

Although promising, many questions remain. Future and ongoing trials will clarify some of these unanswered questions as to the optimal dose, timing, and donor selection for FMT as an

adjunct therapy in various immunotherapy, and how other microbiome-modulating strategies such as probiotics, prebiotics, and diet may be integrated.

Modulation of Chronic Liver Diseases

Observational studies and animal models have unveiled a role for the microbiome in contributing to liver diseases (172). The bidirectional gut-liver axis is implicated in disease pathogenesis and progression to complications (173). The initial inciting event of liver disease varies from alcohol, viral hepatitis, non-alcoholic fatty liver disease (NAFLD), to IBD-associated primary sclerosing cholangitis (PSC). Many studies have found an association between intestinal microbiome disruption and impaired gut barrier function with chronic liver disease (174-176). These inciting events lead to translocation of microbes and microbial products including endotoxins resulting in activation of inflammatory pathways and liver fibrosis, and may progress to cirrhosis, hepatic encephalopathy, and hepatocellular carcinoma. For example, a study found that colonization of specific microorganisms such as Enterococci in the biliary system in PSC is associated with hepatic decompensation, liver transplantation, and death (177). Some of our current treatments already target the intestinal microbiome, such as the use of lactulose and rifaximin for hepatic encephalopathy or vancomycin to improve liver enzymes in PSC, but not all patients respond and the mechanisms of action remain largely unknown. Caring for persons with chronic liver disease is extremely challenging and costly, as there are limited therapeutic options. With increasing understanding of the gut-liver-brain axis, manipulating the intestinal microbiome is a potential therapeutic strategy. FMT has been explored in the context of liver cirrhosis, alcohol-related disorders, HE, NAFLD, and PSC (Table S2 in the supplemental material). Most studies used a single FMT as intervention; however, dose and route of administration differed. A small RCT in 20 patients with recurrent HE reported fewer encephalopathy events over two years following a single FMT by enema compared with the standard of care alone

(178, 179). One small RCT compared FMT with the standard of care in 20 participants with alcohol use disorder and found FMT to be safe and associated with reduced short-term alcohol craving and consumption as well as favorable microbial changes, including higher relative abundance of SCFA-producing taxa. A single RCT compared FMT with prednisone in 112 participants with severe alcoholic hepatitis and found a higher 90-day survival in the FMT group (75%; 45/60) compared with the prednisone group (56.6%; 34/60; *P*=0.044) due to a lower infection rate (180). Other studies in patients with alcoholic hepatitis have reported improved survival and HE and a decrease in alcohol craving (181-184). Three RCTs compared FMT with autologous FMT/probiotics in patients with NAFLD and showed a mixed effect on markers of fat accumulation in the liver (152, 185, 186).

Although the current evidence is quite limited, there are promising preliminary results from these FMT intervention trials in patients with chronic liver diseases. However, it is important to note that most studies were very small, conducted in a few centers in United States and India, and results might not be generalizable to other populations; this highlights the need for more research.

Amelioration of Graft-Versus-Host Disease

Patients with hematologic conditions undergoing hematopoietic stem cell transplant (HSCT) are at an increased risk for infectious complications, primarily due to profound immune suppression with pre-conditioning chemotherapy. To counter this, many HSCT protocols include multiple courses of antibiotic prophylaxis. Selective and total gut decontamination using orally administered antibiotics have been introduced to prevent infections with Gram-negative bacteria and fungi in some countries. The intestinal microbiota, already affected by hematologic disease, undergoes drastic changes in the post-HSCT period, include reduced diversity, shifts in microbial taxa and functionality, and single-taxon domination (187). This disturbed state is associated with multi-drug resistant organism (MDRO) carriage and

infections, increased incidence of CDI, development of graft-versus-host disease (GVHD) and overall mortality (188-190). GVHD is common in patients who undergo allogenic HSCT, responds poorly to current therapeutic interventions (i.e., steroids and immunosuppressive agents), and has a poor prognosis. The pathophysiology of GVHD remains poorly understood (191), but changes in luminal oxygen levels and metabolites may be associated with its development (192), which is further supported by the finding that antibiotics targeting anaerobes are associated with increased risks of acute gut/liver GVHD (193). A small case series of HSCT recipients who received FMT for rCDI showed improvement in GVHD, which further prompted interest in FMT as a therapeutic tool to treat GVHD, specifically GI-GVHD.

A summary of the current literature on FMT in the setting of HSCT/GVHD is presented in Table S3 in the supplemental material. FMT regimens differed significantly, and the donor was related to the patient/autologous in 5 of 14 studies. Most (10/14) did not administer pre-FMT preparation (i.e., bowel lavage). Steroid-refractory GVHD was the indication for all these studies, and response (partial/complete) was the primary outcome. A meta-analysis of pooled data from five studies (n=76) published in 2022 reported a 55.9% complete remission rate for steroid-refractory GVHD and an 82.4% overall response rate after FMT (194). Another 2022 review reported that FMT was associated with a 41% complete response rate and 25% partial response rate (n=242) with a moderate risk of publication bias (195). Lower response rates were observed in prospective studies 64% (95% CI 51%–77%) versus 81% (95% CI 62%–95%) in retrospective studies or case reports. The efficacy of FMT may be reduced in the setting of severe steroid-refractory GVHD due to the uncontrolled disease process that might require systemic intervention. Indeed, two studies used FMT with concurrent ruxolitinib (a selective JAK 1 & 2 inhibitor) for steroid-refractory GVHD and

reported high response rates (4/4 and 16/21), suggesting that this option needs further exploration (196, 197).

The timing and number of FMT interventions are important considerations. Early post-HSCT administration of FMT has been shown to reverse the loss of diversity associated with HSCT-related complications and mortality (188, 189, 198, 199). Although FMT is generally considered safe in immunocompetent patients, the evidence in immunocompromised patients—especially those with profound neutropenia—remains sparse (200). Screening protocols for donors are generally more extended in this setting to prevent transmission of viral diseases such as cytomegalovirus (CMV). Cases of post-FMT bacteremia in patients with severe, steroid-refractory GI-GVHD have been reported; however, the sources of these infections remain unclear. A recent review of 242 patients from 23 studies treated with FMT for steroid-resistant/dependent GVHD patients reported five (2.1%) patients who experienced FMT-related infection events, all of whom responded to antibiotic therapy (195). Autologous transplantation of pre-HSCT fecal material or defined microbial consortia or live biotherapeutics might provide a personalized approach for FMT administration and enhance engraftment (199).

The high response rate across these small studies underscores the potential benefits of microbial interventions to restore the microbial community and function in GVHD after HSCT. Several ongoing randomized controlled trials may provide further confirmation (ClinicalTrials.gov Identifiers: NCT04711967, NCT05067595, NCT04745221).

Multi-Drug Resistant Organism Decolonization

Antibiotic resistance has become a major global threat in health care systems. Intestinal colonization with MDRO such as extended-spectrum beta-lactamase producing Enterobacterales (ESBL-E), carbapenem-resistant Enterobacterales (CRE), or vancomycin-resistant Enterococci (VRE) can precede invasive infections with high morbidity and

mortality, as well as facilitate spread within communities and healthcare facilities (201). European Society of Clinical Microbiology and Infectious Diseases guidelines do not recommend decolonization with nonabsorbable antibiotics, because available evidence for its efficacy is insufficient (202). While strategies to combat MDRO colonization by infection control programs can limit its spread, they do not provide eradication. Although antibiotic use is a risk factor for MDRO carriage, less is known about the degree of gut microbiota disruption in individuals with MDRO colonization than about those who have rCDI. Some studies reported decreased species richness (203-205); however, no differences in diversity parameters or in relative abundance were observed between asymptomatic ESBL carriers compared with non-carriers based on species-level composition in a Dutch case-control study (206). With limited therapeutic options to combat MDRO colonization, novel approaches such as microbial restoration strategies including FMT warrant further consideration given reduced antibiotic resistant genes in rCDI patients after FMT (207, 208).

Table S4 in the supplemental material summarizes the current literature on FMT for MDRO decolonization, including 1 RCT, 15 prospective cohort studies, and 5 retrospective studies. They differed in the number of FMT used, delivery route, use of bowel purge, antibiotic pretreatment, definition of eradication, and follow-up periods. Most studies focused on eradication of ESBL-E, CRE, and VRE. A systematic review in 2021 including seven small nonrandomized cohort studies and five case reports found decolonization rates between 20%–90%, and they were slightly higher for CRE-E than for VRE; this review further found reduced MDRO bloodstream and urinary tract infections (201). A 2022 systematic review including three retrospective studies, six prospective cohort studies, and one open-label RCT reported CRE decolonization rate of 61% (55/90) one month after FMT (209). Several studies have also reported lower antibiotic-resistance genes (210, 211). However, in another RCT Huttner and colleagues randomized 39 patients to either five days of oral nonabsorbable

antibiotics followed by frozen FMT or control and found no statistically significant difference in ESBL-E or CRE decolonization rate (9/22 versus 5/17; OR for decolonization success 1.7; 95% CI 0.4–6.4) (212). A recently published RCT of FMT for MDRO decolonization in renal transplant recipients found that FMT-treated participants took longer to develop recurrent MDRO infection; therefore, time to MDRO recolonization and infection could be included as a clinical outcome in future study designs (213).

Although small cohort studies have shown some effect of FMT for MDRO decolonization, evidence remains limited, and questions remain regarding efficacy given spontaneous decolonization. Ongoing RCTs will provide more conclusive data on its efficacy and safety (214, 215).

Amelioration of Autism Spectrum Disorder

Autism spectrum disorder (ASD) is a heterogeneous neurodevelopmental disorder defined by deficits in social communication and interaction across multiple contexts with repetitive behaviors and restricted interests increasing in prevalence, estimated to be 1% globally (216, 217). The causes of ASD are complex and poorly understood, including genetic risk factors, de novo mutations, gene-environmental interactions, and environmental factors such as in utero exposure and perinatal events (218). Affected individuals commonly experience GI symptoms (219), which correlate with ASD severity (220, 221). Antibiotic exposure has also been associated with ASD onset and chronic diarrhea, which could be transiently ameliorated by oral vancomycin treatment (222), highlighting the disruption in the gut-brain axis and a potential therapeutic target. Because there are no confirmatory laboratory tests, diagnoses are based on multidisciplinary and developmental assessments (216, 217), and current treatment is aimed at behavioral interventions as no approved medical therapy exists (3).

It is difficult to characterize the microbiome in ASD children. A recent systematic review concluded that although the gut microbiota in ASD children was not consistently different

699 from healthy controls based on alpha and beta diversity across all studies, there were some
700 distinguishing patterns. Specifically, there were lower relative abundances of *Prevotella*;
701 Clostridia clusters I, II, and XI; and Fusobacteria in ASD children (218). Several studies have
702 also found a correlation between an increased abundance of Proteobacteria and disease
703 severity (218). *Bifidobacterium* was also consistently found to be lower in counts and
704 proportions in ASD children (218). A more recent study found that a subset of ASD children
705 had an increased lipopolysaccharide-binding protein, positively correlated with IL-8, IL-12,
706 and IL-13, suggestive of disruption of the intestinal barrier and immune dysregulation (223).
707 With emerging data implicating intestinal microbiome disruption in ASD pathophysiology,
708 there is a growing interest in modulating gut microbiota to treat ASD.
709 Several systematic reviews have examined the effect of FMT on ASD children, but it is
710 important to note that the results are based on small open-label or retrospective cohort studies
711 with sample sizes ranging between 18 and 49 and follow-up periods up to 2 months (224-
712 226). A summary of the current literature on FMT for ASD is presented in Table S5 in the
713 supplemental material. There was significant heterogeneity with respect to pre-FMT
714 treatment (from none to daily vancomycin for 2 weeks) and to actual FMT intervention (from
715 six daily FMT via enteral tube to oral frozen capsules or enema daily for 7 or 8 weeks) (227-
716 230). All studies found a statistically significant decrease in the Autism Behavior Checklist
717 and Childhood Autism Rating Scale scores across all studies compared with baseline scores,
718 and one study even found that the positive change correlated with the number of FMT
719 treatments (225). One of the studies, with a 2-year follow up, demonstrated persistent
720 improvement in the study cohort after the initial intervention, consisting of vancomycin pre-
721 FMT followed by daily doses of FMT for eight weeks (230). One study found that after FMT,
722 the gut microbiota of the recipients resembled that of the donors and that of neurotypical
723 children, with increased bacterial diversity and abundances of *Bifidobacterium*, *Prevotella*,

and *Desulfovibrio* (228). Another study found a significantly lower relative abundance of *Eubacterium coprostanoligens* in responders compared with non-responders, and that *E. coprostanoligens* had a negative correlation with serum gamma-aminobutyric acid concentrations (227). Although promising, vigorously conducted RCTs are needed before FMT can be considered for treatment of ASD.

Other Neurodegenerative Diseases

Research on using FMT in humans for other major neurological disorders mainly focuses on multiple sclerosis and Parkinson's disease, with some promising preliminary results (231-233). Several clinical trials with FMT as treatment for these neurological disorders are ongoing, as well as for amyotrophic lateral sclerosis (231). In contrast, promising data from animal models for stroke, Alzheimer's disease, and Guillain-Barré syndrome have not yet translated into clinical studies (234-236); and this warrants further investigation.

736 **Table 2.** FMT indications with promising efficacy requiring further confirmation.

