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Title

Key determinants of success in fecal microbiota transplantation: from microbiome to clinic

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SUMMARY

Fecal microbiota transplantation (FMT) has achieved satisfactory results in preventing the recurrence of *Clostridioides difficile* infection, but they have been hardly replicated in other diseases.

Several factors are known to influence FMT success, including those related to donors and recipients (including diversity and specific composition of gut microbiome, immune system, host genetics), as well as to working protocols (fecal amount and number of infusions, route of delivery, adjuvant treatments). Moreover, initial evidence suggests that the clinical success of FMT may be related to the donor microbial engraftment.

The application of cutting-edge technologies for microbiome assessment, as well as specific mindset shifts aimed at changing the current vision of fecal transplants, are expected to improve FMT protocols and outcomes.

Here we review the key determinants of FMT success and prompt potential insights to enable a close integration of lab-based and clinical approaches aimed at increasing FMT success, with the purpose of advancing the field of microbiome-based therapeutics.

Keywords

Fecal microbiota transplantation; microbiome; engraftment; host immunity

FECAL MICROBIOTA TRANSPLANTATION: CURRENT LANDSCAPE, VIEW, AND CRITICAL ISSUES

Since the first reported sequencing of the human gut microbial ecosystem,[1] the intestinal microbiota has become the object of extensive investigations that led to the discovery of its key role in human health (as it provides essential functions within the human body) and disease (as it is a major pathogenic pathway of several disorders). This gain of knowledge has paved the way for exploring its potential role as a therapeutic tool or target.

Among the therapeutic interventions targeting gut microbiome, fecal microbiota transplantation (FMT) has been rapidly attracting the interest of scientific and clinical communities. FMT can be defined as the transfer of minimally manipulated feces from healthy donors into a recipient's gut with the aim of treating a disorder associated with gut microbiota alterations.

FMT has been investigated in mainstream medicine for several communicable and non-communicable diseases with heterogeneous outcomes. Based on early successful reports on pseudomembranous colitis,[2] FMT was first investigated as a potential treatment for recurrent *Clostridioides difficile* infection (rCDI), where it has shown a consistent success rate of nearly 90%, as reported in several systematic reviews and meta-analyses,[3–5] and has been proven to be a reliable therapeutic alternative to vancomycin[6,7] and fidaxomicin.[8] In this setting FMT has also gained other clinically relevant achievements, including the management of severe and severe-complicated CDI,[9] the increase of overall survival in patients with rCDI,[10] the reduction in CDI-associated bloodstream infections and in CDI-related surgery,[10,11] and it was shown to be a cost-effective strategy for this disorder.[7]

These findings, consistently confirmed by a large body of evidence, have primed the progression in the field of FMT in both clinical practice and research.

First, FMT has been firmly embedded among the recommended treatment options for rCDI and has been considered as a potential rescue strategy for severe and refractory CDI.[12–14] Due to the increased need for CDI cure in clinical practice, FMT has gradually evolved over years through actions aimed at establishing the methods of this procedure and disseminating its provision. The use of frozen material over fresh feces was the first step into FMT modernization.[15] This transition allows managing large volumes of patients without the risk of stool shortages, and has also increased the safety of donor screening by the application of measures, including the quarantine of stored feces[16,17] and/or molecular testings,[18] that were able to prevent the theoretical transmission of multi-drug resistant (MDR) bacteria, recently claimed as a known risk of FMT by the Food and Drug Administration (FDA) of the USA.[19] More recently, the stool donor screening has been updated to prevent the theoretical risk of COVID-19,[20] with satisfactory safety outcomes.[21] Both the increase in clinical request for FMT to prevent rCDI and the high level of required quality controls have led to the development of stool banks, that have established as entities able to provide a widespread and equitable access to FMT for patients together with high safety, quality and traceability of the workflow.[22] Capsulized FMT appeared as another critical step toward the modernization of this procedure, allowing FMT services to disentangle from the need of a structured endoscopy unit to provide fecal transplants.[23]

The increasing interest toward FMT and understanding of its potential have fostered its regulation and classification (Table 1). After an early state of uncertainty, several authorities have attempted to define the essence of gut microbiome, with different lines of thought. In several Countries, including USA, Canada and France, FMT is defined as an investigational drug and must be released in the context of clinical trials, with specific exceptions e.g. rCDI (USA) or use in hospital settings (France).[24] Notably, the U.S. FDA has recently limited this exception only to FMT provided within establishments that treat their own patients (e.g.

hospital laboratories), excluding products released by stool banks. The change in the FDA enforcement discretion policy was based on the safety concerns related to specific characteristics of stool banks, including centralized manufacturing practices and to the number of patients who could be exposed to a specific donor. (REF)

In another view, however, stool can be hardly compared to a standardized and reproducible mixture of microorganisms, being composed of a heterogeneous consortium of microbes (bacteria, viruses, micro-Eukaryotesetc), human cells, water, mucus, metabolites, antigens, and its composition varies in time depending on several donor-related factors (age, lifestyle, drugs, etc.).[25] Based on this principle, and in line with the concept of microbiome as a human organ,[26] other countries, including Italy, the Netherlands and Belgium, have assimilated FMT as a tissue transplant.[27] Moreover, the European Commission has identified stool as a substance of human origin (SoHO),[28] which did not fulfil the requirements for being regulated under the European Union Tissues and Cells Directive (EUTCD), because the mechanism of action is not mediated by human cells.[29,30] However, the European Commission has recently updated its view and adopted the proposal for a regulation on standards of quality and safety for substances of human origin intended for human application (including microbiota), which are going to be incorporated in the EUTCD.[31] Finally, in some other Countries FMT is regulated more flexibly as a medicinal product (United Kingdom) or as a practice of medicine (e.g. Australia, Germany).[27] This variety in regulating FMT may reflect the uncertainty about the active components and mechanisms of microbiome transfer. The regulatory landscape of FMT has recently become even more varied after the recent receipt of licensure by two commercial fecal microbiota based live biotherapeutics. Indeed, over the past few months the U.S. FDA has approved REBYOTA, and the Australian Register of Therapeutic Goods has licensed BIOMICTRA, for commercial use limited to the prevention of rCDI. Both products have been labelled by these authorities as biological products. This

classification paves the way for a future differentiation between the regulatory pathways of standard FMT and commercial microbiome therapeutics.

After the positive outcomes on CDI, FMT has been investigated in a number of chronic disorders that have been associated with dysbiosis, including inflammatory bowel disease (IBD),[32] irritable bowel syndrome (IBS),[33] metabolic syndrome,[34] neuropsychiatric disorders,[35] and others. However, despite the increasing number of clinical trials and body of evidence, several issues still prevent the translation of FMT from research to clinical practice for chronic noncommunicable conditions to date. First, most of these investigations have achieved alternate results, not comparable with outcomes of FMT in rCDI. CDI is a relatively simple condition primarily driven by gut microbiota alterations,[36] which is much easier to treat than complex chronic disorders in which the gut microbiome is only one among the pathogenic pathways contributing to the disease. This concept is supported by the evidence that the high efficacy rate of FMT in rCDI is not observed in other conditions where the characteristics of donors,[25] recipients,[40] and working protocols,[41] appear to influence more the clinical outcomes. Finally, as chronic diseases are likely to need sustained therapy to maintain remission, also a mindset shift toward considering FMT as a chronic treatment to be incorporated among other therapeutic strategies has been advocated,[42] and tools to make microbiome modulation sustainable in the long-term, such as capsulized FMT (to increase the patient compliance) or microbiome therapeutics (to guarantee the standardization, reproducibility and precision of the treatment) have already come out as effective options.[43] Here we discuss the key determinants of FMT success, including the role of donor-recipient microbiome engraftment, the variables related to donors, recipients, and working protocols, that influence gut colonization and clinical success. Finally, we prompt potential mindset changes and technology insights (including diagnostic and therapeutic tools) that may prime the advancement of microbiome therapeutics.

FACTORS THAT INFLUENCE OUTCOMES OF FMT

Donor-related factors

The screening and selection of donors is the most challenging step of the FMT process, and encloses different layers of complexity, including those related to the safety and those that may influence the efficacy of fecal transplants (Figure 1).

Short-term and long-term safety of FMT

Current guidelines for FMT in clinical practice[14] recommend specific protocols for donor screening aimed at assuring that the transplanted material is safe, primarily by avoiding the transfer of infectious agents. This issue has become extremely timely after the transmission of an extended-spectrum β-lactamase-producing *Escherichia coli* strain from a stool donor to two immunocompromised recipients who developed bacteremia, that was fatal in one case.[44] Recipients received FMT in the context of two independent clinical trials where the donors had not been screened for multi-drug resistant organisms (MDRO). Then, the FDA required the inclusion of MDRO screening into FMT-based study protocols,[19] although it was already recommended by international guidelines[14] and included in the working protocols of all major stool banks, without any reported case of MDRO transmission,[45] by molecular screening of fecal batches for pathogens, or through quarantine of batches.[46] Then the FDA has released a similar alert rapidly after the start of the COVID-19 pandemic,[47] promptly followed by the definition of guidelines to prevent the FMT-related transmission of SARS-CoV-2,[20] that proved effective.[21] Notably, in a recent systematic review all FMT-related serious adverse events occurred in patients with damage of the mucosal barrier.[48] As the gut barrier is involved in the pathogenesis of several gastrointestinal and extraintestinal disorders,[49] its injuries should be considered while evaluating potential recipients.

As soon as the assessment of clinical outcomes was coupled with the evaluation of gut microbiome in FMT trials, and along with the discovery that some microbial strains or signatures may be associated with the risk of specific disorders,[50] the risk of transferring microbial signatures that could be potentially threatening in the long term has become a matter of discussion. The durable transfer of three potentially procarcinogenic bacterial strains (enterotoxigenic *Bacteroides fragilis*, *Fusobacterium nucleatum,* and colibactin-producing *Escherichia coli*, also known as polyketide synthase-positive [pks+] *E. coli*) was determined in a small cohort of pediatric patients receiving FMT for rCDI and followed up to six months.[51] The sustained acquisition of at least one potentially procarcinogenic strain was shown in four of 11 patients (36%), but three patients experienced their clearance or decrease after FMT from a negative donor. In another retrospective cohort of 49 patients with rCDI, eight of nine patients (89%) with pks+ *E. coli* who received FMT from donors also positive for pks+ *E. coli* kept this strain, while 13 of 18 positive patients (72%) who underwent FMT from a negative donor eradicated it, while the transmission from positive donors to negative patients was unlikely (one of five patients, 20%).[52]

Although preliminary, these two studies suggest that FMT may be a double-edged sword that can transmit or eradicate potentially procarcinogenic bacteria and, more generally, unfavorable microbial signatures. The consistency and durability of these shifts have not yet been clearly assessed, and may depend on several factors, including the age of the recipient, as the pediatric microbiome is still unconsolidated and more likely to be durably modified,[51] or the number of fecal infusions, as sustained microbial engraftment after single FMT is unlikely.[53] Current guidelines for donor screening recommend excluding individuals with cancer and chronic inflammatory or metabolic disorders,[14] but whether these criteria are sufficient to avoid the transfer of harmful microbial signatures is still unknown. To enhance the long-term safety of FMT, future studies aiming at assessing the real risk of transferring

specific microbial signatures by metagenomic strain tracking, as well as those focused on understanding how much the transmission of these signatures is likely to trigger the development of specific disease phenotypes, are advocated.

Efficacy of FMT

To date, internationally recommended donor screening protocols are focused on granting the safety of procedures mainly by minimizing the risk of infection, and apply general criteria to exclude donors with perturbation of gut microbiome (e.g. by discarding those with chronic gastrointestinal disorders, systemic autoimmune disorders, cancer, neurological/neurodegenerative disorders, psychiatric/neurodevelopmental conditions, obesity and/or metabolic disorders, recent exposure to systemic antimicrobials, immunosuppressant agents, chemotherapeutics, chronic treatment with proton pump inhibitors). However, these protocols have not yet focused on assuring clinically effective donor feces, for a number of reasons. First, the transfer of a healthy intestinal microbiome, regardless of its composition, is consistently effective in rCDI,[3–5] so there was no need to refine donor screening for this disorder, which is still the only indication for FMT in clinical practice. Moreover, at the time of early FMT investigations, microbiome sequencing was less effective and more expensive than nowadays, so most studies did not assess donor microbiota,[6,15] and the microbial characteristics that make a clinically effective donor were not available. Finally, as the donor selection process is already highly selective, the addition of other parameters beyond the safety ones is highly challenging in terms of feasibility.

However, in recent years several investigators have associated donor microbiome characteristics with clinical success in FMT studies, starting from *Clostridioides difficile* infection, as it is the most diffused indication for FMT, but also in noncommunicable disorders, where the boost to identify effective strategies is given by the inconsistency of

results achieved through classical FMT approaches. An increasing body of evidence suggests that both ecological parameters and the taxonomic composition of donor microbiome can influence clinical success.

Higher donor richness was repeatedly associated with clinical success of FMT in nonrandomized cohort of patients with IBD refractory to medical therapy.[54,55] In another randomized trial of capsulized FMT in patients with UC, the subjects who received donor fecal batches with higher evenness were more likely to respond to FMT, suggesting that this parameter may be more relevant as it could indicate that the donor microbiome has an established niche and enough stability, so it does not fluctuate with dietary changes.[56] More extensively, higher donor alpha diversity was identified as a predictor of response in a systematic review of 25 studies evaluating FMT in patients with UC.[57]

As alpha diversity is a marker of host health and FMT aims at restoring the diversity of patient microbiome to resemble that of healthy subjects,[58] these findings have a reliable biological background. However, alpha diversity alone is a poor and aspecific indicator of host health and these results were not confirmed in other trials [59.] In a meta-analysis of FMT studies across different disorders, donor alpha diversity had a significantly positive association with donor strain engraftment, although it did not influence clinical success.[60] Finally, in two randomized controlled trials of patients with UC, responders to FMT showed a higher similarity to their donors than nonresponders.[61,62] This last finding anticipates conceptually the importance of donor microbiome engraftment for clinical response to FMT.

Beyond ecology parameters, several studies have evaluated if the composition of donor microbiome, including bacteria, bacteriophages and fungi, was associated with clinical success, in communicable but mainly in noncommunicable disorders, where the need to find an optimal donor microbiome signature was higher, due to the alternate results obtained by FMT in this setting. As expected, donor feces with higher abundance of supposedly

beneficial bacteria were more likely to provide clinical success after FMT, in several disorders. According to different studies, high relative abundance of several donor taxa has been associated with clinical success (induction of remission) of FMT in UC, including Lachnospiraceae,[62] Ruminococci,[55,62] *Akkermansia muciniphila*,[55] or Bacteroides,[63] while donor fecal Streptococci were associated with lack of response.[63] Moreover, in a small cohort of patients with IBS treated with FMT, clinically effective donors showed a higher abundance of Bifidobacteria than non-effective donors or patients.[64] In a more recent randomized trial of patients with IBS, a so-called "super-donor", selected with clinical predictors of a healthy microbiome (healthy, young, lean, born by vaginal delivery, breastfed, etc) and with a favorable microbiome profile rich in Dorea, Lactobacillus, and Ruminococcaceae spp, was associated with the highest ever success rate of FMT in this setting (nearly 90%).[65]

Such an association was found also in extra-GI disorders, although with more preliminary evidence. In three recent studies, mouse models of melanoma experienced a satisfactory response to immune checkpoint inhibitors (ICI) after receiving feces from patient who responded to these drugs, whose microbiome was characterized by high alpha diversity and high abundance of beneficial bacteria, including Ruminococcaceae, Faecalibacterium, *Bifidobacterium longum*, *Akkermansia muchiniphila*.[66–68] Additionally, patients with advanced melanoma refractory to ICI experienced increased response after receiving FMT from long-term responders to nivolumab, whose feces were abundant in Bifidobacteria and Ruminococci.[69]

Also the composition of donor gut virome may play a role in the success of FMT. In a cohort of patients with CDI, cure after FMT was associated with a high colonization from donor-derived Caudovirales taxa in the recipient gut, mostly when donors had a higher Caudovirales richness than patients.[70] Finally, dysbiosis of the donor mycobiome, specifically an overgrowth of *Candida albicans,* has been associated with reduced efficacy of FMT in

patients with CDI.[71] This association is supported by increasing evidence that C. albicans worsen the CDI course both in pre-clinical models[72] and in patients.[73]

In conclusion, available evidence suggests that metrics and composition of donor microbiome may influence the clinical outcomes of FMT in different disorders and may represent a promising target to improve FMT efficacy. However, current data also suggest that single taxa can be hardly considered as consistent predictors of clinical outcomes, and that the role of microbiome in the natural course of diseases is more complex than previously believed.[74] Specific matching models, based on analytic hierarchy process[75] or machine-learning approaches,[76] have preliminarily shown to be able to identify the most suitable donor for a specific recipient, supporting the advancement of efforts in this field.

Recipient-related factors

The intestinal ecosystem is a complex niche shaped by a combination of host and environmental factors. These driving forces combine to achieve a steady state that underlies the resilience of gut microbiota composition and structure after an acute perturbation such as FMT. Among the key determinants of the mucosal landscape are host genetics and immunity. After FMT these host factors will persist and contribute to remodeling the transferred microbial population to a new host-microbial equilibrium.