Indications	Highest level of evidence	Clinical efficacy and durability	Dose/formulation/route and frequency of administration	Patient preparation and effect	Donor selection and effect	Serious adverse events	Clinical applications	Potential strategies to enhance efficacy and safety	Potential mechanisms of action
Fulminant *C. difficile* infection (fCDI)	Mostly case series (237-240); one small RCT comparing single versus multiple FMT (99); systematic reviews (100, 101).	Durable response (240).	Multiple dosing more efficacious than single dosing (99). Most studies evaluated colonoscopy-delivered FMT (99, 240).	Bowel prep with colonoscopy-delivered FMT (240). Most studies continued *C. difficile*-directed antibiotics with FMT (240).	Single donor.	Colectomy. Death but likely related to underlying disease rather than FMT. Aspiration pneumonia. Colonic perforation may be procedure related rather than FMT.	Potential benefit in those who are not surgical candidates (84).	Likely similar to rCDI.	Likely similar to rCDI.
Induction of remission in mild to moderate ulcerative colitis (UC)	Multiple RCTs comparing FMT to a comparator with various dosing regimens and routes of administration (111-116). Multiple systematic reviews, and meta-analyses (84, 117-120).	Range from 30 to 50% (114, 116). Most studies assessed remission at 8-12 weeks after initiating FMT without long-term follow-up (113, 114). Response not durable since disease flare is common without FMT maintenance (112).	Most studies use multiple doses with varying donor stool weights, intervals as well as routes of administration (111). Majority of studies used aerobically manufactured FMT (111). Most studies used fresh or frozen FMT (111); emerging evidence may support lyophilized FMT use (114).	Most studies did not include antibiotic pre-treatment or bowel preparation prior to FMT(111-113, 116). Bowel preparation necessary with colonoscopy-delivered FMT (113).	Some studies have used pooled multiple-donor FMT (112, 113) instead of single-donor FMT (111, 114) without obvious increased efficacy. Some studies suggest potential donor effect (111, 112).	IBD flare, hospitalization and colectomy, likely related to underlying IBD rather than FMT (112).	Not recommended in clinical practice. Should be done in the context of clinical trials.	Pre-FMT treatment with antibiotics to "open up microbial niches" and enhance bacterial engraftment (114). Addition of anti-inflammatory diet in recipients to prolong response (241). Matching donor and recipient with similar microbial profiles and dietary patterns (242, 243). Anaerobic FMT	Bacterial engraftment associated with remission, but relatively low degree of engraftment compared to rCDI indication (244).

								manufacturing to preserve strict anaerobes.	
Irritable bowel syndrome (IBS)	Multiple RCTs (127-133) and systematic reviews (84, 135-137).	Most studies have not shown a clinical benefit in stool frequency, consistency, or abdominal pain (127, 129, 133).	Variable dosing, number of sessions, and routes of administration. Majority of studies used frozen material.	Most studies used bowel preparation prior to FMT administration. Most studies did not use antibiotics.	Most studies used single-donor FMT.	Death by suicide, diverticulitis, likely related to underlying disease rather than FMT.	Not recommended in clinical practice. Should be done in the context of clinical trials.	Selection of "super-donors".	Unknown.
Metabolic syndrome (MetS)	Multiple phase 1-2 RCTs (Table S1).	Potential early positive signal for lowering insulin resistance; not durable without maintenance FMT (245, 246). No efficacy on weight loss (247, 248).	Various administration routes were used, with no clear indication of preference, although some evidence exist for the upper route administration in regard to insulin sensitivity. Most studies use a single FMT session with various doses. Fresh/frozen material were used in most studies.	Most studies used bowel preparation prior to FMT. Most studied did not use antibiotics.	Material from both single and pooled donors were used.	No significant SAE were reported.	Not recommended in clinical practice. Should be done in the context of clinical trials.	Addition of anti-inflammatory diet or fiber supplementation.	Bacterial engraftment.
Immune checkpoint inhibitor (ICI) -induced colitis	One large case series of 12 patients and various smaller case series and case reports (164-167).	Efficacy in more than 80% of the reported case series.	Various routes of administration and various doses (1 to 3).	No specific preparations.	Single healthy unrelated donors.	Infectious SAEs mostly related to prolonged immune suppression.	Not recommended in clinical practice (except in the Netherlands). Should be done in the context of clinical trials.	Important to exclude GI pathogens due to immunosuppression.	Increase in the proportion of Tregs within the colonic mucosa.

Supporting immune checkpoint inhibitor (ICI) therapy in malignancy	Two large case series and one phase 1 study (168, 169, 171).	Potential early positive signal.	Two case series: a single FMT administered colonoscopically together with PD-1 blockade. Phase 1 study; single FMT with capsules.	Varied from orally ingested antibiotics as pretreatment to only bowel lavage.	Case series used donors treated with anti-PD-1 with ongoing PR or CR. Phase 1 study used healthy donor stool to prepare FMT capsules.	Not reported.	Not recommended in clinical practice. Should be done in the context of clinical trials. Possibly donor effect.	May have clinical benefit in 30-65% of the patients.	Response to anti-PD-1, with changes in immune cell infiltrates and gene expression profiles in the gut lamina propria and blood.
Alcoholic hepatitis	Several retrospective cohorts, one prospective cohort, and a single RCT reported by a single group of researchers (Table S2).	Potential early positive signal for improvement of short-term survival (30-50% reduction in mortality). One study suggests durable response up to three years.	Most studies used seven sessions of 30 g fresh material via nasoduodenal tube.	No bowel preparation was given. Most studies did not use antibiotics.	Single donor.	GI bleeding and spontaneous bacterial peritonitis related to the underling disease.	Not recommended in clinical practice. Should be done in the context of clinical trials.	NA	Unknown.
Hepatic encephalopathy (HE)	One prospective cohort; three small RCTs reported by a single group of researchers (Table S2).	Potential early positive signal for improvement in cognitive function and prevention of HE events at six months.	Early studies with enema and later studies with oral capsules. Most studies used ~24-27 g of frozen stool in a single session.	Early studies used antibiotics (enema) while later studies (oral capsules) did not.	Single donor.	ESBL-producing *E. coli* bacteremia and hospitalization.	Not recommended in clinical practice. Should be done in the context of clinical trials.	NA	Bacterial engraftment.
Graft-versus-host disease (GVHD)	Multiple retrospective and prospective cohort studies and case series; one small RCT (Table S3).	Potential early positive signal for clinical response in acute steroid-refractory/dependent GI-GVHD.	Variable dosing, number of sessions (1 to 8) and routes of administration, using fresh/frozen material.	Stopping prophylactic antibiotics prior to FMT. Some studies used bowel preparation.	Most studies used single related/unrelated donor.	Bacterial and viral infections, thrombotic events, respiratory failure likely related to underlying hematologic disease/therapy rather than FMT.	Not recommended in clinical practice. Should be done in the context of clinical trials.	Possibly stopping prophylactic antibiotics prior to FMT.	Unknown.

Eradication of multi-drug resistant organism (MDRO) carriage	One small phase 1 trial and multiple prospective cohort studies (Table S4).	Potential early positive signal in MDRO eradication, although definition of eradication varies. Single RCT did not show a benefit in eradicating CRE or ESBL infections.	Majority of studies used 1-2 fresh/frozen FMT treatments, most frequently delivered by the upper route.	Most studies used bowel preparation prior to FMT when performing colonoscopy. Most studies used gastric acid suppression (PPI). Most studies did not use antibiotics.	Single donor.	Infections, probably related to underlying medical state.	Not recommended in clinical practice. Should be done in the context of clinical trials.	Avoiding the use of antibiotics in the pre-FMT period.	Unknown.
Autism spectrum disorder (ASD)	Case series (Table S5).	Potential early positive signal.	Multiple dosing by either oral or rectal route with varying dosing intervals.	Most studies did not use antibiotics or bowel preparation.	Single donors.	Not reported.	Not recommended in clinical practice. Should be done in the context of clinical trials.	Antibiotic pretreatment.	Unknown.

CR, complete response; CRE, carbapenem-resistant Enterobacterales; ESBL, extended-spectrum beta-lactamase; *C. difficile*, *Clostridioides difficile*; FMT, fecal microbiota transplantation; GI, gastrointestinal; GI-GVHD, gastrointestinal GVHD; ICI-induced colitis, immune checkpoint inhibitor-induced colitis; PD-1, programmed cell death protein 1; PPI, proton pump inhibitor; PR, partial response; NA, data not available; rCDI, recurrent *Clostridioides difficile* infection; RCT, randomized controlled trial; Tregs, regulatory T cells.

MECHANISMS UNDERPINNING THE EFFICACY OF FECAL MICROBIOTA TRANSPLANTATION

In understanding the mechanisms underpinning FMT actions, most progress has been made in the context of treating rCDI, which has been extensively reviewed elsewhere (243, 249, 250). Briefly, proposed mechanisms may involve 1) restored colonization resistance through bacterial engraftment and modulation of non-bacterial components, 2) direct effect on *C. difficile* through modulation of microbial ecology by the virome/phageome, 3) inhibition of *C. difficile* growth and germination through bacteria-derived metabolites, or 4) modulation of host immune responses and epigenetic responses (242). The main recognized mechanisms contribute to the restoration of gut microbial functionality. With the increasing application of FMT for other conditions, there is emerging evidence describing the mechanistic role of FMT in IBD and MetS. This section will focus on results from human studies in IBD, contrasted with results from treatment of rCDI. The potential mechanisms underpinning the interactions between intestinal microbiota and the immune system in IBD as a model are depicted in Fig. 5.

Functional Restoration of Gut Homeostasis through Bacterial Engraftment

Studies have consistently demonstrated restored microbial composition and diversity to resemble that of a healthy donor following successful FMT in rCDI patients, with durable engraftment (251). Engraftment is also assumed to result in a desired outcome in other conditions associated with microbial disruption, such as IBD. Indeed, the microbiota in UC patients who had a response following FMT showed significantly higher diversity and were significantly more similar to their donors (111-113). One study found a decrease in patient-derived *Bacteroides* spp. and an increase in donor-derived *Prevotella* spp. and *Bacteroides* spp. following FMT. Another study found that UC patients who had achieved remission following FMT had enriched *Eubacterium halli* and *Roseburia inulivorans* compared with

767 those who did not achieve remission (252). However, the degree of engraftment in IBD is
768 much lower than in rCDI (253), and is not as durable. For example, one study found that the
769 abundance of engrafted microbes was not maintained at 12 months (113). Furthermore,
770 although there does not appear to be any donor effect in rCDI, some FMT for IBD studies
771 have shown a "donor" effect. In an RCT, Moayyedi and colleagues noted that most patients
772 who responded to FMT had received donations from a donor whose microbiome had higher
773 diversity and abundance of family Lachnospiraceae and genus *Ruminococcus* compared with
774 other donors, and they introduced the notion of "super donors" (111). In another RCT,
775 Paramsothy and colleagues conducted pooled multi-donor FMT and found that UC patients
776 had a higher response rate to FMT using samples from a particular donor, although the
777 overall microbial diversity was higher in the pooled FMT products than in that of single
778 donors: 14/38 (37%) patients treated with FMT from this donor responded compared with
779 7/40 (18%) patients whose FMT did not include material from this donor (P=0.054) (112).
780 However, the rates of induction of remission by FMT in RCTs for UC patients to date are not
781 higher in studies that used pooled multi-donor FMT (3–7 donors) compared with those using
782 single donors; all rates of remission are in the range of 30%–50% relative to the control of
783 5%–20% (111-114, 116).
784 Factors that determine engraftment are complex and not fully understood. Pretreatment of
785 recipients with vancomycin orally was needed to establish engraftment of a live
786 biotherapeutic product (LBP) of eight commensal *Clostridia* strains (254). Patients with rCDI
787 are usually on vancomycin prior to receiving FMT, and the degree of their microbial
788 disruption is much more profound than for the other diseases for which FMT is being applied.
789 Recent strain-level metagenomic analyses provide an ecological framework for the effect of
790 FMT (244); these analyses support the importance of deterministic, niche-based processes for
791 post-FMT microbiome assembly, specifically the competition between and exclusion of

closely related recipient and donor strains. The outcome of such competition is determined by the fitness of the strains and the relative fitness differences of the incoming and recipient strains. Priority effects, favoring early-arriving strains at an ecological site, generally supports recipient strains in undisturbed communities and provides an explanation for the low levels of strain engraftment in UC patients without the antibiotic-induced microbiota disruption seen in rCDI (244, 255). Recent evidence also suggests that metabolic independence is yet another important determinant of engraftment, because the "good colonizers" are enriched in metabolic pathways for biosynthesis of essential nutrients (256). Furthermore, while the gut microbial ecosystems of healthy individuals include microbes with both low and high metabolic independence, IBD primarily selects for microbes with high metabolic independence (256), which may, in part, explain the much lower levels of donor microbial engraftment observed following FMT in IBD than in rCDI (244).