Genetic and immune factors

Genetic factors are drivers of the gut microbiota composition, as demonstrated by the greater similarity between the gut microbiota of homozygotic twins compared to dizygotic or unrelated individuals.[77] Specific associations between gene polymorphisms and taxa have been identified by multiple genome-wide association studies from different countries and

cohorts.[78–80] Interestingly, many of the highlighted genetic loci were involved in the host immune response, such as polymorphisms of genes encoding sensor molecules of innate immunity including C-type lectins or the vitamin D receptor.[79,80] In inflammatory bowel disease (IBD), associations between polymorphisms in innate immunity genes and gut microbiota composition were even more striking and corroborated by mechanistic studies in mice such as for *Nod2* or *Card9*.[81,82]

Timing is everything

In the cross-talk between mucosal immunity and gut microbes, specific periods of plasticity have been identified, such as during weaning and neonatal periods, while host lymphocytes and gut bacteria colonize the intestinal mucosa and expand.[83] FMT during this period has shown distinct and durable engraftment in mice. For example, FMT performed during the neonatal period in mice shows very distinct engraftment compared to FMT from the same donor performed at the adult age with overexpansion of flagellin-producing bacteria depending on the neonatal expression of the Toll-like receptor 5, an innate receptor for flagellin.[84] In human adults, after allogeneic hematopoietic stem cell transplantation, the gut microbiota and mucosal immunity undergo a similar resetting of immune-microbial cross-talk in the context of intensive antibiotic treatment, chemotherapy-induced mucosal injury, and re-colonization of the mucosa by donor-derived immune cells. Dysfunction in these new host-microbial interactions can participate in the severity of gastrointestinal Graft-versus-host disease, which induce an acute intestinal inflammation for which FMT constitutes a promising therapeutic approach, yet to be evaluated in large randomized trials.[85] Data from these upcoming studies should allow to better understand the processes involved in this period of high plasticity of the intestinal ecological niche.

In another way, timing regarding the inflammatory state of the gut mucosa impacts donor strains engraftment. Indeed, an expansion of the *Enterobacteriaceae* family in inflammatory settings is observed in various diseases, in accordance with the particular fitness of this family of bacteria, including the ability to exploit aerobic metabolic pathways via nitrate metabolism optimally, competitive nutritional advantages over the use of carbohydrates and iron and resistance to antimicrobial peptides.[86]

In turn, *Enterobacteriaceae* will promote mucosal inflammation contributing to a vicious circle.[87] For example, following FMT for Crohn's disease, engraftment of *Haemophilus parainfluenzae* and the *Enterobacteriaceae* member *Escherichia coli* were associated with the likelihood of disease relapse.[38] In the case of inflammatory bowel disease, performing FMT after controlling mucosal inflammation with conventional immune-targeting therapy such as corticosteroids may foster the engraftment of taxa adapted to a non-inflammatory environment that can stabilize the mucosal healing such as the anti-inflammatory bacteria *Faecalibacterium prausnitzii* or *Roseburia intestinalis* which are obligate anaerobes rapidly eliminated in an acute mucosal inflammatory state.[88]

This finding may suggest that, in presence of mucosal inflammation, FMT may be performed when the ecosystem is tuned the closest to the targeted non-inflammatory steady-state with the use of non-microbial therapy.

Recipient gut microbiota composition and functions

The resilience of the recipient gut microbiota, i.e. their ability to compete against microbial colonization, constitutes another key parameter determining FMT engraftment and thus success. When exposed to an intestinal pathogen, the barrier function of the intestinal microbiota depends on the stability of the bacterial community, its competitiveness in nutrient supply and its ability to adapt to the new redox status of the mucosal environment.[90] In FMT,

prediction of donor strains engraftment based on basal recipient's gut microbiota composition remains a challenge. As with many ecological systems, baseline diversity (i.e. here the number and relative abundance of different taxa in the ecosystem) has been suggested to constitute a marker of the system's robustness to external disturbances and FMT biological success. However, depending on the disease, basal microbiota diversity in recipients can be associated with various FMT clinical outcomes. In metabolic syndrome, lower initial fecal microbiota diversity in recipients was associated with improvement of insulin sensitivity after FMT,[39] whereas in UC and IBS FMT clinical success was associated with a higher basal bacterial diversity.[63] These discrepancies suggest (i) that bacterial diversity is a highly imperfect tool for predicting gut microbiota plasticity and (ii) that depending on the disease, diversity does not presume microbiota function.

More specifically, according to studies and diseases, specific taxa and strains were associated with FMT outcomes. Abundances of *Fusobacterium* and *Sutterella* species in the recipient were associated with FMT failure in UC,[91] whereas in CD the colonization of the recipient by members of the *Enterobacteriaceae* family such as *Klebsiella* was associated with poor clinical outcomes.[92] In metabolic syndrome, responders had lower relative abundances of *Eubacterium ventriosum* and *Ruminococcus torques* and higher relative abundances of *Subdoligranulum variabile* and *Dorea* species.[39] In IBS, a higher baseline abundance of *Streptococcus* species was associated with FMT clinical success.[93] From these various works, often based on small cohorts with conflicting results a clear microbial signal predictive of FMT success is difficult to figure out even within the same disease condition. Two recent meta-analyses highlighted the importance of baseline abundance of specific taxa in FMT recipients to predict post-FMT engraftment.[76,94] This suggests that quantitative aspects and prior bacterial load in the recipient may be of specific importance in predicting the success of FMT. Absolute quantification of species of interest in the recipient microbiome may help

resolve discrepancies observed between studies. Nonetheless, the predictive factors for the success of FMT may be considered more at the functional level of the gut microbiota than at the taxonomic level, assessed by a multi-omics approach. As an example, fecal levels at baseline of 5-aminovalerate and N-methylphenylalanine were associated with positive outcome after FMT in UC.[63] However, systematic meta-analysis and powerful comparative studies are needed to overcome inter-study variability and internal bias.

Apart from bacteria other microbial kingdoms that inhabit the intestine should be taken into account when considering predictors of post-FMT microbial transfer. In CDI, positive FMT outcome was associated with a low abundance of faecal *Candida albicans*, in recipients at baseline.[71] On the contrary, in UC a high abundance of *C. albicans* in recipient at baseline was associated with a higher bacterial engraftment and FMT clinical success.[95] Low baseline of *Caudovirales* bacteriophages in recipients and low eukaryotic virus richness were also associated with FMT success in UC.[96,97]

In another way, donor-recipient similarity has been recently shown as a potential robust predictor of engraftment, suggesting that the pre-existence of an ecological niche adapted to one species (i.e. ecosystem preconditioning) might help donor strains of the same species to engraft.[38,98,99]

Notably, high-resolution taxonomic analyses are essential to understand microbial dynamics. Indeed, in a trial of FMT in CD, patients that were previously identified as FMT failure based on analysis at the species level showed clear evidence of long-term microbial engraftment by strain replacement and/or coexistence.[38,92] More recently, He and colleagues proposed classification of recipient's microbiota into a *Bacteroides*-dominated or a *Enterobacteriaceae*-dominated enterotype using various clustering methods on different FMT studies in CD, UC and CDI. According to the basal recipient enterotype, distinct levels of taxa engraftment were observed.[98] In contrast, in a recent meta-analysis of microbiome

dynamics at the strain level of more than 300 FMTs in 10 different diseases, Schmidt and colleagues showed that for most species, higher relative abundance in the recipient was negatively correlated with engraftment of phylogenetically related species, suggesting an exclusion effect.[94] This donor-recipient compatibility may also be undertaken by viral particles as shown in CDI where a lower diversity of *Caudovirales* bacteriophages in the recipient basal microbiota compared to the donor's was associated with FMT positive outcome.[70] Thus, microbial determinants of donor-recipient complementarity may vary with diseases with subsequent different engraftment patterns and clinical outcomes.[100] In the competition between donor and recipient taxa, quantitative aspects regarding absolute and relative abundance are likely to be of great importance.

Environmental co-factors

Additionally, despite a stable core structure, the gut microbiota composition presents temporal dynamics depending on diet, lifestyle (exercise, travels, etc.), and drug treatment. The precise control of these factors right before and after FMT can optimize strain engraftment and shift the whole microbiome structure and function to a healthy condition. Recently, such approach has shown some efficacy in improving insulin sensitivity in patients with severe obesity and metabolic syndrome by combining a single-dose oral FMT with daily low-fermentable fiber supplementation.[41]

Factors related to details of FMT working protocols

Beyond the screening of donors and the manufacturing of fecal material, the infusion of feces is a key component of FMT working protocols, and includes potentially several steps, from

patient preparation to adjuvant dietary post-FMT interventions, that have been investigated more extensively in very recent years (Table 2).

Antibiotic priming

Antibiotic priming has been being a key part of the patient preparation for FMT since early investigations. It plays a well-defined role in patients with rCDI, where vancomycin is used as the preferred antibiotic, as it represents a bridging therapy during the wait for FMT and it also contributes lowering the *C. difficile* load before FMT, therefore it has been recommended by the international guidelines of FMT in clinical practice.[14]

The use of antibiotics before FMT in noncommunicable disorders has been mistrusted for a long time, as it could in theory worsen microbiome alterations,[101] and early trials did not include antibiotics in their working protocols.[64] However, the concept that antibiotics may act as a pre-conditioning therapy to favor the colonization of donor microbiome, has emerged in recent years, due to several reasons. First, there is evidence that low patient diversity at baseline is associated with clinical success of FMT, as shown in patients with metabolic syndrome.[39] Moreover, a similar approach is well established in the hematology field, where pre-conditioning with immunosuppressants is used to prevent graft-versus-host disease after allogenic bone marrow transplantation.[102] Also, there is evidence that pre-FMT antibiotics improve the likelihood of donor microbiome engraftment in mice.[103] Overall, in a metagenomic metanalysis of 24 studies, the use of antibiotics before FMT was associated with higher levels of donor strain engraftment after FMT, which correlated with clinical success.[76] This finding was confirmed in another contemporary metagenomic metanalysis of 14 FMT trials.[60] Moreover, in a metanalysis of 28 studies investigating FMT for IBD, the antibiotic priming was associated with higher rates of clinical response and remission after FMT, compared with no antibiotic conditioning.[104] However, as already shown in mouse

models,[105] the class, dosage and duration of antibiotic pre-conditioning could influence the donor engraftment rate after FMT. Antibiotic pre-treatment appears to be more effective when including systemic antibiotics or antibiotics with a profound impact on gut bacteria. A 3-day combination of two antibiotics covering both Gram positive bacteria – vancomycin- and Gram negative bacteria - neomycin - was associated with engraftment of donor microbiome and clinical success in patients with melanoma undergoing immune checkpoint inhibitors.[69] Moreover, in a successful randomized trial of patients with UC, capsulized FMT was preceded by a 2-week priming with amoxicillin, doxycycline, and metronidazole.[106] Contrarily, the use of rifaximin as conditioning antibiotic was not associated with increased rates of engraftment,[107,108] probably as it does not decrease significantly microbial diversity.[109] However, some reports did not find any significant association between antibiotic use and FMT success,[107] and currently there is no agreement among experts on which antibiotic to use, and for how long before FMT.

Therefore, the identification of an effective priming antibiotic protocol, by randomized controlled trial aimed at assessing microbiome engraftment and clinical success in different disorders, is advocated to increase the chances of increasing the efficacy and expanding the role of FMT.

Bowel cleansing

Bowel cleansing is a mandatory component of the FMT working protocol when colonoscopy is the preferred route of delivery. By removing the colonic content, it also reduces the bacterial load, being helpful in the eradication of *C. difficile*.[14] An inadequate bowel cleansing at the time of FMT has, indeed, been identified as a predictor of CDI recurrence after FMT, with an increased risk of more than 11 folds.[110]

Emerging evidence suggests that bowel cleansing could also be exploited also to pre-condition the patient microbiome before FMT, due to its impact on gut microbiota.

In several reports bowel preparation before colonoscopy has been associated with a decrease in the alpha diversity[111-115] and the bacterial load[115] of the patient microbiome. This consequence, that has been generally considered detrimental, could be in theory exploited to favor microbiome engraftment and clinical success, by providing a low baseline diversity of the patient[39] and reducing, therefore, the competition between the microbial communities of the patient and of the donor. This concept has been shown in a metagenomic metanalysis of 14 studies, where bowel cleansing was able to enhance donor microbiome engraftment.[60] This approach has already been proposed to increase the effectiveness of probiotics in patients with IBS, and a follow-up in silico simulation found that the use of laxatives may increase the likelihood of Clostridium cluster XIVa to shift the microbiota.[116] Further randomized studies are needed to clarify if bowel cleansing, with or without antibiotics, may influence the rate of donor microbiome engraftment and/or clinical success after FMT.

Number of fecal infusions, amount of infused feces, multi-donor approach

As FMT is basically a warfare between the donor and the patient microbes, in theory the increase in the amount of donor microbes may enhance its likelihood to colonize the recipient gut. This result may be achieved by increasing the load of microbes (that is equivalent to the amount of feces) for single FMT, or by increasing the number of fecal infusions.

The repeat of FMT after a failure is a well-established way to increase overall efficacy rates in patients with CDI, especially in specific conditions. In an early randomized trial of patients with CDI, Cammarota and colleagues observed that patients with pseudomembranous colitis were less likely to be cured after single FMT.[6] This observation was replicated also by Fischer and colleagues, who found that sequential FMT plus vancomycin was a successful

strategy in patients with severe CDI.[117] Finally, this evidence was firmly established in a randomized trial, where a single fecal infusion was less effective than repeat FMT in curing severe CDI,[9] and in a later systematic review and metanalysis of 240 patients and 10 studies.[118]

As expected, response to FMT was not sustained long term in chronic noncommunicable disorders. Therefore this sequential approach has been applied also in this setting, and most trials have used repeat FMT, with promising results,[39,61,62,91] that advocate a deeper investigation of the potential of sequential FMT in this setting.

Interestingly, a long-standing FMT approach has been successfully experienced in two recent randomized[106, 119] and nonrandomized[120] trials of patients with UC and in another nonrandomized study of patients with IBS,[121] suggesting that a chronic modulation of the patient microbiome may be beneficial in noncommunicable chronic disorders.

There is evidence that also the amount of infused feces could influence the clinical effectiveness of FMT. In a systematic review and meta-analysis of 15 studies and 1150 subjects, a fecal amount ≤50 g was associated with lower efficacy of single FMT at meta-regression analysis.[5]

The relationship between high quantities of feces and clinical success was confirmed also in disorders beyond CDI. In a randomized controlled trial of FMT in patients with IBS, the increase of infused feces from 30 grams to 60 grams increased the success rate from 76.9% to 89.1%.[65] Moreover, in a systematic review and meta-analysis of 9 trials and 425 patients with UC, the use of more than 300 grams of feces was associated with a higher likelihood of clinical remission after FMT.[122]

However, the importance of fecal weight has been recently scaled back, based on the impossibility to evaluate the actual presence of live microorganisms in the fecal material with this parameter. The estimate of the total viable organisms present in the fecal product has

been proposed as a metric able to measure the product potency. Several approaches, including the assessment of total viable colony-forming units (CFUs) per dose through a dilution series and plating assay, the use of membrane-excluded dyes to differentiate live versus dead microorganisms, or the application of qPCR-based techniques, have been highlighted as potential methods to assess practically the presence of viable microorganisms.

Finally, a multi-donor approach has been investigated, based on the rationale of increasing the diversity of the infusate by mixing feces from different donors. In two randomized controlled trials of patients with UC, multi-donor FMT was more effective than placebo in inducing clinical remission,[91,123] being also able to decrease inflammatory markers,[124] and this benefit was confirmed also in a systematic review and metanalysis[122] and in a pilot trial investigating multi-donor FMT as a long-term treatment.[120] Interestingly, in a randomized controlled trial of patients with obesity, multi-donor FMT was able to sustainably alter the patient microbiome, and two of four donors dominated the microbial engraftment of the recipient.[125] However, currently there are no studies providing a direct comparison of single-donor FMT with mixed-donor FMT. As variations in several factors (e.g. manufacturing process, donor pool, or patient population) may influence current results of multi-donor approaches, randomized trials designed to clarify this specific issue are advocated.

Although interesting, the multi-donor approach could also have some drawbacks, including the higher risk of transmitting detrimental species and even pathogens. Moreover, it limits the ability to understand the mechanisms underlying FMT success and strain engraftment rules.

Routes of delivery

FMT has been delivered by several routes, including upper endoscopy and nasogastric/nasoduodenal tube,[39 61,65] enema,[15,62] colonoscopy,[6,9,10] or capsules.[23,106] Although

some metanalyses have assessed efficacy rates of different routes,[3,5] a direct comparison has been provided only by few studies. In a pilot open-label randomized trial, frozen FMT by nasogastric tube appeared to be as effective as colonoscopic administration in resolving CDI-associated diarrhea. However, as this study involved a small sample of patients, well-sized trials are advocated to confirm this finding.

More solid evidence of efficacy is available, instead, for capsulized FMT. In a large non-inferiority RCT, frozen capsules obtained similar efficacy rates than colonoscopy in preventing rCDI.[23] This result was confirmed also later in a large nonrandomized study of >300 patients with rCDI.[126] Finally, FMT capsules have also achieved promising results in other disorders.[106] (109). Capsulized FMT is sustainable also in the long-term, especially in its lyophilized form,[106] and therefore paves the way for a chronic approach to microbiome modulation.

Notably, FMT using combined routes was associated with higher likelihood of microbial engraftment than single-route infusion in a metagenomic metanalysis of 24 studies.[76] Based on this indirect finding, combination FMT may represent an interesting target of future research.

Dietary adjuvants

The importance of diet in influencing outcomes of FMT has been increasingly acknowledged in recent years.

Generally, a healthy diet is linked with a healthy microbiota, and donors adhering to a healthy diet have shown to be effective in specific settings,[65] suggesting that a dietary questionnaire may be included in the donor screening process to increase FMT efficacy, although this hypothesis needs to be clearly confirmed.