Virome/Phageome Modulating Effects

The effect of the gut virome on the efficacy of FMT therapy has received little attention until recently (257, 258). One study found a higher abundance but a lower diversity of Caudovirales bacteriophages in stool samples of rCDI patients prior to FMT (94); a similar pattern was found in IBD patients where there was a significant expansion of Caudovirales bacteriophages compared with healthy controls (51, 52). Following successful FMT for rCDI, there was a significant decrease in the abundance of Caudovirales and an increase in donor-derived Caudovirales in the recipient virome (94). Another pilot study examining fecal filtrate to treat rCDI found the recipient's virome composition to resemble that of the donor, further suggesting viral engraftment (259). In a study examining FMT in UC patients, Conceicao-Neto and colleagues found lower richness of the eukaryotic virome in healthy donors and in UC patients who were responders (260), and identified nine donor-derived phage operational taxonomic units in a responder. They went on to suggest that eukaryotic

817 virome richness could be used as a potential diagnostic marker for UC and response to FMT,
818 although this was based on a small sample size of nine patients (260). Likewise, a
819 resemblance in the virome profile of pediatric UC patients toward the donor profile was
820 reported following successful FMT in another study, although this shift is less pronounced
821 than the shift in the bacteriome (261). While interpreting the sole impact of the fecal virome
822 on the recipient microbial community and clinical outcome is difficult because of the
823 presence of various other components in human stool, fecal virome transplantation has been
824 demonstrated to potentially contribute to microbiota restoration in pre-clinical and clinical
825 models, including MetS (262-266). Engrafted donor virome could adhere to the gut mucus
826 layer and prevent bacterial attachment and colonization. In addition to modulating the
827 bacteriome, bacteriophages could regulate bacterial function, metabolism, and virulence
828 (267). Further studies should investigate bacterial alterations upon phage predation and how
829 they can be exploited to improve clinical outcomes.

830 **Microbial Metabolites**

831 The best-characterized bacteria-derived metabolites mediating the efficacy of FMT in rCDI
832 are bile acids and SCFAs, and restored metabolism and increased levels of secondary bile
833 acids and SCFA are observed following successful rCDI (95, 268-270). The relevance of
834 these metabolites is not as well described or known in IBD. For example, Paramsothy and
835 colleagues found increased levels of SCFAs and secondary bile acids following FMT in UC
836 patients who are responders (252). In contrast, Costello and colleagues found no significant
837 differences in butyrate and other SCFAs between baseline and 8 weeks after intervention, or
838 even between FMT or placebo groups; more importantly, SCFA concentrations were not
839 associated with any observed FMT treatment effect (113).

840 Other bacteria-derived metabolites may be important to consider in IBD. Khalessi Hosseini
841 and colleagues examined fecal metabolic alterations in UC pediatric patients following FMT,

and found that indole-3-acetate, 2,6-diaminopimelic acid, and ricinoleic acid were primary metabolites associated with a response; these metabolites continued to increase for six months (271). Another study by Nusbaum and colleagues reported that the metabolic profile of pediatric UC patients clustered into a disease-associated group that was distinctly different from their donors, with higher levels of putrescine and 5-aminovaleric acid at baseline; in addition, the post-FMT metabolic profile clustered toward their respective donors, with increased levels of xanthine, oleic acid, and butyrate (261). Compared with non-responders, energy-related pathways and bacterial cell surface components increased in CD patients who responded after FMT in one study (272), while heme, lipopolysaccharide/lipid A, peptidoglycan, ubiquinone and lysine, and oxidative phosphorylation biosynthesis pathways decreased in UC FMT responders in another study (252). Substantial alterations in 151 serum metabolites were observed in UC patients after FMT administration, and the most significant increases were in eight different metabolites associated with vitamin B6 metabolism and aminoacyl-tRNA biosynthesis pathways (273).

Host Immunity

In patients with IBD, FMT reduces the prevalence of CD8+ and CD4+ T cells and prevents the accumulation of proinflammatory cytokines (274). Further, FMT in animal models of acute colitis led to augmented anti-inflammatory cytokine production, promoted aryl hydrocarbon receptor activation, and alleviated inflammation (275). Likewise, SCFAs regulate the size and activity of the colonic Treg population that directly ameliorates colitis (276).

Additional pre-clinical studies in IBD indicate other immunoregulatory actions occur in response to FMT, including increased antimicrobial peptides such as cathelicidin, S100A8, specific defensins, secretory IgA, and mucin; these changes are coupled with reduced neutrophils, macrophages, and proinflammatory cytokines and a downregulation of major

867 histocompatibility complex (MHC)-II dependent presentation of bacterial antigens (277).
868 FMT is also associated with an upregulation of Tregs, IL-10–secreting CD4 T cells, and
869 circulating gut-homing T cells. Collectively, these findings are associated with amelioration
870 of colonic inflammation. In contrast, immunological changes have been very poorly
871 described in human IBD FMT studies except for immune checkpoint inhibitor–associated
872 colitis, where immunological response is associated with a significant reduction in colonic
873 mucosal $CD8^+$ T cell density and an increase in $FoxP3^+$ CD4 cells (278).

874 ## FECAL MICROBIOTA TRANSPLANTATION MANUFACTURING

875 ### Donor Screening and Selection

876 #### General Considerations

877 The multi-staged donor screening process is first and foremost to ensure the safety of FMT
878 products. To date, transmission of infections through FMT occurred as a result of inadequate
879 donor screening, such as the use of microbiological tests with suboptimal test characteristics
880 or not testing for certain pathogens (86, 279). A secondary objective is to select an “optimal”
881 donor, although this may vary with indications of interest; furthermore, defining an ideal
882 donor based on the gut microbiota composition is problematic and not yet possible (280).
883 The criteria applied to exclude donors and the extent to which donors are screened vary
884 considerably, partly because of different regulations around the globe and uncertainty
885 surrounding the short- and long-term safety of FMT. Most studies do not report the details on
886 how donors are selected or tested. Many screening guidelines are driven by regulatory
887 requirements and expert opinions (78, 79, 281), not based on data obtained from experimental
888 human gut challenge models. Individuals with certain characteristics such as high-risk
889 behaviors, morbid obesity, autoimmune conditions (e.g., IBD), malignancy (e.g., colon
890 cancer), and neurodegenerative and psychiatric disorders are excluded as donors (17, 282,
891 283); however, there is less certainty whether other characteristics should constitute exclusion

criteria, such as age or BMI cutoffs, a family history of the aforementioned conditions, or recreational drug use.

The risk of infectious transmission by FMT is based on transmission capabilities and presumed viability of the pathogen in the gut, in combination with recipient host immunity and comorbidity. Each microbiological test also has different performance characteristics, sensitivity, and specificity. Furthermore, a positive test result may indicate the mere presence of genetic material (e.g., PCR) instead of a viable pathogen (e.g., culture), depending on the testing method. In addition to blood-borne infections (e.g., hepatitis viruses and *Treponema pallidum*), the screening tests focus on excluding fecal-oral transmitted GI pathogens, multi-drug resistant bacteria, or pathogens with a systemic impact. Although some blood-borne pathogens are included in many screening programs, there is a considerable lack of clinical data. For example, hepatitis C is known to be transmitted by blood, not by the fecal-oral route, and yet it is considered an absolute exclusion criterion for a stool donor. There are other unresolved questions as to what constitutes absolute versus relative donor exclusion criteria. For example, although the parasite *Blastocystis hominis* has potential enteropathogenic properties (284), Terveer and colleagues found that FMT containing *B. hominis* subtype 1 (ST1) or ST3 resulted in intestinal engraftment in approximately 50% of 31 rCDI patients without developing GI symptoms or diminishing treatment efficiency (285). Notably, in patients receiving *Blastocystis*-positive donor feces, a significant improvement in self-rated defecation pattern was observed at long-term follow up. *Blastocystis* sp. has also been found to correlate with a more diverse microbiome in some studies, which is a desirable characteristic of a good donor (286). As such, there continues to be debate as to whether *Blastocystis* should be considered an exclusion criterion for donors in all programs. Furthermore, it is very likely that other common enteric parasites such as *Dientamoeba fragilis* (287) have similar properties as *B. hominis*, and their presence may become part of

the exclusion criteria for some donor programs. Grosen et al. could not detect *Helicobacter pylori* transmission in a cohort of 26 recipients of FMT via capsules from *H. pylori*–positive donors screened by an *H. pylori* feces antigen test (288). Similarly, not all donor programs test for *H. pylori* because the viability of *H. pylori* in feces materials is questionable.

The potential transmission of certain viruses through FMT also deserves further consideration. In a large Swiss cohort of 500 healthy donors with 36% CMV seropositivity, no fecal shedding was observed with PCR, even in the presence of CMV IgM in 2.3% of the 182 CMV seropositive donors (289). In contrast, CMV may have been transmitted through FMT from an unscreened related donor to a recipient with active UC, although CMV was more likely transmitted via saliva/household contact from son to father in this donor and recipient pairing (290). Thus, screening for CMV may need to be considered if a recipient is (severely) immunocompromised. In the peak of the coronavirus disease 2019 (COVID-19) pandemic, viable severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) was detected in stool samples, especially in patients with concomitant GI symptoms, on rare occasions of hospitalized symptomatic COVID-19 patients (291-294); however, it is unknown if the presence of viable virus is still a possibility in healthy asymptomatic donors. Despite the fact that transmission by fecal-oral route or FMT has not been documented, safety alerts have been issued by regulatory agencies requiring stool donor programs to be screened for SARS-CoV2 (295).

Recently, monkeypox (Mpox) virus highlighted a gap in the screening of sexually transmitted infections. With the numbers of Mpox-infected individuals rising among men who have sex with men prompting a further safety alert in 2022 (296), this infection is, fortunately, relatively easy to detect with screening guidelines. Careful screening for the presence of prodromal non-specific symptoms, newly appeared skin lesions, or close contact with proven or suspected infection within the previous 30 days can identify individuals at risk of being

infected, and these questions have been added to many screening guidelines (297). Most donor programs do not include screening for other sexually transmitted infections such as herpes simplex virus or lymphogranuloma venereum subtypes of *Chlamydia trachomatis*. Therefore, it is important to subject donors to the screening questionnaire, including questions on sexual behavior or complaints, and to repeat screening with every donation with a short questionnaire on recent health status. Furthermore, it is important to have appropriate and secure data management and storage to guarantee traceback and anonymity of donors. Some argue that donors should be unpaid to reduce the risk that applicants withhold sensitive information (17). Examples of donor screening questions are provided in Table 3.

Screening Processes

Once a donor has been identified, the screening process can be divided into three phases: donor history, physical exam, and laboratory tests. History can be obtained through an in-person interview or, more commonly, through a questionnaire that covers six domains: baseline characteristics, relevant medical history including recent antibiotic use, relevant family history, occupational exposures, high-risk behaviors, and travel history. Variations may exist in exclusion criteria, where some of these may be considered absolute while others may be relative, such as being a healthcare worker or within an age range (e.g., 18–50 or 18–65 years).

After meeting the inclusion and exclusion criteria, selected individuals undergo a physical exam followed by lab-based screening of stool and serum samples (Table 4). Variations exist with the extent of donor testing and the intervals of screening. For example, Health Canada requires testing donor stool for Mpox (79), while expert consensus from Europe (297) and Australia (298) suggest that a questionnaire may be sufficient to exclude potentially infectious donors. Finally, negative results on lab-based tests lead to donor acceptance. It is

966 interesting to note that after the stringent and rigorous screenings, the overall acceptance rate
967 for stool donors tends to be low, ranging from <5% to 25% (299-302).
968 Ultimately, it is crucial to ensure completeness of donor screening based on guideline
969 recommendations and regulatory requirements. Donor programs also need to quickly respond
970 and update screening processes based on reported transmission events, emerging pathogens,
971 or pandemics, such as enteropathogenic *E. coli*, MDROs, SARS-CoV2, or Mpox. The best
972 available tests should be used, given the rapidly evolving field, in consultation with local
973 expert medical microbiologists. Comorbidity, including immune status of a recipient, may
974 require additional consideration. For example, CMV status of a donor may have relevance for
975 a severely immunocompromised recipient who is CMV negative. There also needs to be an
976 appropriate response to a positive screening result should it arise. The length of the
977 (temporary) exclusion of the donor/donor feces depends on the expected course of the
978 infection and colonization of the pathogen in healthy donors and possible treatment or side
979 effects; testing should be repeated until a negative result to accept the donor, in dialogue with
980 a medical microbiologist/infectious disease specialist.

981 **TABLE 3** Examples of donor screening questions

Themes	Examples	References
Donor baseline characteristics	• Ages between 18–65 years during the donation period? • BMI be between 18.5–25 kg/m²? • Currently taking any medications including vitamins, supplements, antibiotic, prebiotic or probiotic, birth control, etc.? • Feeling healthy and well today? • Is the consistency of stool normal (smooth and shaped like a sausage)? • Has daily bowel movements?	(112, 282, 303-313)
Relevant medical history	**Chronic disease** • GI disorder and/or chronic liver disease? • Neurological, autoimmune, or atopic conditions? • Metabolic syndrome? • Cancer? • Psychiatric history? **Medication use** **Hospitalizations in the last 3 months** **Infection risk** • History of a blood transfusion or other blood products? • History of being tested positive for HIV/AIDS virus or viral hepatitis? • History of being treated for syphilis or gonorrhea? **Vaccination history**	(57, 68, 69, 282, 303-311, 313)
Relevant family history	• First-degree relative with GI malignancy <60 years old? • Family history of genetically driven cancer? • First-degree relatives with IBD?	(57, 68, 112, 113, 282, 283, 303-312)

Occupational exposure	• Come into contact with someone else's blood? • Had an accidental needle stick? • Risk factors for MDROs including: - Work in clinical environment or long-term care facility? - Regularly attend outpatient medical or surgical clinics? • Work or has worked with animals in an environment where transmission of zoonotic infections is likely? • A member of the United States military, a civilian military employee, or a dependent member of the United States military?	(303, 305, 309, 311)
High-risk behaviors	• Have received a tattoo or a body piercing in the last 6 months? • Have used or injected drugs into the vein, muscle, or skin? • Have had sexual intercourse for money or drugs in the past 12 months? • Had sex with any person suspected or known to have HIV/AIDS or viral hepatitis? • Have a history of incarceration or held in a correctional facility? • Have received anal intercourse in the past 12 months? • Male donors: Have ever had sexual contact with another man? • Female donors: Had sexual contact with a male who has ever had sexual contact with another male? • Had sexual contact with anyone who has hemophilia or has used clotting factor concentrates?	(57, 66-69, 112, 113, 282, 283, 303-309, 311, 312, 314)
Travel history	• Been outside the United States or Canada? Where? • Spent time that adds up to three months or more in the United Kingdom? • Spent time that adds up to five or more years in Europe? • Have ever been to Africa? • Have been admitted and/or treated at a hospital or clinic abroad in the past 12 months? Duration? • Have traveled to high-risk areas of infectious diarrhea in the last 3 months?	(57, 66-69, 112, 113, 304-309)

AIDS, acquired immunodeficiency syndrome; BMI, body mass index; GI, gastrointestinal; HIV, human immunodeficiency virus; IBD, inflammatory bowel disease; MDROs, multi-drug resistant organisms.