More specifically, the addition of specific diets, either for the donor or for the recipient, has been recently experienced in FMT studies. The first FMT trials to use such approach were those involving patients with cardiometabolic disorders, probably for cultural and biological affinity, as the diet is the first treatment option for these disorders. Mouse models have shown that donor diet may influence FMT outcomes regardless the baseline metabolic conditions of the donor, as FMT from high caloric-fed donors was able to disrupt glucose metabolism in recipient mice regardless adiposity,[127] and autologous FMT from a lean state enhanced the effects of caloric restriction effects in obese mice.[128] These findings have been confirmed also in humans, as autologous FMT, collected during a lean phase and re-infused during a regain phase in obese or dyslipidemic patients, was able to attenuate weight gain and insulin rebound.[129] However, a dietary priming of donors was not effective in improving the FMT success in inducing remission of patients with UC.[130]

While a diet given to the donor has mainly the aim of shifting her/his microbiome toward a more beneficial composition, a diet designed for recipients may have also other purposes, including the priming of the engraftment and the fostering of the newly settled microbiome, or a synergistic action with FMT in ameliorating symptoms.

The synergistic effect of FMT and diet was explored in a randomized trial of FMT versus placebo plus Mediterranean diet in patients with metabolic syndrome, without any significant difference between the two groups, although this trial was not specifically designed to assess the value of this diet to FMT.[131]

In another randomized trial of patients with UC, the combination of FMT plus an anti-inflammatory diet (AID) was more effective than standard medical therapy in inducing disease remission, and AID was also more effective than SMT in maintaining remission.[123] This specific AID included an increased intake of foods that nourish the colonic microbiota, including fresh fruits and vegetables (especially aryl hydrocarbon receptor/AhR ligand-rich

vegetables), and fermented foods, that could have fostered the settlement of the donor microbiome in the large bowel of recipients. The combination of FMT and high-fiber diet also attenuates the development of emphysema by downregulating inflammation and apoptosis in a mouse model.[132] Notably, in a randomized controlled trial of patients with severe obesity and metabolic syndrome, the post-FMT supplementation of low-fermentable fibers, (specifically microcrystalline cellulose fiber) was more effective than high-fermentable fibers in promoting microbial engraftment and improving insulin sensitivity; potential explanations of these results included the bulking/binding effect of cellulose toward microbes and metabolites, as well as a shift of gut microbiota due to its prebiotic properties. [41] Overall, these results pave a fascinating way for applying dietary products (mainly fibers) as adjuvants of FMT and, more widely, toward a combined approach of therapeutic microbiome modulation.

HOW TO ESTABLISH THE SUCCESS OF FMT FROM MICROBIOME TO CLINIC: THE KEY ROLE OF ENGRAFTMENT

Need for comprehensive evaluation of gut microbiome to establish engraftment: the advantages of whole genome sequencing

Surveying microbiome composition is crucial to obtain first insight on what processes take place when a stool of a donor is transferred into a recipient. Mechanistic hints on what determines FMT success in different diseases and conditions can be obtained by understanding microbiome ecology and dynamics: the gut microbiome of patients with an infectious disease, like *C. difficile*, is likely to respond differently to a pool of incoming health-associated bacteria from that of patients with non-infectious diseases. FMT protocols

across conditions, though, seek to enhance donor's engraftment as this could determine clinical success.[76,133,134] To this end, the composition of the gut microbiome of the patient before and after FMT, together with the FMT inoculum need to be profiled. As an individual's gut microbiome typically harbors >200 microbial species,[135] a great majority of which have never been cultivated,[136] high-throughput sequencing methods are required. Indeed, the high-throughput techniques that permit studying microbial communities as a whole revolutionized the study of the microbiome.[137] Metagenomics consists in sequencing the whole DNA pool extracted from a microbial community, which allows assessing which microorganisms are present, their abundance, their genome, as well as their functional potential. While earlier studies were mostly based on 16S rRNA gene sequencing, i.e. amplification by PCR and sequencing of hypervariable regions of the ribosomal RNA gene that is universally present in Bacteria and Archaea, the technique is being replaced by shotgun metagenomics, which bypasses amplification steps and provides higher taxonomic resolution and insight into the functions encoded by the microbial community.[138] Shotgun metagenomics is now very cost effective and the increased sequencing depth enabled by cheaper sequencing costs is lowering the limit of detection for the taxa present in a microbiome. Particularly for the gut microbiome, large-scale cultivation projects and massive metagenomic assembly efforts are quickly expanding the reference databases needed by taxonomic profiling tools, although tasks such as precise functional profiling and virome characterization remain challenging.

Different individuals with similar diets, lifestyle and health status can display overall similar gut microbiome composition by convergence to a comparable set of species. In contrast, bacterial strains, rather than species, are highly subject-specific, which makes them exploitable to assess microbiome engraftment.[76,139,140] If a certain strain that was present in the transferred sample is then detected in a recipient's fecal sample, engraftment of that strain in

the patient's gut can be inferred. As only shotgun metagenomics reaches the resolution of single strains and has the throughput of allowing to survey hundreds of strains in a sample, this is arguably the technology of choice when studying the microbiome in the context of FMT.

Microbial strain engraftment to assess the transfer of the donor's microbiome to the recipient

A key assessment in FMT is determining the extent to which the microbial strains in the inoculum engraft in the recipient's gut. Strain engraftment can be seen as a proxy for FMT efficiency, and quantifying it is a first step toward identifying the conditions that allow maximizing it. However, strain engraftment calculation is not yet standard in the field. A first limitation is that a consensus definition of strains in the microbiome context is missing. [139,140] While many agree in defining strains as "microbial entities that, despite a limited genetic heterogeneity, have the same phenotype under different conditions",[139] this is in practice difficult to establish: with the a vast fraction of the microbiome remaining so-far uncultured,[141,142] phenotypes cannot be tested in a straight-forward manner. Because of this, thresholds on (phylo)genetic variation have been employed,[143-145] but these remain somewhat arbitrary. Operational definitions of strain that are species-specific to best capture species' different evolutionary rates and overall variability were recently-proposed[146] and used to assess strain engraftment upon FMT.[76] We believe that while the specific definitions can be fine-tuned as more data becomes available and strain-level profiling tools keep improving, such informed definitions based on strains' individual specificity and persistence allow more accurate assessment of microbiome engraftment.

Second, while fecal samples provide a non-invasive survey of the microbial community in the patient's gut, with microbial densities reaching as much as ~10^{11} cells per gram of stool

sequencing techniques do not capture the entire community.[147] The number of strains detected to engraft in the FMT recipient is thus dependent on sequencing depth, and this needs to be accounted for to avoid biased estimates. Strain engraftment metrics have been defined by normalizing the number of engrafting strains by the total detected strains in the donor and recipient or the (detected) strains in the FMT inoculum that could potentially engraft in the recipient (i.e. with a representative of the species being detected by sequencing) among others.[60,76] While a consensus strain engraftment rate metric would facilitate comparability across studies in the future, it is important to keep in mind the calculation method and its nuances (e.g. were any strains shared by the donor and the recipient at baseline excluded from the calculation?) when interpreting the results of single studies.

Correlation between microbial engraftment and clinical success

Since early experiences of evaluating both clinical and microbiological success in FMT trials, the relationship between these two variables has been investigated. In the TURN trial, the post-FMT microbiome of recipients who experienced clinical success was more similar to the microbiome of their corresponding donors than to their microbiome before FMT, with a shift in their beta diversity.[60] In this setting, beta diversity could have been assumed as an elementary surrogate of microbiome engraftment. The more recent exploitation of whole genome sequencing (WGS) has allowed evaluating microbial engraftment more precisely, with an assessment based on strain tracking, as explained above. With this approach, microbial engraftment has been associated with clinical success, regardless of disease, in a large metagenomic metanalysis of 24 FMT trials and almost 1400 fecal samples.[76] This finding, however, was not replicated in another similar study, probably due to the heterogeneity of cohorts, diseases, FMT working protocols, and differences in microbiome analytical methods.[94]

Beyond the absolute relationship between microbial engraftment and clinical success after FMT, another unsolved question is whether the longitudinal persistence of microbial engraftment may influence the duration of clinical success. With high-throughput strain-level resolution microbiome analyses being only recently available, the persistence of engrafted strains in the recipient's microbiome over time remains an open question. Strains are rather persistent in time in an individual's microbiome in the absence of perturbations (87% strain persistence at around 6 months).[146]Antibiotic treatments, while eliminating both disease- and health-associated bacteria (which might be well adapted to the patient's gut environment and help mitigate the effects of the disease) as a side effect, reduce colonization resistance, thereby favoring engraftment.[76,148] Antibiotic preconditioning has been thus recommended for inclusion in FMT clinical protocols, but the persistence of the engrafted strains has not yet been explored in this context.[76]

Overall, the observed relationship between engraftment and clinical success of FMT deserves further and dedicated investigations through well sized and designed studies (e.g. with well detailed definition of clinical success).

Moreover, longitudinal sampling at multiple time points after FMT is thus warranted specially to unveil the association between persistence of engrafted strains and disease remission. This will in turn inform on whether repeated FMT courses should be performed for different conditions and if so, how long they should be spaced in time.[149]

CULTURAL SHIFTS AND FUTURE INSIGHTS

Beyond improvements in technologies, some mindset shifts are, in our belief, needed to let FMT advance as a potential treatment option for noncommunicable disorders. To date, two main cultural obstacles prevent the progression of FMT outside infectious disorders,

including the lack of microbiome analyses coupled to FMT procedures, and the consideration of FMT as an acute, single-use therapy (Figure 2).