985

986 **TABLE 4** Recommended donor screening tests

Type of specimen	Type of pathogen	Examples	Suggested tests	Canada (2022) (79)[#]	Australia (2020) (281)	UK (2018) (62)	Denmark (2021) (315)	Netherlands (2017) (283)	USA (OpenBiome) (2021) (305)	UEG (2020) (17)	International consensus (2019, 2020) (304, 316)
Stool	Bacterial agents, toxins, or products	*Salmonella* spp.	PCR combined with enrichment culture	✓	✓	✓	✓	✓	✓	✓	✓
		Plesiomonas shigelloides	PCR	✓				✓	✓	✓*	
		Vibrio spp.	Culture	✓				✓	✓	✓	✓
		Shigella spp.	PCR combined with enrichment culture	✓	✓	✓	✓	✓	✓	✓	✓
		***Escherichia coli* pathotypes**								✓@	
		ETEC	PCR				✓		✓		
		EPEC	PCR	✓			✓		✓		
		EIEC	PCR				✓		✓		
		EAEC	PCR						✓		
		VTEC/STEC	PCR	✓			✓	✓	✓	✓	✓
		E. coli O157-H7	Culture	✓					✓		
		MDROs									
		VRE	Enrichment culture confirmed by PCR	✓	✓		✓	✓	✓	✓^	✓
		CRE	Enrichment culture (with non-selective broth)	✓	✓	✓	✓	✓	✓	✓^	✓
		ESBL-E	Enrichment culture (with non-selective broth)	✓	✓	✓	✓*	✓	✓	✓^	✓
		MRSA	Enrichment culture confirmed by PCR	✓		✓		✓		✓^	✓
		Others									
		Clostridioides difficile	PCR (target toxin B)		✓	✓	✓	✓	✓	✓	✓

		Helicobacter pylori	Stool antigen test	✓	✓			✓	✓	✓	✓
		Yersinia pseudotuberculosis, *Y. enterocolitica*	PCR	✓		✓	✓	✓	✓	✓	✓
		Campylobacter jejuni, (*C. coli*)	PCR	✓	✓	✓	✓	✓	✓	✓	✓
		Neisseria gonorrhoeae	PCR	✓							
		Chlamydia trachomatis	PCR	✓							
		Aeromonas spp.	PCR confirmed by culture and further subtyping of toxins/virulence factors	✓				✓			
		Listeria	PCR	✓							
	Viral agents	Norovirus	PCR	✓	✓	✓	✓	✓	✓	✓	✓
		Astrovirus	PCR				✓	✓	✓	✓*	✓
		Sapovirus	PCR				✓	✓	✓	✓*	✓
		Rotavirus	PCR	✓	✓	✓	✓	✓	✓	✓	✓
		Adenovirus 40/41	PCR	✓			✓	✓	✓	✓*	✓
		Enterovirus	PCR				✓	✓	✓	✓*	
		Parechovirus	PCR				✓	✓		✓*	
		HEV	Serology (only in case of seroconversion PCR of feces)					✓			
		Mpox	PCR	✓							
		SARS-CoV-2	PCR	✓			✓		✓	✓	✓
	Protozoa, parasites, and others	*Giardia lamblia*	PCR		✓	✓	✓	✓	✓	✓	✓
		Entamoeba histolytica	PCR		✓		✓	✓	✓	✓	
		Cryptosporidium parvum, *C. hominis*	PCR		✓	✓	✓	✓	✓	✓	✓
		Isospora belli	PCR			✓		✓	✓		✓
		Cyclospora cayatenensis	PCR			✓		✓	✓		
		Microsporidium (Enterocytozoon	PCR					✓	✓	✓*	✓

		bieneusi, *Encephalitozoon intestinalis)*									
		Strongyloides stercoralis	PCR feces (in combination with serology)					✓	✓	✓	✓
		Ova, cysts, larvae, parasites, and helminths	Microscopy	✓		✓		✓	✓	✓	✓
		Protozoa	Microscopy	✓#							✓
Serum	**Bacterial agents**	*Treponema pallidum* (syphilis)	Serology; TPHA	✓	✓	✓	✓	✓	✓	✓	✓
	Viral agents	HIV-1 and HIV-2	Serology; P24 antigen and HIV antibodies	✓	✓	✓	✓	✓	✓	✓	✓
		HTLV-1 and HTLV-2	Serology; IgG	✓	✓	✓			✓		
		HAV	Serology; IgM/IgG**		✓	✓	✓	✓	✓	✓	✓
		HBV	Serology; HbsAg and preferably anti-HB core	✓	✓	✓	✓	✓	✓	✓	✓
		HCV	Serology; Ig Total***	✓	✓	✓	✓	✓	✓	✓	✓
		HEV	Serology; IgM/IgG**			✓		✓	✓	✓	✓
		EBV	Serology IgM/IgG*		✓	✓*	✓	✓		✓*	✓
		CMV	Serology IgM/IgG**		✓	✓*	✓	✓		✓*	✓
	Protozoa, parasites, and others	*Strongyloides stercoralis*	Serology; IgG		✓	✓		✓	✓		✓
		Entamoeba histolytica	Serology; IgG			✓					
		Toxoplasma gondii	Serology, IgM							✓*	

CMV, cytomegalovirus; CRE, carbapenem-resistant Enterobacterales; EAEC, enteroaggregative *Escherichia coli*; EBV, Epstein-Barr virus; EIEC, enteroinvasive *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*; ESBL-E, extended-spectrum beta-lactamase producing Enterobacterales; ETEC, enterotoxigenic *Escherichia coli*; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HIV, human immunodeficiency virus; Mpox, monkeypox; MRSA,

991 methicillin-resistant *Staphylococcus aureus*; PCR, polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TPHA, *Treponema pallidum*
992 hemagglutination assay; VRE, vancomycin-resistant Enterococci; VTEC/STEC, verotoxigenic *Escherichia coli*/Shigatoxigenic *Escherichia coli*.
993 # Canadian guidelines do not indicate the specimen type for the testing various infectious agents.
994 * Tested only in immunosuppressed individuals.
995 ^ Tested by culture.
996 [@] May be considered in some countries.
997 [&] Recommends use of validated standard of care test methods according to nationally and locally approved guidelines.
998 ** To detect seroconversion the follow-up screening can be limited to IgG testing.
999 *** Consider both antigen and antibody testing.
1000

Manufacturing and Storage

Manufacturing of FMT is poorly standardized, with significant variation in terms of the ratio of stool to a diluent, what diluent is used, whether anaerobic condition is applied, and if or which cryoprotectant is added. Three formulations can be produced—fresh, frozen, and lyophilized FMT products—which can be administered as a slurry or in a capsular form. Given the inherent batch effect in the donor microbiome, coupled with the variations in manufacturing practices, it is challenging to produce FMT treatments that have relative consistency to meet regulatory standards as a drug or as a biologic. Although there do not appear to be significant differences in clinical efficacy in preventing rCDI with different manufacturing processes or formulations (70), this may or may not hold true for other indications. Below, we briefly discuss preparation and handling of each FMT formulation and the advantages and disadvantages of each.

Stool is generally manufactured within 24 hours of collection. Fecal material is mixed with a diluent, such as saline or water, homogenized, and filtered—typically under aerobic conditions for convenience—to produce a fecal slurry. Anaerobic processing is more cumbersome and requires an anaerobic chamber, but has been shown to preserve obligate anaerobes and butyrate-producing bacteria (317), potentially an important consideration if the viability of these microbes is crucial (318).

The fresh fecal slurry can be administered immediately or can be stored frozen. A cryoprotectant, such as glycerol at 5%–10% final concentration, is commonly added, allowing the product to be stored at −20 °C, or preferably −80 °C, for up to 12 months without diminishing bacterial viability (304). Prior to usage, frozen FMT formulation is thawed and used within 6–8 hours.

For capsule manufacturing, minor differences exist between protocols, which follow similar principles in two steps. First, fecal slurry is centrifuged at low speed for a short duration (e.g.,

400*g* for 2 min), discarding the pellet and retaining supernatant. Second, the supernatant undergoes high-speed centrifugation for a longer duration (e.g., 3000*g* for 25 min) to precipitate a pellet which contains microorganisms (69, 319). For frozen capsules, glycerol is commonly used as a cryoprotectant (69); 100 grams of stool would produce approximately 40 capsules, which are stored at −80 °C (69). For lyophilized capsules, trehalose is commonly added to preserve bacterial viability (70); 80 grams of stool would produce five capsules, each containing 1.6 g of lyophilized product, which can be stored at −80 °C for up to 36 weeks with preserved bacterial viability (319).

Early clinical studies in the management of rCDI used fresh FMT formulations (66). This type of formulation has the least amount of manipulation but is also logistically the most challenging, has a limited shelf life, and does not allow quarantine during donor interval testing. Frozen and lyophilized FMT formulations have a longer shelf life, are particularly suitable for stool banks, and allow for a quarantine model, i.e., donor samples can be stored until the screening results from two time points flanking the quarantine period have returned (308). Frozen and lyophilized FMT formulations can also be prepared into capsules and offer the least invasive way of delivery. Furthermore, lyophilized products likely can remain viable even stored at 4 °C or room temperature as long as they are kept dry, and can facilitate shipping and transport as well as treatment dosing in an office setting or at home (70). The main disadvantage is the required equipment and infrastructure.

Safety

Adverse events (AE) associated with FMT are dependent on donor screening, indication for FMT, route of delivery, and recipient immune status. Although most side effects are generally mild and self-liming, severe adverse events (SAEs), including death and hospitalization, have been reported following FMT, and may be under-reported. Systematic reviews with studies including up to 5000 patients have found the overall rates of reported

adverse events to be as high as 39.3%; however, the majority are minor and transient, including abdominal pain/cramping, bloating, nausea, vomiting, fever, constipation, and diarrhea (320-323). Transient diarrhea and abdominal pain occur in ≤10% of FMT procedures. Rare SAEs directly attributed to FMT include transmission of multi-drug resistant *E. coli* from a single donor to two recipients. One of these patients received FMT following allogeneic hematopoietic cell transplant to prevent GVHD and died of the infection, while the other patient received FMT for HE and required hospitalization and recovered (86). Other FMT-associated transmission of infectious agents from OpenBiome products include Enteropathogenic *E. coli* and Shiga toxin–producing *E. coli*, resulting in six patients who required hospitalization and two subsequent deaths (279). All these events were related to inadequate donor screening. Unique to IBD patients, hospitalization and colectomy have been reported when FMT was used to treat concurrent rCDI or to induce UC remission. A recent systematic review and meta-analysis with 777 patients focusing specifically on the use of FMT to treat rCDI in IBD patients (86) demonstrated an SAE rate of 12%, with the most common being hospitalization, IBD-related surgery, or IBD flare. However, the causal link to FMT remains uncertain because these events may reflect the worsening progression of IBD itself. Regardless, these possible effects highlight the need for awareness and thorough consent when treating IBD patients with FMT. Colonoscopy-administered FMT has also been linked to sedation related aspiration pneumonia and perforation (89). The variability in the pooled rates of AEs and SAEs in systematic reviews stem from differing inclusion criteria, with the highest rates originating from a study that included only prospective, randomized studies (320); this review suggested a possible degree of under-reporting in studies with less rigorous methodology, as well as a lack of recognition and microbiological examination.

Beyond infectious pathogens, theoretical long-term risks may exist concerning the transmission or precipitation of non-infectious conditions including autoimmune,

neuropsychiatric, and neurodegenerative diseases, obesity, or malignancy (324). Developing new medical conditions after FMT has been reported; however, causality in these cases cannot be firmly established (325-328). Two studies described the transmission or persistence of potentially procarcinogenic *E. coli* in FMT recipients whose donors were positive for the same organism (329, 330).

Overall, FMT appears to be a safe therapy even among special populations including pediatric, immunosuppressed, and cirrhotic patients (200, 331-333). However, the evidence for severely immunocompromised individuals remains sparse. The majority of risks can be mitigated by adherence to rigorous donor screening and surveillance protocols, as outlined in several consensus guidelines (304). Standardized reporting through efforts such as the FMT Registry from the AGA and the European FMT working group will help bolster the knowledge of short- and long-term adverse effects (311).