Donor selection: the role of microbiome sequencing and machine learning

Currently many clinicians are still not familiar with microbiome sequencing and analysis. While the infusion of a healthy microbiome is clinically effective regardless of its composition in rCDI, the composition of donor microbial communities appears to play a key role in treating noncommunicable disorders,[56] probably mediated by the achievement of successful microbial engraftment.[76] Therefore, clinicians should get ready (and be adequately trained) to interpret the results of microbiome sequencing to modulate precisely gut microbiome in the near future, despite clear rules to regulate this approach are currently missing.

Identifying the microbiome characteristics that maximize strain engraftment in the FMT will allow selecting the best donor for each single patient. While some studies supported the existence of shared characteristics that make up "super-donors" (e.g. high microbial species richness and diversity),[54,134] others found that the optimal donor is much more patient-specific,[76,150] thus calling for personalized selection strategies rather than a "one stool fits all" approach. For example, while a family or household member donor was found associated with higher strain sharing at baseline (possibly a consequence of microbiome transmission favored by being in close contact), this did not result in higher strain engraftment rates once subtracting prior sharing.[76] By providing a detailed view of engraftment on each FMT instance, strain-level resolution microbiome profiling thus unravels the donors microbiome characteristics that optimize FMT efficiency. Meta-analyses of such data then allow moving from scattered cohort-specific observations to solid global

and disease-specific findings. Finally, prediction models can be built from such associations to inform donor selection in a personalized manner.

While microbiome data is highly complex and multidimensional, the high numbers of samples that are currently available open the way for developing models that allow pinpointing the optimal donor for each recipient given their microbiome composition. Xiao *et al.*[151] (2020) assessed microbial dynamics in FMT using an ecological modeling framework (based on the generalized Lotka-Volterra model), and found that the optimal donor was most personalized when the recipient had higher microbiome diversity before FMT. However, most studies use machine learning approaches that are then validated on independent data. With random forests followed by validation with a leave one out (LOO) approach, Kazemian *et al.*[150](2020) identified genera in the FMT inoculum and in the recipient pre-FMT associated that could interact to determine FMT success. These included endospore-forming bacteria in the inoculum that might aid survival until the engraftment in the new host. Also Zou *et al.* [152] (2019) found relatively high predictability of the recipient microbiome composition after FMT (AUC>85%) based on bacterial taxa together with host parameters (e.g. immune markers and gastrointestinal symptom scores). He *et al.*[98] (2022) followed a similar approach to predict the efficacy of FMT and obtained comparable accuracy (AUROC=80%). Smillie *et al.*[99] (2018) used a random forest classification model (incorporating clinical data together with microbiome features of both the host and the recipient) to predict which bacterial species engraft in a recipient, followed by a random forest regression model to predict their abundances, which also performed well (AUC=92%). However, the small numbers typically used in single FMT studies might result in overfitting (i.e. overestimating the model's performance), thus limiting their applicability across cohorts. A more recent study developed a random forest model to predict species engraftment upon FMT in 226 FMT instances from 24 different cohorts using 16 host and microbiome

features.[76] This resulted in an AUROC (area under the receiver operating characteristic curve) of 85% with a fivefold cross-validation setting. The authors then predicted the abundance of species post FMT (with a random forest regression model), and while the prediction potential was found partially dependent on the cohort, substantial predictability was maintained across datasets.

FMT: a chronic therapy for chronic disorders? Potential and limitations

The other main cultural drawback includes the consideration, by patients and clinicians, that FMT is mainly a single-use treatment option, while other drugs are commonly used in the long-term to cure chronic disorders. As described in this review article, there is emerging evidence that FMT may act as a chronic therapy.[106] However, in order to implement FMT on a large scale in the routine management of chronic diseases, several issues must be addressed. First, stool materials are inherently variable from donor to donor and even over time in the same donor, depending on diet, habits and other uncontrollable environmental factors. This will limit the reproducibility of the FMT product from batch to batch, with a potential uncontrollable source of variability in efficacy and safety. To limit this source of uncertainty, one may want to develop functional benchmarks that can be used to demonstrate FMT reliability between different batches. However, complete characterization of stool composition is so far impossible to achieve, even with the most recent multiomic approaches. Based on what is known and what is feasible, 20-50% of shotgun metagenomic reads cannot be mapped, 30-50% of identified genes are of unknown function, and most strikingly, 80-90% of metabolites cannot be identified.[141,153] This was without mentioning virus and human cells material that are present in FMT material with mostly unknown functions.

Second, treating chronic diseases requires chronic treatment, which may rise unexpected safety issues. FMT has shown a very good safety profile in CDI after one or few

transplantations even in immunocompromised and fragile patients.[48] However, repeated administration of live microbes may be associated with specific complications. The likelihood of transmission of known and unknown infectious agents will mechanically increase with the number of FMTs, despite careful and extensive donor screening for pathogens. Indeed, two cases of multi-drug resistant bacteria transmission with life-threatening complications have been described in patients with cirrhosis and hematological malignancy after repeated FMT.[44] Moreover, repeated FMT may also confer a deep and stable shift of the gut microbiota that may transfer noninfectious chronic disease. Long-term prospective safety cohorts are needed to address this issue, but with much uncertainty about success because of the long time period needed to discover such potential effects and the many confounding factors that will interfere.

Third, donor recruitment in routine care and clinical trials is tremendously challenging. With 2-20% of potential candidates ultimately eligible[154] and the inherent constraints of fecal collection and processing, industrialization of the process will face major cost and logistical obstacles.

Fourth, the drivers of the therapeutic effects of FMT are mostly unknown and are likely to be different from one disease to the other, and even from one patient to the other. A striking example of the complexity of industrializing the FMT product is the arrested development of SER-287, a "purified" bacterial preparation derived from the fecal microbiota of healthy subjects that shows effects in inducing remission in UC in a positive phase 1b trial, that was not confirmed in phase 2b in 2021.[155] (Henn et al., 2021).

Today FMT trials seem mandatory to explore the potential therapeutic effects of gut microbiota intervention in chronic diseases. However in view of all these difficulties, research on FMT must still aim to develop post-FMT microbiota-based treatment such as new live biotherapeutic products composed of synthetic microbiome consortia, or of specific

combinations of metabolites and microbes that can be settled in a highly controlled, scalable and reproducible way.

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AUTHOR CONTRIBUTIONS

GI conceived the review article. NS, AG, GC, HS, and GI designed the structure of the review article. SP, NB, MV-C, HS and GI wrote the initial draft of the manuscript. SP and GI built the tables. NB and HS draw the figures. All authors provided critical revision of the manuscript and approved its final version for submission.

DECLARATION OF INTERESTS

AG. reports personal fees for consultancy for Eisai S.r.l., 3PSolutions, Real Time Meeting,

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FIGURES

Figure 1. Factors that influence FMT success. To successfully perform FMT in various diseases, the optimal procedure and microbial determinants of efficacy should be identified for each condition. Among the factors that will need to be better defined, donor-recipient compatibility criteria and donor or recipient pre-conditioning will be crucial to ensure donor's strain engraftment and FMT clinical success. The use of analytical models and artificial intelligence (AI) discovery tools on large-scale datasets is a cornerstone in developing our knowledge of the precise factors that determine FMT outcomes.