FECAL MICROBIOTA TRANSPLANTATION IN CHILDREN

FMT in children deserves special consideration, as one size may not fit all. Generally speaking, the gut microbiota in children differs substantially from that of adults, and one should be careful extrapolating efficacy and safety data from adult populations. Furthermore, diagnostics of CDI in young children is difficult because of the asymptomatic presence of both *C. difficile* and its toxins in the intestinal tract of neonates and young children. Many laboratories exclude *C. difficile* testing in children with diarrhea below the age of two years. If testing is indicated, the likelihood of *C. difficile* colonization and coinfection with other intestinal pathogens and the presence of alternative diagnosis should be considered (334). Once a diagnosis of rCDI is certain, FMT appears safe and effective in children, similar to what is seen in adult patients, and has been recommended by practice guidelines (335). Questions arise regarding selecting the most appropriate donor for children who require FMT: Should it be from a sibling or an unrelated donor of similar age, or from a parent or

unrelated adult donor? How does the age of the donor impact clinical efficacy and, more importantly, how does it contribute to the long-term development of the microbiota of a child? Although some of these questions remain unanswered, in practice, heathy, unrelated adult stool donors have been used for convenience, supplied by stool banks. Most evidence for FMT in pediatric rCDI patients comes from uncontrolled studies (336-340). The largest retrospective cohort study with 335 patients (aged 11 months to 23 years) from 18 pediatric centers in the United States found that 87% of the recipients had a successful outcome following at least one FMT from an unrelated adult donor (337), comparable to what is observed in adult patients. Similarly, concurrent IBD does not negatively affect treatment success rate (341), and in one study, the risk of an IBD flare was also low (4%) following FMT with adult stool donors (337). Pediatric patients with compromised immunity pose a particular challenge because of limited safety data.

The route of FMT delivery also merits additional consideration. Although evidence in adult recipients found that efficacy varies somewhat by different routes, an individualized approach is required in children, which would vary depending on patient factors and preferences and on provider expertise, and would need to weigh the benefits versus risks of each option.

OMICS TECHNOLOGIES AND BIOINFORMATICS PIPELINES FOR FECAL MICROBIOTA TRANSPLANTATION

Omics technologies—such as genomics, transcriptomics, proteomics, and metabolomics—have revolutionized our capacity to investigate biological systems on a large scale. Advanced molecular methods such as amplicon sequencing (targeted) and shotgun (untargeted) metagenomics can capture differences at the DNA level, while others can detect changes at the level of mRNA (transcriptomics), or final gene products (proteomics and metabolomics). The pros and cons of omics technologies exploited in microbiome analysis of FMT research are summarized in Table 5. To extract meaningful insights from this data, the utilization of

bioinformatics pipelines is essential. These pipelines consist of a series of computational stages, encompassing data preprocessing, alignment, variant calling, annotation, data integration, and visualization. Through the application of bioinformatics pipelines, researchers can effectively process, integrate, and interpret omics data, facilitating the elucidation of complex biological processes and mechanisms. While single-omics using a reductionist approach (e.g., amplicon sequencing) can demonstrate association, integration of multi-omics data with in vitro (e.g., organ-on-chips) and in vivo (animal studies) data has the potential to reveal causality. When integrated, the omics technologies can be used to analyze changes following FMT.

1136 **TABLE 5** Advantages and disadvantages of omics technologies utilized in microbiome analysis of FMT research

Omics technology	Advantages	Limitations	Advancements and future directions
Amplicon sequencing	Detailed microbial community characterization at high taxonomic resolution in both donors and recipients. Cost-effective target region amplification. High-throughput analysis of multiple samples. Utilization of established data analysis tools (e.g., Mothur (342), Qiime 2 (343)) and reference databases (SILVA (344), Greengenes (345), NCBI (346)). Enhancing study comparability for meta-analyses and multi-cohort investigations. Diversity indices, such as alpha and beta diversity, help researchers analyze the ecological dynamics within microbial ecosystems.	Sensitivity tied to factors such as DNA preservation, extraction quality, and primer efficacy (347, 348). Need for optimization, including bead-beating DNA extraction and contamination detection controls (347, 348). Require downstream bioinformatic tools (e.g., SourceTracker (349), Decontam (350)) for identifying and removing contaminants (351). Impact of amplicon region choice on diversity (V4, V5–V6 of 16S rRNA) (352-355). Need to account for copy number variation within the 16S rRNA gene and preferential amplification of certain taxa (356). Unsuitable for virus and bacteriophage characterization due to lack of conserved genomic regions. The choice of alpha and beta diversity metrics can	Ability to sequence the complete V1–V9 16S gene, enabling species-level identification (357). Incorporation of additional target regions (ITS, 23S) in conjunction with long-read sequencing for improved resolution (357).

		influence results. Interpretation might be sensitive to sampling effort and sequencing depth.	
Shotgun metagenomics	Holistic sequencing of microbial genomes of donors and recipients for comprehensive insights. Enhanced taxonomic resolution, facilitating precise strain identification. Functional and community assessment potential, encompassing viruses and fungi alongside bacteria. Strain-level identification through single copy marker genes like PhyloPhlAn (358). Evolution toward species abundance estimation tools: protein-based (e.g., Kaiju (359)), k-mer-based (e.g., Kraken (360)), marker gene-based (e.g., MetaPhlAn2 (361)), and single nucleotide polymorphism-based (e.g., StrainFinder (362)). Diversity indices, such as alpha and beta diversity, help researchers analyze the ecological dynamics within microbial	DNA-based approach may capture inactive microbial DNA, resulting in potential misrepresentation of active microbial population due to DNA persistence (363). Taxonomic identification does not reflect functional activity. Inclusion of host DNA requires filtering tools [e.g., Bowtie2 (364), BWA (365)]. Challenges in identifying microbial dark matter lacking in reference databases (366). The choice of alpha and beta diversity metrics can influence results. Interpretation might be sensitive to sampling effort and sequencing depth. Costly.	Integration with other molecular techniques (transcriptomics, proteomics, metabolomics) for comprehensive insights. Long-sequencing technologies: Harnessing the capabilities of long-read sequencing to enhance genome assembly, particularly for complex microbial communities, thereby improving taxonomic and functional profiling. Advancements in computational analysis: Development of innovative algorithms and tools for metagenomic data analysis, including improved assembly, binning, and taxonomic assignment methods. Reference database expansion: Continued efforts to expand reference databases, encompassing a broader range of microbial diversity, including previously uncharacterized species and strains.

	ecosystems.		
Metatranscriptomics	Bridges gap between metagenome and community phenotype through RNA profiling (367). Reveals host-microbiome interactions post-FMT and offers dynamic insights into microbial community shifts. Availability of functional annotation tools: Utilizes various tools for functional annotation, including read-based packages such as MetaCLADE (368), UProC (369), or assembled-contig packages such as Prokka (370), and MG-RAST (371). Differential gene expression analysis: Facilitated by tools like EdgeR (372) and DeSeq2 (373), enabling identification of genes with variable expression.	Limited exploration of transcriptional activity in human microbiota (374-376). RNA instability and microorganism adaptability affecting data quality. Confounding factors: gene copy number and shared genes among closely related organisms. Post-translational regulation impacts gene expression and functional activity. Very costly.	Dual RNA sequencing (dual RNA-seq): Simultaneously measures host and microbial genome-wide transcriptional changes, providing insights into disease processes and host responses to microbial therapeutics. Single-cell RNA sequencing (scRNA-seq): Analyzes gene expression at the single-cell level, overcoming limitations of population-level analysis. Integration with other methods: Combination with techniques measuring final gene products (proteomics, metabolomics) to mitigate limitations and provide comprehensive insights.
Metaproteomics	Comprehensive functional insights: Proteomics directly measures protein levels, revealing real-time functional activity and accounting for post-transcriptional modifications (377). Various software packages are available for	Detection sensitivity: Low abundance protein detection limitations may miss key components, leading to incomplete functional understanding (380). Data complexity: Abundant proteomic data demands advanced tools for accurate analysis, especially in multi-omics integration.	Overcoming sensitivity limitations: Addressing low abundance protein detection challenges to achieve a more comprehensive proteomic profile. Advances in the field of single-cell proteomics (383, 384).

	exploring metaproteomic data, including MaxQuant (378). Specific open-source software programs such as MetaProteomeAnalyzer offer additional tools for data analyses and interpretation (379).	Sampling variability: Sample handling variations affect reproducibility, emphasizing the need for standardized protocols. Quantitative challenges: Quantifying protein abundance accurately, especially in label-free approaches, may face instrument-related hurdles (381, 382). Costly.	Developing techniques to capture post-translational modifications (e.g., phosphorylation) affecting protein function. Integrated phenotype analysis: Combining proteomics with genomics and transcriptomics to bridge genetic, epigenetic, and phenotypic variations(380).
Metabolomics	Comprehensive molecular profiling (385): Metabolomics provides a holistic view of small molecule metabolites, enabling a deep understanding of microbial and host metabolic activities (386). Early disease indicators: Metabolomic changes serve as early indicators of disease remission post-FMT, facilitating effective treatment assessment (252, 387, 388). Phenotypic clues: Metabolomics reveals phenotypic variations resulting from FMT, contributing to a comprehension of treatment outcomes (261, 389).	Metabolite identification constraints: Identification of all metabolites remains challenging, with an estimated identification rate of up to 30% (390). Sample handling sensitivity: proper sample collection and preservation are crucial due to metabolite turnover and susceptibility to handling conditions (390). Selective approach trade-offs: Choosing between untargeted and targeted methods involves trade-offs between comprehensive coverage and specific focus (391, 392). Costly.	Integrated multi-omics approaches: Future research entails integrating metabolomics with metaproteomics and metagenomics to overcome limitations and achieve more comprehensive insights. Precision disease monitoring: Metabolomics paves the way for personalized biomarker development, facilitating precise monitoring of FMT treatment outcomes. Functional network elucidation: Advances in metabolomics enable the construction of functional interaction networks, revealing intricate molecular crosstalk within FMT

			scenarios. Temporal dynamics analysis: Longitudinal metabolomic studies unveil dynamic changes in microbial functions and their correlation with FMT responses.

CHALLENGES AND OPPORTUNITIES

Factors to Consider in Fecal Microbiota Transplantation Research

Although there have been many advances in this field, important challenges remain, such as: 1) minimizing the risk for recipients, 2) optimal dosing, 3) confounders that affect analyses downstream of FMT, 4) duration of a clinical response and, arguably the most crucial, and 5) role of recipient characteristics in FMT success. Given the inherent heterogeneity in current FMT treatments, it is very challenging to compare across trials, even within specific indications. It is also unclear how one can predict which patients may respond to an FMT intervention.

Cost-effective and rigorous donor screening as well as quarantine models can mitigate but not eliminate all risks of transmitting communicable and non-communicable diseases. For rCDI, it appears that treatment by FMT is forgiving toward differences in methodology. However, this may not be the case for other indications, and dosing parameters (such as the frequency of administration, route of delivery, single versus pooled donor material, aerobic versus anaerobic processing, and formulation of the product) may determine treatment success.

Importantly, the role of potential confounding factors in microbiome research, such as diet, environmental factors, ethnicity, comorbidities, and medication other than antibiotics have not been systematically investigated in most clinical trials to date. For published clinical studies, follow-up durations are typically short, in the range of 8–12 weeks, and this makes it difficult to determine the durability of a positive clinical response. To address this, long-term follow-up data of FMT recipients is necessary. Additionally, most studies focus on stool samples and do not provide information on the mucosa/crypts–associated flora and the microbiota in the small intestinal tract. The development of smart robotic capsules to analyze the length of the whole gut can be used to collect tissue biopsy and gut microbiota samples for in-depth analysis with FMT intervention.

Finally, it remains to be established to what extent recipient characteristics contribute to FMT success. Such characteristics may relate to genetic factors, immune parameters, dietary patterns, or microbiome composition. FMT combined with an anti-inflammatory diet has shown promise in UC (115, 241). FMT plus fermentable fibers improved insulin sensitivity compared with FMT or fiber alone in a RCT (246) with MetS participants, and autologous FMT following a "green Mediterranean" diet (Mediterranean diet supplemented with green tea and a shake containing *Wolffia globosa*) prevented weight regain after the initial weight loss when compared with controls in a RCT in MetS (393). Pairing of donors and recipients based on similarity of gut microbiota should be tested in future trials.

Evaluating Microbial Engraftment and Functional Alterations Following Fecal Microbiota Transplantation as They Pertain to Efficacy and Durability of Response

In the prevailing view, engraftment of donor species is important for efficacy of FMT treatment (139, 243, 244); therefore, treatment might benefit from increasing FMT dose and frequency, or using other strategies that may lead to improved engraftment. It appears that multiple treatments may be required for a response in chronic conditions, and this response may wane without ongoing maintenance therapy, as seen in UC and MetS. Capsulized FMT would make this approach feasible (114, 148, 394).

Another strategy to increase microbial species richness and diversity is through the use of multi-donor products. For example, one study using pooled multi-donor FMT found that UC patients who had a response received treatment with material from a particular donor, and the overall microbial diversity was higher in the pooled FMT products compared with that from single donors (112). However, the rates of remission in RCTs for UC patients to date have not conclusively been higher in studies that used pooled multi-donor FMT (3–7 donors) compared with those using single donors (112, 113). However, because of variations in trial

design between studies, there is currently no clear evidence to support the use of multi-donor over single-donor FMT.