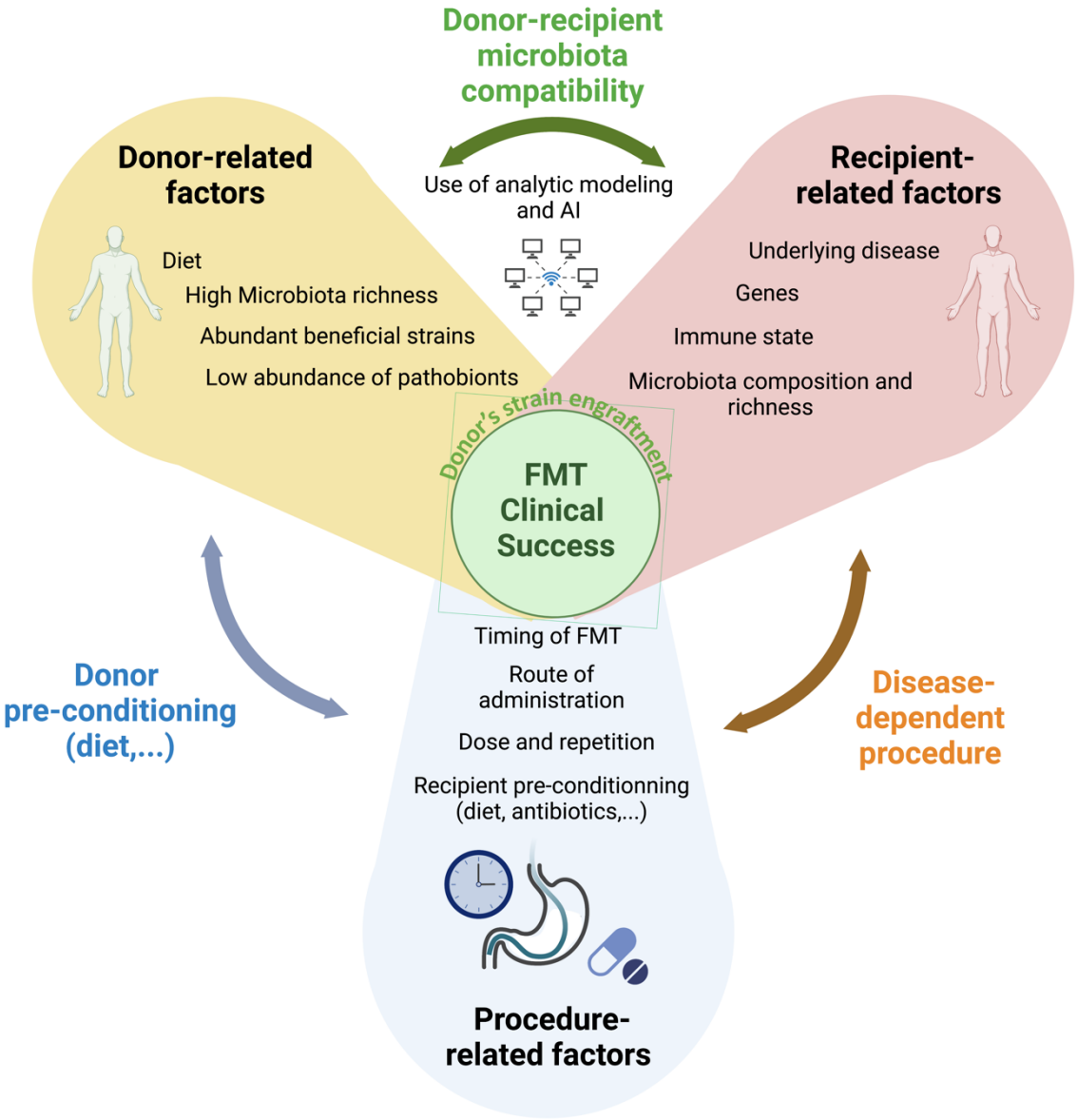
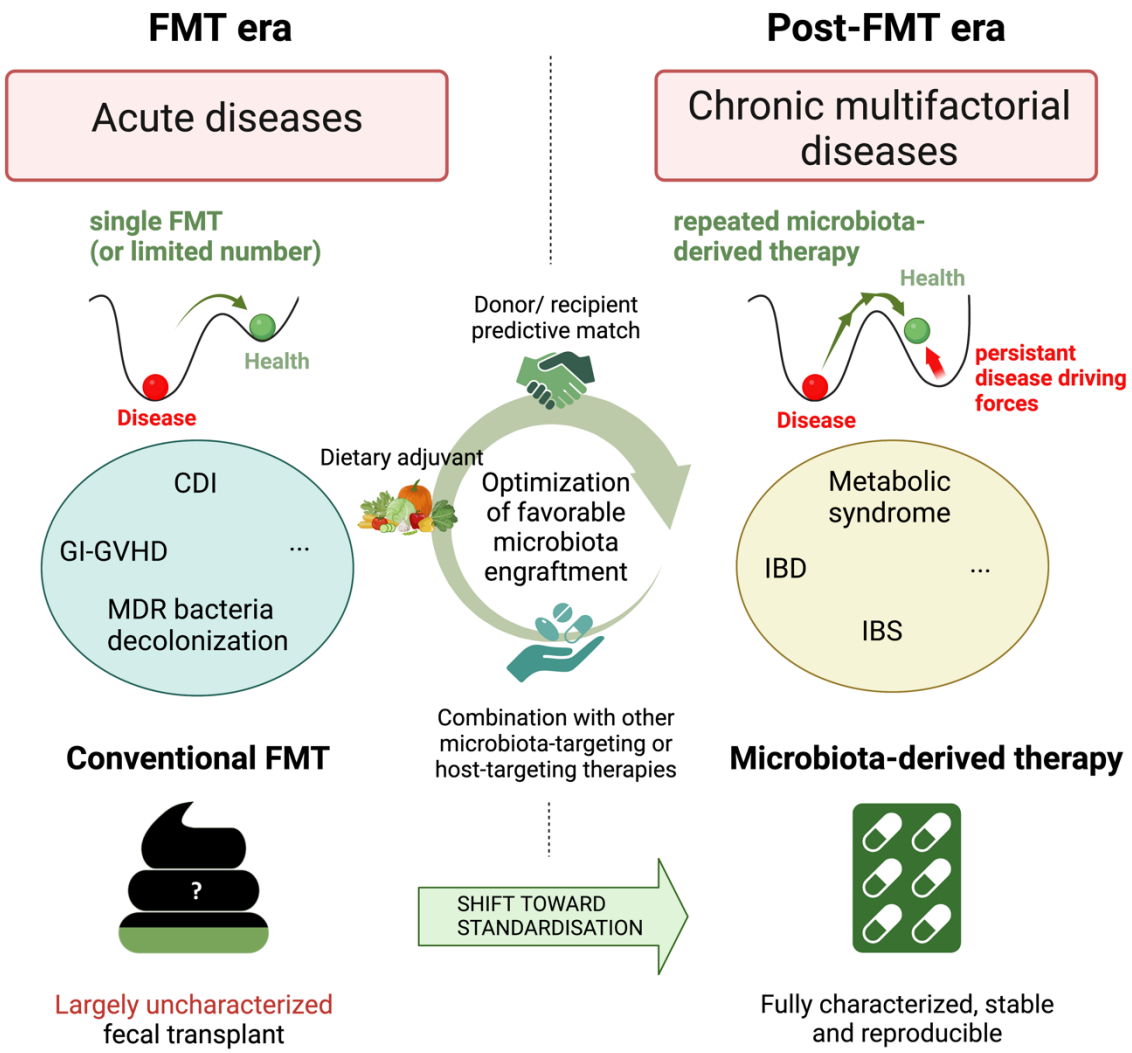


Figure 2. Cultural shifts and future insights for FMT and post-FMT microbiota-derived therapies. Since the gut microbiota is only one factor in the pathogenesis of most diseases, combining classical host-targeting therapy with microbiota-targeting approaches will enhance therapeutic efficacy. A dietary adjuvant or other treatment targeting the microbiota could also help optimize microbiological success and engraftment of donor's strains. Going forward, the development of FMT will need to overcome two structural pitfalls. The first is the questionable long-term efficacy of FMT-related microbiological changes in chronic multifactorial diseases where persistent disease driving forces will still affect the gut ecological niche after FMT. In such diseases, repeated treatment may be necessary and raises the question of the stability and reproducibility of FMT material. Second, the variability and largely unknown nature of fecal transplants precludes the industrial development of a scalable and fully characterized product over time. This suggests that for chronic treatment, post-FMT microbiota-derived therapies represent a more reliable long-term approach. CDI: *Clostridioides difficile* infection; GI-GvHD: gastrointestinal Graft-versus-Host Disease; IBD: inflammatory bowel disease; IBS : irritable bowel syndrome; MDR bacteria: multi-drug resistant bacteria.


FMT era
Post-FMT era
Acute diseases
Chronic multifactorial diseases
single FMT
(or limited number)
repeated microbiota-derived therapy
Health
Disease
Donor/ recipient predictive match
Health
persistant disease driving forces
Disease
Dietary adjuvant
Optimization of favorable microbiota engraftment
CDI
GI-GVHD
...
MDR bacteria decolonization
Metabolic syndrome
IBD
...
IBS
Combination with other microbiota-targeting or host-targeting therapies
Conventional FMT
Microbiota-derived therapy
?
SHIFT TOWARD STANDARDISATION
Largely uncharacterized
fecal transplant
Fully characterized, stable
and reproducible

TABLES

Table 1. Classification of fecal microbiota transplantation across countries

Country	FMT classification
USA, Canada	Investigational drug used in context of Clinical Trial or to treat rCDI
France	Investigational drug used in context of Clinical Trial or use in hospital setting
Italy, Netherlands, and Belgium	Considered as a tissue transplant. (European Commission has identified stools as a SoHO and his regulation will be under the EUTCD)
United Kingdom	FMT is regulated as a medicinal product.
Australia, Germany	FMT is regulated as a practice of medicine

FMT: Fecal microbiota transplantation; rCDI recurrent *Clostridioides difficile* infection;
SoHO: substance of human origin; EUTCD: European Union Tissues and Cells Directive.