Engraftment may depend on compatibility or exclusion between the donor and recipient microbiota, and bowel preparation or antibiotic pretreatment may be necessary (139, 394) to "open up microbial niches." Of note, vancomycin pretreatment, a common practice prior to FMT for rCDI (90), appears to be needed for engraftment of the LBP VE303 (93). Using dedicated computational tools, persistent engraftment of donor-derived strains is shown to be associated with elimination of host-strains of the same species (395). Notably, this computational approach can introduce biases at every step, including the choice of bioinformatic pipeline and reference database used to analyze the data. Additionally, most studies focus on taxonomic compositions of the metagenomes without examining functional gene prediction profiles, neglecting the fact that some important functionalities are conserved across many different microbiota species, such as SCFA producers (396).. Integrated multi-omics output can overcome some of these challenges but require substantial interdisciplinary expertise.

Regulatory Challenges

FMT poses a challenge for regulatory bodies in terms of how to classify or regulate the product, as existing regulatory frameworks are developed for different classes of products. For example, in the United States and Canada, FMT is considered a biological product and drug. In the United Kingdom, it is regulated as a medicinal product. In Australia, it is considered a biologic, whereas in Italy, the Netherlands, and Belgium, it is classified as a tissue and regulated under the European Union Tissues and Cells Directive. In many countries, such as in Finland, India, and China, FMT is not clearly regulated.

Given the therapeutic benefits in rCDI and its potential benefits in an even wider range of microbiota disruption–associated states, it is essential that regulatory agencies balance

protecting the public with equitable access. This field is evolving quickly, which may not always favor patients. With the recent regulatory approvals of RBX2660 (a donor stool-derived microbiota suspension, also known as live-jslm or Rebyota) (398) and SER-109 (a donor stool-derived spore suspension, also known as live-brpk or Vowst) (399) in the United States, for example, the FDA has limited its enforcement discretion policy to establishments under which FMT is used to treat local patients. Centralized stool banks now require an Investigational New Drug (IND) application in order to continue to supply such products for clinical use (84); such a move will add complexity and costs to stool bank operations. There is a clear need to establish regulatory processes that fit the unique challenges of FMT, and can accommodate and adapt to microbial-based therapeutics in the future. At the same time, regulations should not further impede access to FMT, because significant barriers already exist in many countries (400): only 10% of patients with rCDI had access to FMT in a recent European survey (401).

Ethical Considerations

Ethical considerations need to consider both patient and donor perspectives. Informed consent is a critical element prior to FMT, and is even more important when the indication is beyond rCDI. The therapeutic benefit of FMT for rCDI is well established, but is less clear in other conditions. Short- and long-term risks, known and unknown, need to be disclosed and framed around donor selection, screening processes, and limitations. Patients who have specific religious beliefs/dietary restrictions may need special accommodations in their donor selection that may not be feasible to accommodate. Patients' autonomy may be compromised by their stress and desperation, affecting their ability to give informed consent. Thus, it is crucial that a provider clearly weighs the risks and benefits with their patients, provides alternative treatment options, and does so in a rational, compassionate, nonjudgmental, and nondirectional manner (402).

It is still not known what constitutes an ideal donor or a healthy donor. Several studies have shown the difficulty in recruiting donors based solely on simple criteria to mitigate risks. If further factors known to affect or be associated with the microbiome are to be considered and applied, such as diet or psychological wellness, it will be even more challenging to recruit and retain donors. Additionally, the invasive nature of stringent and repeated screening process may lead to concerns over donors' privacy and autonomy (403). Given the commitment required, should donors be compensated for their altruism, similar to sperm and egg donation? Or would compensation potentially encourage dishonesty and compromise the safety of donor products? Furthermore, if a donor who is healthy today develops a non-infectious condition of concern years later, does this information need to be disclosed to all the recipients of FMT products from this donor? Who bears the responsibility of tracking donors and recipients in the long term?

Integration of Multi-Omics Approaches

Generally, there are two ways to analyze multi-omics data: top-down and bottom-up. When researchers use genomics or transcriptomics data as a basis to predict phenotypic responses, variations in key proteins, and metabolic pathways (404), this is referred to as the top-down approach. An advantage of a top-down approach is that the researchers are working with genomics and transcriptomics data, which generally have higher coverage and, therefore, changes in the host may be captured more easily (404). However, the relationship from gene to metabolites is not always proportional, and DNA/RNA variations may not always correlate to functional variations (405). An alternative to the top-down approach is the bottom-up approach, in which metabolites are used to guide other omics analyses; changes in metabolites are more likely to be representative of phenotypic differences (404, 406). Combined analyses of multi-omics data with host physiology and mechanistic experiments in humans and animal models is promising, particularly in personalized medicine.

Experimental limitation and opportunity for improvement include (i) understanding the complexity and statistical behavior of output from each omics approach in isolation, (ii) being aware of possible covariate and cofounder relationships that might exist, and (iii) detection limitation and resolution differences in abundance between the omics data (407, 408). These intricacies mean that it is critical to develop advanced computational methods that efficiently extract key information from heterogeneous and complex multi-omics data. Machine learning, deep learning, language processing, and cognitive commuting—collectively known as artificial intelligence (Fig. 6)—hold great promise to explore and integrate multi-omics data to discover hidden patterns and find models that can accurately predict phenotypes (409).

One limitation of multi-omics approaches is that the mechanisms and direction of host-microbial interactions are still not clear, because the evidence is largely correlational. Moreover, the human microbiome is inherently complicated and heterogenous, with many factors that can have direct effects; as a result, traditional study designs may not have enough statistical power to extract causation from multi-omics data. Thus, there is a demand for finding alternative approaches that can be integrated with human studies (410), such as animal studies, organ-on-chips (organ chip), organoids, and cell studies (411). The organ-on-chip has been used for numerous cancer studies (412, 413), and recently an intestinal cell line–on-chip (414) has been developed for human clinical trials. These innovative approaches provide opportunities to investigate host-microbial interactions in a controlled and reproducible manner. Ultimately, results from association studies can generate hypotheses that can inform validation studies in other model systems; these results will need to feed back to human trials to confirm causality.

Pharmacomicrobiomics

The term pharmacomicrobiomics refers to the study of the interactions between drugs and the microbiome and analyzes how the composition and activity of the microbiome can influence the pharmacokinetics (PK) and pharmacodynamics (PD) of drugs. Although most of the studies target the gut microbiome through gut-active supplements such as probiotics and prebiotics, FMT presents new opportunities for improving therapeutic efficacy by mediating PKs/PDs. This is of special importance for metabolic diseases (such as diabetes mellitus), psychiatric diseases, IBD, autoimmune diseases, and various form of cancers treated with ICI. Microbial metabolism and its metabolites profoundly affect both the efficacy and toxicity by converting drugs to bioactive, inactive, or toxic compounds (415). Beyond immunomodulatory activities, the engrafted microbiota can influence the extent of drug absorption, PKs, and PDs. Changes in the microbiome may have consequences for the PKs of drugs such as levodopa (the mainstay of treatment of Parkinson's disease), because bacterial tyrosine decarboxylase in the gut microbiota influences the metabolism of levodopa (416, 417). An excellent example is the interaction of the microbiota with anti-cancer drugs (160). A topoisomerase I inhibitor, irinotecan, is converted into active metabolite SN-38 to prevent cancer cells from nucleotide biosynthesis (418). High concentrations of SN-38 can result in severe diarrhea, while SN-38 can be detoxified into SN-38 glucuronide (SN-38G) by liver uridine diphosphate glucuronosyltransferases (UGTs). However, bacterial β-glucuronidase, mainly produced by *Clostridium*, *Eubacterium*, and *Ruminococcus*, can potentially convert SN-38G back to SN-38 (419, 420). Another example is bacterial vitamin B6 and B9 metabolism, which is mandatory for 5-fluorouracil (5-FU) activation into cytotoxic 5-fluorouridine triphosphate to impede tumor cell division (421). Despite the elimination of distinct host-microbiome-drug interactions, a holistic view of this sophisticated interplay is still missing. Logical questions to consider here are: 1) How could the gut microbiota influence PKs and PDs of drugs and their metabolism in different organs such as the liver and

kidney? 2) How might FMT contribute to the rational modification of the gut microbiota, orchestrating favorable host-microbiome-drug interactions? Resolving these questions could lead to the development of more efficient combinations of conventional drug treatments with microbiome-based therapeutics such as FMT administration and cancer immunotherapy.

CONCLUSIONS AND FUTURE PERSPECTIVES

FMT is an important research tool for the future development of microbial therapeutics given its long track record, especially in rCDI. It will remain a useful tool in other indications to determine the causal relationship between microbial disturbance and a particular disorder.

Since 2012, FMT has developed from an experimental intervention to guideline-recommended treatment for rCDI. Spurred by this success, it is being explored as an intervention for many other indications, with varying success that can in part be attributed to heterogeneity in methodology and rapid developments in processing and formulation, sampling, and analyses. FMT research will benefit from using carefully designed large-scale studies with extensive metadata collection and consistent biospecimen collections to minimize noise in downstream multi-omic analyses, and from long-term follow up to address potential safety concerns. The results from such experiments can guide targeted experiments that address the underlying mechanisms of clinical outcomes of FMT, and may lead to personalized medicine in which donor and recipient characteristics are matched for optimal success.

The identification of microbial signatures from data obtained from FMT-treated trial participants suggests that interventions in the microbiome may be possible with defined microbial consortia or LBPs that may address most if not all of the drawbacks associated with FMT, such as the inherent variability of a product derived from minimally manipulated fecal material. Pharmaceutical industry is developing LBPs for conditions such as rCDI, IBD, and

cancer therapy, particularly focusing on rCDI given the long history of FMT treatment for this condition (93, 422).

Considering the above, are FMT's days numbered? We feel that FMT will remain an important tool for the exploration of the role of the microbiota in health and disease. After all, mechanistic investigations and the development of LBPs require hypotheses derived from associations between microbiota and clinical outcomes; these in turn follow from complex and heterogeneous data, such as those from FMT treatments.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1

ACKNOWLEDGMENTS

DK has received research funding from Canadian Institutes of Health Research, University of Alberta Hospital Foundation, Alberta Health Services, and Department of Medicine at the University of Alberta; consulting fees from Ferring. EMT and EK received an unrestricted research grant from Vedanta Biosciences. TM has received support from the National Institute for Health Research (NIHR) Nottingham Biomedical Research Centre based at the Nottingham University Teaching Hospitals NHS Trust and University of Nottingham, the Medical Research Council, UK Development Pathway Funding Scheme (grant reference: MR/S019103/1), and the Cystic Fibrosis Trust; consulting fees from Takeda Pharmaceuticals; served as an advisor for CHAIN Biotechnology.

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FIGURE LEGENDS

FIG 1 Multi-modal impact of indigenous and environmental factors on the gut microbiota. Several factors contribute to the structure and maintenance of a healthy gut microbiota (genetics, diet, birth mode, and lifestyle), while others could disrupt the microbial composition (medications, stress, western diet, and diseases) and trigger inflammatory responses. Microbiome disturbance reduces the thickness of the mucus layer and stimulates the production of inflammatory cytokines IFN-γ, TNF-α, and IL-1β. Intestinal inflammation and microbial disturbance further disrupt the indigenous composition of the host microbiome.

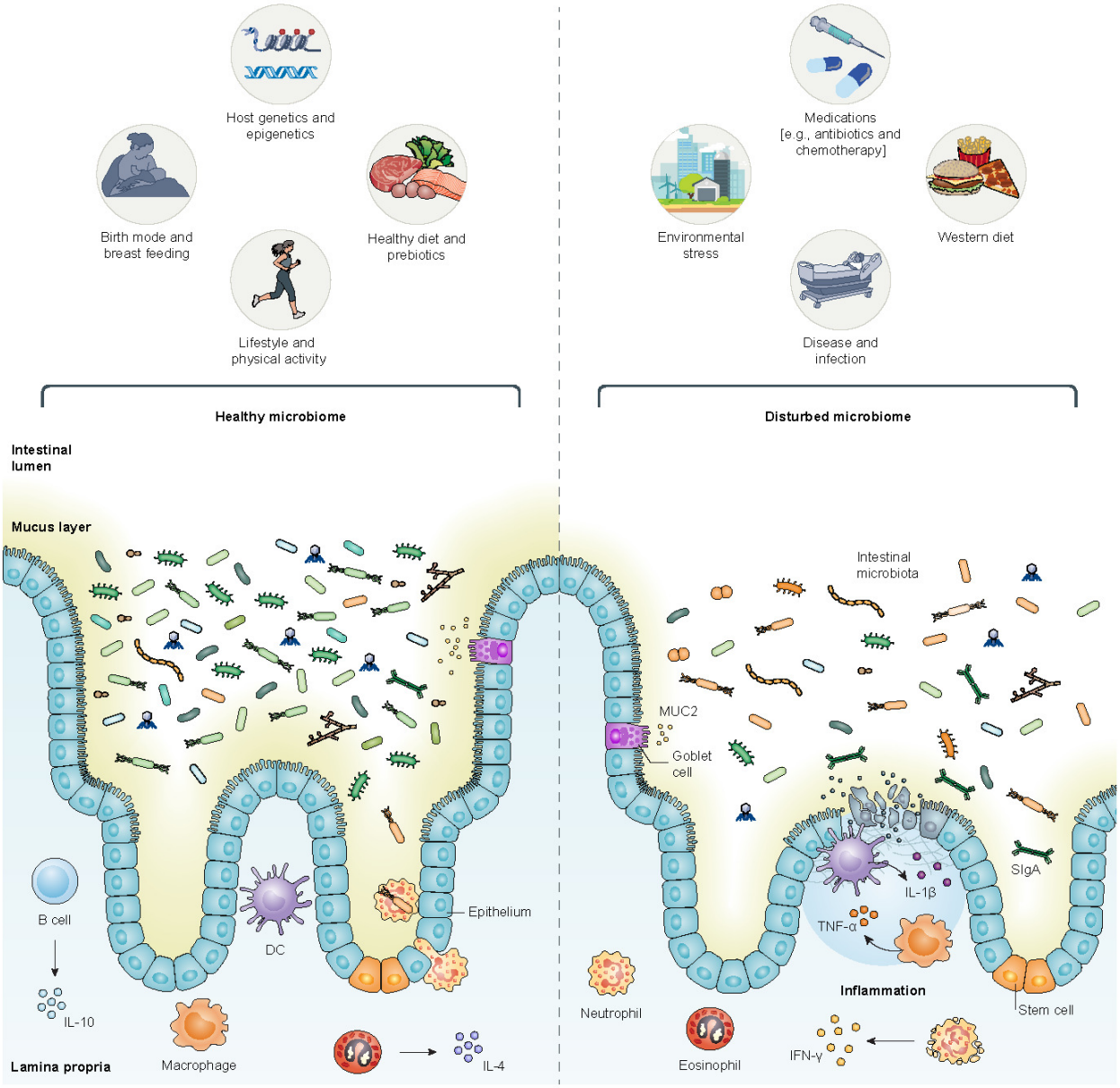
FIG 2 Main pathogenic mechanisms of *C. difficile* infection. TcdA binds to the host colonic epithelial cells by glycans and sGAGs, while cognate receptors for TcdB include glycans, Nectin 3, CSPG4, and FZD1/2/7 (423). The CDT toxin binds to LSR and undergoes proteolytic cleavage, and CDTa accelerates actin cytoskeleton breakdown and may ultimately facilitate *C. difficile* adherence (424). *C. difficile* cell wall PG can stimulate CXCL1 production and neutrophil infiltration in a NOD1-dependent manner (425). *C. difficile* SLPs are involved in DC maturation and stimulation of inflammatory responses through TLR4 activation (426). Moreover, *C. difficile* flagellin detection by TLR5 stimulates the activation of MYD88 in the host epithelial cells (427). CSPG4, chondroitin sulfate proteoglycan 4; CXCL1, CXC chemokine ligand 1; FZD1, Frizzled 1; LSR, lipolysis-stimulated lipoprotein receptor; NOD1, nucleotide-binding oligomerization domain 1; PG, peptidoglycan; sGAG, sulfate glycosaminoglycan; SLP, surface layer protein.

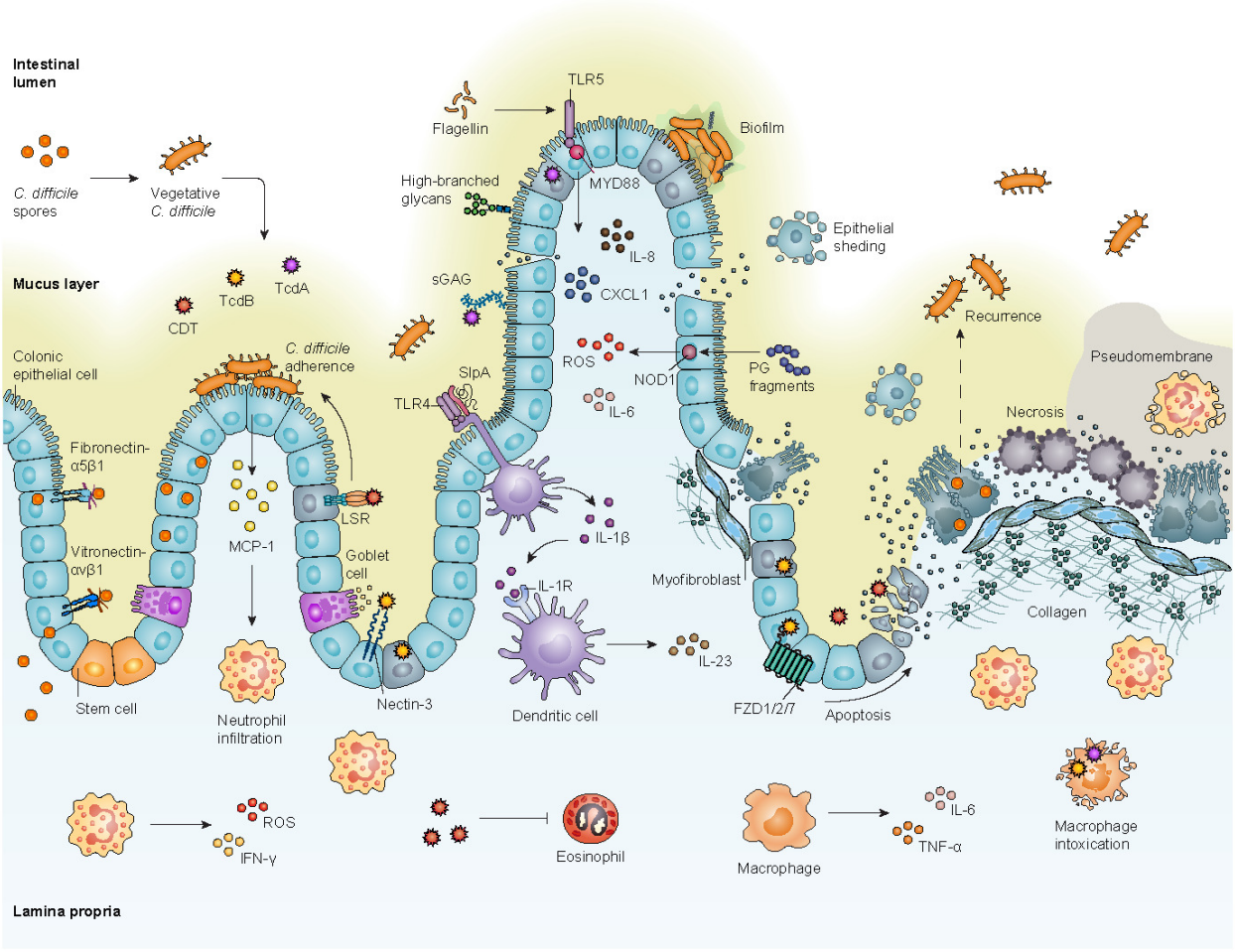
FIG 3 Evolution of FMT in clinical practice and research. The timeline describes the history of FMT-based therapy and key clinical studies for different disorders.

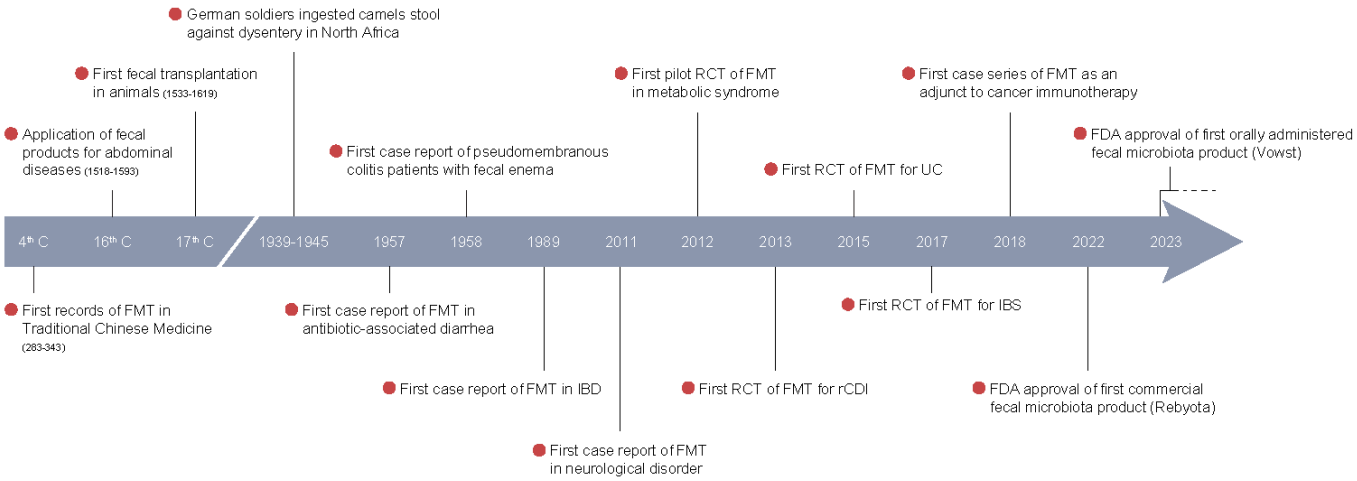
FIG 4 Registered clinical trials of FMT application as of July 2023. IBD, inflammatory bowel disease; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplant; MDRO, multi-drug resistant organism.

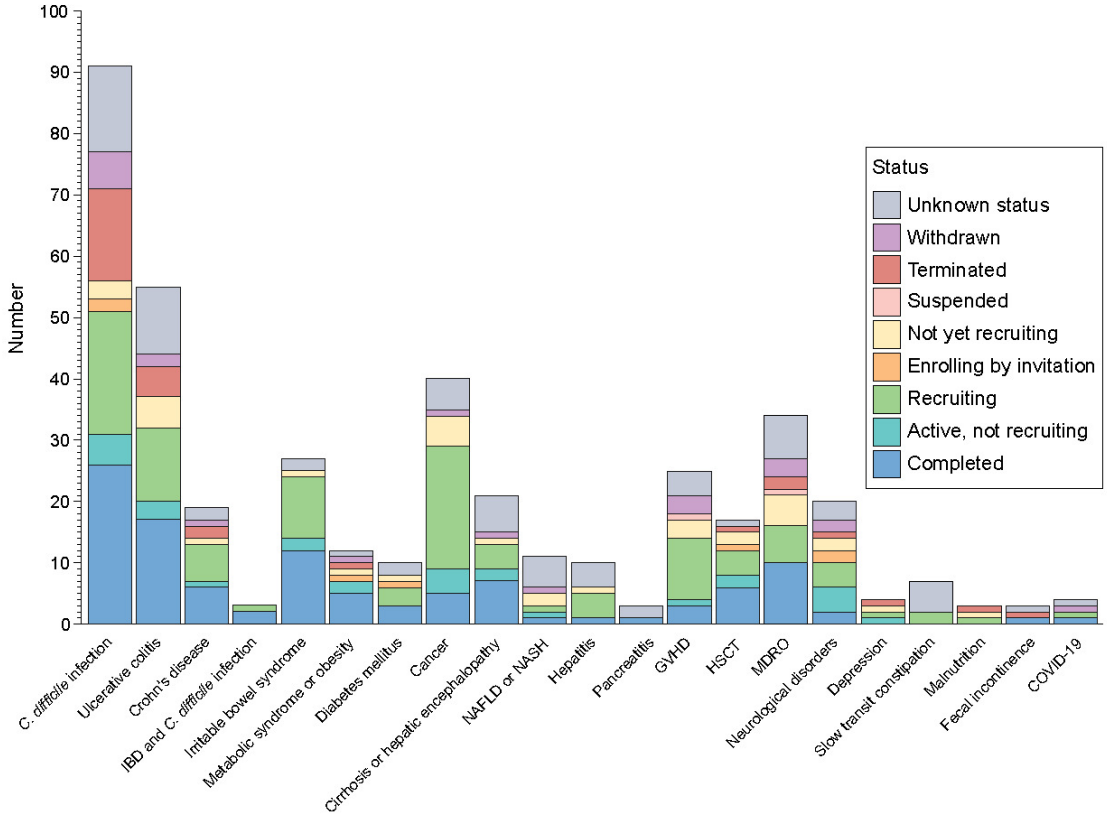
FIG 5 Pre- and post-FMT mechanisms underlying the interplay between microbiota and immune system. Before FMT administration, disturbed microbiota can stimulate immune responses that eventually lead to chronic inflammation. Following FMT, microbial restoration is accompanied by high production of anti-inflammatory cytokines, SCFAs, IgA, IgG, and antimicrobial peptides. Immune and metabolite homeostasis results in inflammation amelioration and repair of mucosal layer and epithelial barriers.

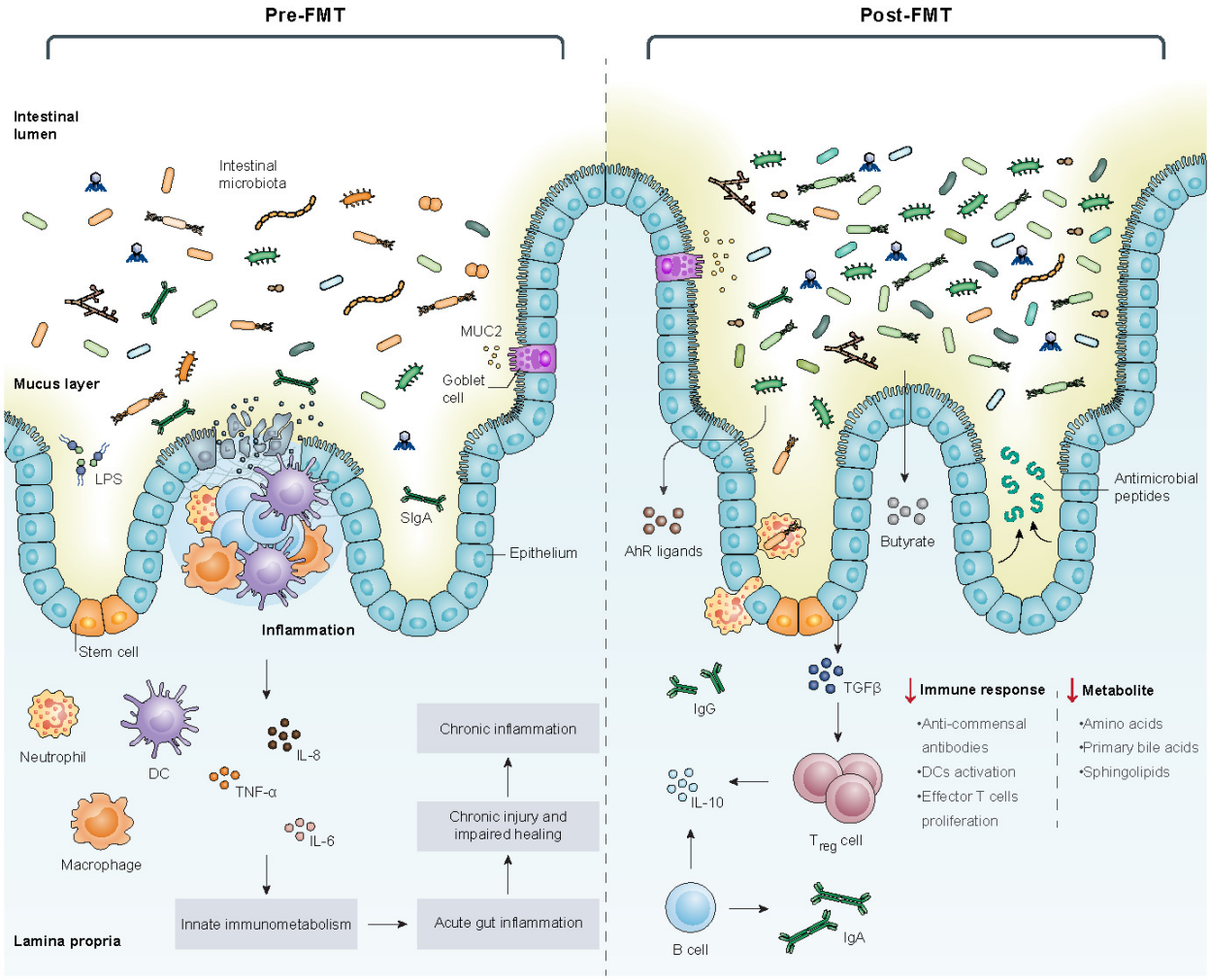
FIG 6 Multi-omics approaches and their application in future studies. (A) FMT procedure from healthy donor microbiota to clinical outcomes of the recipient. (B) 1. In a reductionist approach, only one organ is considered, while a holistic approach considers multiple organs at the same time. 2. Due to genetic and environmental variations between human and animal models, organ-on-a-chip can provide new approaches in microbiome studies. 3. Types of artificial intelligence strategies currently used for omics data analysis and interpretation. 4. An example of data integration by multi-omics approach.

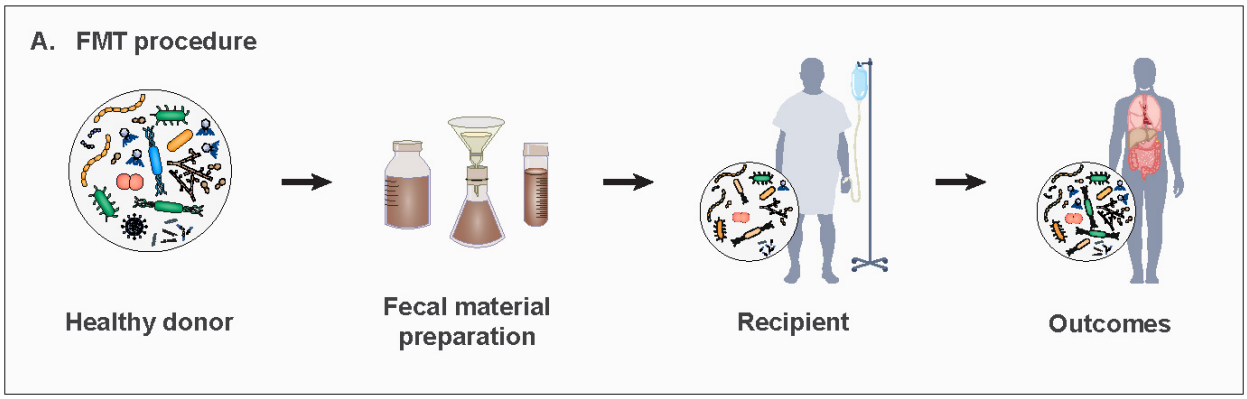

Host genetics and epigenetics
Birth mode and breast feeding
Healthy diet and prebiotics
Lifestyle and physical activity
Medications [e.g., antibiotics and chemotherapy]
Environmental stress
Western diet
Disease and infection
Healthy microbiome
Disturbed microbiome
Intestinal lumen
Mucus layer
B cell
IL-10
DC
Epithelium
Macrophage
Lamina propria
IL-4
Intestinal microbiota
MUC2
Goblet cell
SIgA
IL-1β
TNF-α
Inflammation
Stem cell
Neutrophil
Eosinophil
IFN-γ

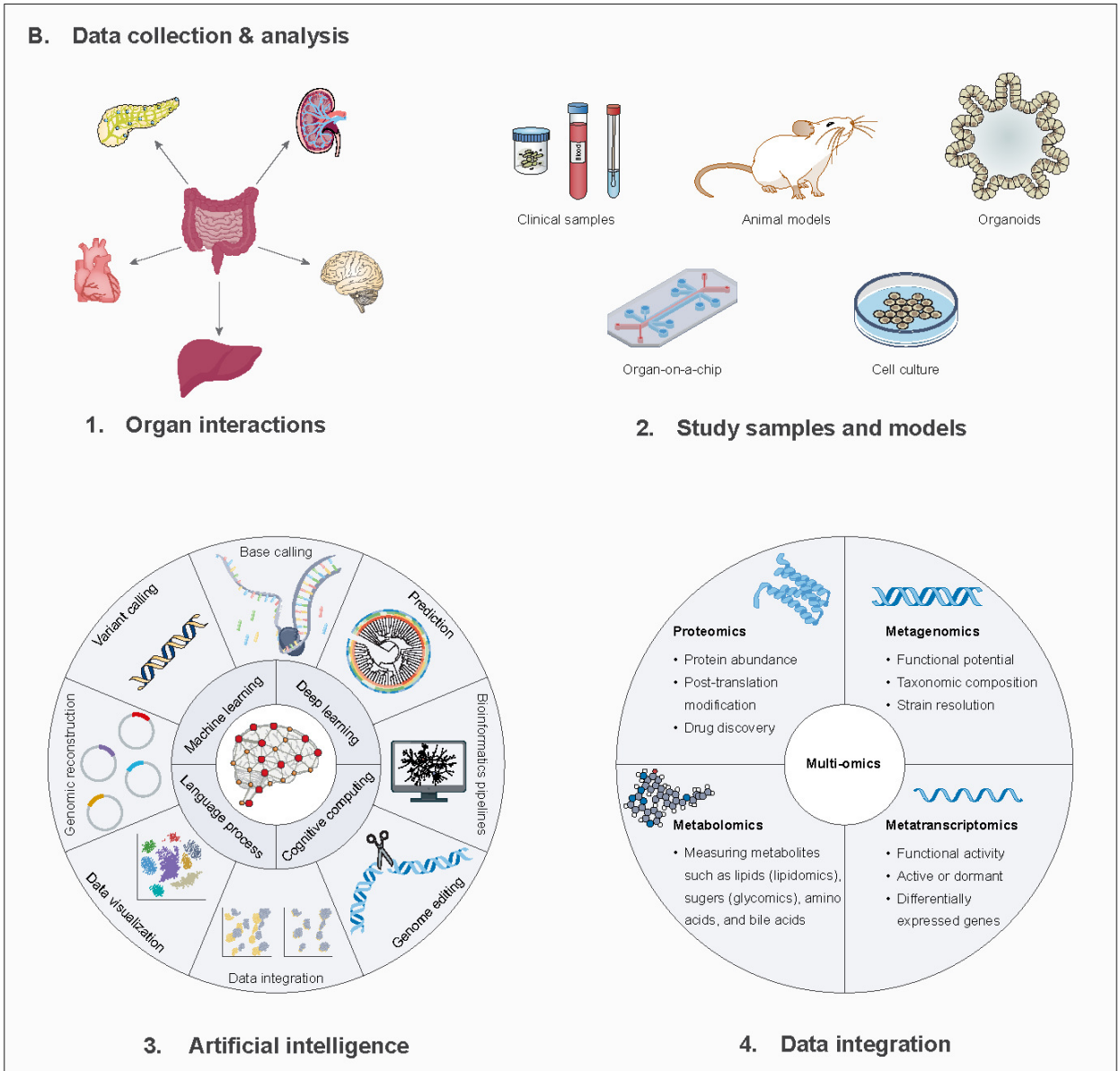

Intestinal lumen
C. difficile spores
Vegetative C. difficile
Mucus layer
TcdB
TcdA
CDT
C. difficile adherence
Colonic epithelial cell
Fibronectin-α5β1
Vitronectin-αvβ1
LSR
MCP-1
Goblet cell
Stem cell
Neutrophil infiltration
Nectin-3
Flagellin
TLR5
Biofilm
High-branched glycans
MYD88
IL-8
sGAG
CXCL1
SlpA
TLR4
ROS
NOD1
IL-6
PG fragments
IL-1β
IL-1R
Dendritic cell
IL-23
Myofibroblast
FZD1/2/7
Apoptosis
Epithelial sheding
Recurrence
Pseudomembrane
Necrosis
Collagen
ROS
IFN-γ
Eosinophil
Macrophage
IL-6
TNF-α
Macrophage intoxication
Lamina propria


German soldiers ingested camels stool against dysentery in North Africa
First fecal transplantation in animals (1533-1619)
Application of fecal products for abdominal diseases (1518-1593)
First pilot RCT of FMT in metabolic syndrome
First case series of FMT as an adjunct to cancer immunotherapy
FDA approval of first orally administered fecal microbiota product (Vowst)
First case report of pseudomembranous colitis patients with fecal enema
First RCT of FMT for UC
4th C
16th C
17th C
1939-1945
1957
1958
1989
2011
2012
2013
2015
2017
2018
2022
2023
First records of FMT in Traditional Chinese Medicine (283-343)
First case report of FMT in antibiotic-associated diarrhea
First RCT of FMT for IBS
First case report of FMT in IBD
First RCT of FMT for rCDI
FDA approval of first commercial fecal microbiota product (Rebyota)
First case report of FMT in neurological disorder


Number
100
90
80
70
60
50
40
30
20
10
0
Status
Unknown status
Withdrawn
Terminated
Suspended
Not yet recruiting
Enrolling by invitation
Recruiting
Active, not recruiting
Completed
C. difficile infection
Ulcerative colitis
Crohn's disease
IBD and C. difficile infection
Irritable bowel syndrome
Metabolic syndrome or obesity
Diabetes mellitus
Cancer
Cirrhosis or hepatic encephalopathy
NAFLD or NASH
Hepatitis
Pancreatitis
GVHD
HSCT
MDRO
Neurological disorders
Depression
Slow transit constipation
Malnutrition
Fecal incontinence
COVID-19


Pre-FMT
Post-FMT
Intestinal lumen
Intestinal microbiota
Mucus layer
MUC2
Goblet cell
LPS
SIgA
Epithelium
Stem cell
Inflammation
Neutrophil
DC
IL-8
TNF-α
IL-6
Macrophage
Lamina propria
Innate immunometabolism
Acute gut inflammation
Chronic injury and impaired healing
Chronic inflammation
AhR ligands
Butyrate
Antimicrobial peptides
IgG
TGFβ
IL-10
T reg cell
B cell
IgA
Immune response
•Anti-commensal antibodies
•DCs activation
•Effector T cells proliferation
Metabolite
•Amino acids
•Primary bile acids
•Sphingolipids


A. FMT procedure
Healthy donor
Fecal material preparation
Recipient
Outcomes


B. Data collection & analysis
1. Organ interactions
Clinical samples
Animal models
Organoids
Organ-on-a-chip
Cell culture
2. Study samples and models
Base calling
Prediction
Bioinformatics pipelines
Genome editing
Data integration
Data visualization
Genomic reconstruction
Variant calling
Machine learning
Deep learning
Language process
Cognitive computing
3. Artificial intelligence
Proteomics
• Protein abundance
• Post-translation modification
• Drug discovery
Metagenomics
• Functional potential
• Taxonomic composition
• Strain resolution
Multi-omics
Metabolomics
• Measuring metabolites such as lipids (lipidomics), sugers (glycomics), amino acids, and bile acids
Metatranscriptomics
• Functional activity
• Active or dormant
• Differentially expressed genes
4. Data integration