Table 2. Influence of different steps of working protocols on microbiological and clinical efficacy of FMT

First Author	Study Design	Details of Intervention	Study Setting	Results of the intervention
ANTIBIOTIC PRE - TREATMENT				
Amorim N (2022) [103]	Pre-clinical study	- Ampicillin - Vancomycin - Neomycin - Metronidazole	Mouse model	Antibiotic treatment for at least 7 days effective in gut decontamination; Following gut decontamination, FMT provided successful engraftment of donor microbiota. Donor microbiome engraftment persisted for 5 weeks after FMT
Ianiro G (2022) [76]	Systematic review and metagenomic meta-analysis	-Vancomycin -Rifaximin -Colistin -Colistin + Neomycin -Vancomycin + Neomycin	Several communicable and noncommunicable diseases	Antibiotics before FMT associated with higher donor strain engraftment rate
Podlesny D (2022) [60]	Systematic review and metagenomic meta-analysis	-Vancomycin -Fidaxomycin -Metronidazole -Lincosamide -Fluoroquinolone -Colistin -Neomycin -Cefepime -Paramomycin -Nystatin	Communicable and noncommunicable diseases	Antibiotics before FMT associated with higher donor strain engraftment rate
Baruch EN (2021) [69]	Phase I clinical trial	-Vancomycin -Neomycin	Immunotherapy-refractory melanoma	Clinical response in three patients Favorable immune changes in the gut lamina propria and the tumor microenvironment after FMT
Mocanu V (2021) [104]	Systematic Review and Meta-Analysis	-Amoxicillin -Fosfomycin -Metronidazole -Vancomycin -Paromomycin -Nystatin	IBD	Repeat FMT and antibiotic pre-treatment improved clinical response and remission rates
Haifer C (2022) [106]	RCT	-Amoxicillin -Metronidazole -Doxycycline	UC	FMT effective in inducing steroid-free remission at 8 weeks in 73% of patients
Suskind DL (2015) [108]	Prospective open label study	-Rifaximin	CD	-Donor microbiome engraftment in 7/9 (78%) patients. -7/9 (78%) patients in remission at 2 weeks -5/9 (56%) patients in remission at 6 and 12 weeks
Singh P (2022) [107]	RCT	-Ciprofloxacin and Metronidazole -Rifaximin	IBS-D	-Higher donor microbiome engraftment in the FMT alone arm than in the pre-FMT antibiotic groups
BOWEL CLEANSING				
Gorkiewicz G (2013) [111]	Prospective study	-PEG	Healthy subjects	-Decrease of microbial richness -Shift in the phyla Bacteroidetes and Firmicutes, increase of mucosal Proteobacteria
Harrel L (2012) [112]	Prospective study	-PEG (Low volume based bowel preparation)	NA	-Standard bowel preparation altered the diversity of mucosa-associated microbiota. - Taxonomic classification did not reveal significant changes at the phylum level, but at genus level
Powles STR (2022) [113]	Prospective study	-PEG	NA	-Reduction in alpha diversity between samples taken at baseline and three days following bowel cleansing
Kim J (2021) [114]	Prospective study	-PEG + with 20 g ascorbic acid	-	-5/24 (21%) subjects reported minor complications -Firmicutes/Bacteroidetes ratio before bowel preparation was higher in complication group than in that without complications. - Firmicutes/Bacteroidetes ratio after bowel preparation decreased in complcation group.
Jalanka J (2015) [115]	RCT	-PEG (single dose 2L or split-dose 1L x 2)	-	-Decrease of the total microbial load by 31-fold after bowel preparation and 22% of the participants lost the subject-specificity of their microbiota. -Consumption of the purgative in a single dose had a more severe effect on the microbiota composition than that of a double dose (In patients treated with single dose there were an increase in the levels of Proteobacteria, Fusobacteria and bacteria related to Dorea formicigenerans)
Podlesny D (2022) [60]	Systematic review and metagenomic meta-analysis	PEG	Communicable and noncommunicable diseases	-Bowel cleansing depleted the resident microbiota and prepare patients for donor microbiota engraftment
Li M (2020) [116]	RCT	Laxative bowel preparation or CB probiotic	IBS	Laxative use created a favourable opportunity for Clostridium cluster XIVa to shift the microbiota
NUMBER OF FECAL INFUSIONS				
Cammarota G (2015) [6]	RCT	Repeat FMT in patients with PMC	CDI	-All five patients with PMC were cured received a faecal infusion procedure every 3 days until the resolution of colitis was achieved
Fisher M (2015) [117]	Prospective study	Repeat FMT	CDI	The overall treatment response of endoscopic sequential FMT was 93% (27/29), with 100% (10/10) for severe CDI and 89% (17/19) for severe/complicated CDI
Ianiro G (2018) [9]	RCT	Single vs repeat FMT	CDI	Repeat FMT achieved significantly higher cure rates than single FMT (100% vs 75%, P = 0.01)
Song YN (2021) [118]	Retrospective study	Single or multiple FMT	IBD + CDI	-Initial FMT successful in 53/67 (79%) -8 patients received a second infusion; in 6 of them FMT cured effectively CDI -The overall success rate to clear CDI was 90% after repeat FMT.
Paramsothy S (2017) [91]	RCT	-Donor FMT -Placebo FMT FMT vs placebo colonoscopic infusion were followed by multi-donor enemas 5 days per week for 8 weeks	Active UC	Steroid-free clinical remission with endoscopic remission or response at week 8 was achieved in 11/41 (27%) donor-FMT patients vs 3/40 (8%) in patients who received placebo (p=0.021).
Moayeddi P (2015) [62]	RCT	Repeat FMT (by enema)	Active UC	9/35 (24%) who received donor FMT (24%) and 2/35 (5%) who took the placebo group were in remission at 7 weeks

				Stool from patients receiving FMT had higher microbial diversity, compared with baseline, than that of patients given the placebo (P =0.02,).
Rossen NG (2015) [61]	RCT	Repeat donor FMT vs placebo	Active UC	7/17 (41.2%) of patients who underwent donor FMT vs 5/20 (25%) controls achieved clinical combined with ≥1-point decrease in the Mayo endoscopic score at week 12 (P = 0.29).
Kootte (2017) [39]	RCT	2 different arms of treatment: Autologous FMT (12) and Allogenic lean donor FMT (26)	MetS	At 18 weeks after FMTs metabolic changes were not observe. Insulin sensitivity at 6 weeks after allogenic FMT was significantly improved, accompanied by altered microbiota composition. We also observed changes in plasma metabolites such as γ-aminobutyric acid and show that metabolic response upon allogenic FMT (defined as improved insulin sensitivity 6 weeks after FMT) is dependent on decreased fecal microbial diversity at baseline.
AMOUNT OF INFUSED FECES				
Ianiro G (2018) [5]	Systematic review and meta-analysis	Studies offering multiple infusions if a single infusion failed to cure rCDI were included.	CDI	< 50 gr of feces are associated with a lower response to single FMT
El-Salhy M (2020) [65]	RCT	Donor FMT vs placebo	IBS	The increase of infused feces from 30 grams to 60 grams increased the FMT success rate
Wei Z (2022) [122]	Systematic review and meta-analysis	FMT vs control group	UC	300 gr of feces improved FMT efficacy
MULTI-DONOR APPROACH				
Paramsothy S (2017) [91]	RCT	Repeat donor FMT vs Placebo FMT	Active UC	Steroid-free clinical remission with endoscopic remission or response at week 8 in 11/41 donor FMT patient vs 3/40 in those who received placebo
Sood A (2019) [120]	Pilot study	Repeat donor FMT vs placebo FMT	UC patients in clinical remission achieved after multi-session FMT were	No differences between placebo and FMT
Kedia S (2022) [123]	RCT	FMT plus AID vs standard of care	UC	FMT-AID was superior to SMT in inducing clinical response, remission and deep remission at 8 weeks.
Wilson BC (2021) [125]	RCT	Donor microbiota vs placebo	Obesity	4.5-fold reduction in the prevalence of MetS
Wei Z (2022) [122]	Systematic review and meta-analysis	FMT by multiple donors	UC	FMT from multiple donors was more effective than single donor FMT
ROUTE OF DELIVERY				
Quraishi MN (2017) [3]	Systematic review with meta-analysis	FMT	CDI	Lower GI delivery more effective than upper GI delivery in curing CDI
Wei Z (2022) [122]	Systematic review and meta-analysis	FMT	UC	Lower GI tract FMT had a more beneficial effect than Upper GI FMT
Ianiro G (2022) [76]	Systematic review and metagenomic meta-analysis	-Combined lower and upper GI route of delivery -Lower GI FMT -Upper GI FMT	Different communicable and noncommunicable diseases	Combination of FMT routes associated with higher microbial engraftment
Ianiro G (2018) [5]	Systematic review and meta-analysis	FMT by different routes	CDI	Capsules obtained similar cure rates of CDI than colonoscopy.
Vaughn BP (2022) [126]	RCT	Capsule FMT vs colonoscopy FMT	CDI	No differences in CDI cure rates
Haifer C (2022) [106]	RCT	-Lyophilized FMT capsules vs placebo	UC	Lyophilized capsules were effective in induce clinical remission
DIETARY ADJUVANTS				
El-Salhy M (2020) [65]	RCT	Super donor with healthy diet (dietary supplements rich in proteins, vitamins, fibre and minerals, proteins, creatine, vitamin C, vitamin E, vitamin B6, vitamin B12,Vitamin D, zinc Magnesium, desloratadine	IBS	FMT more successful than placebo in curing IBS
Zoll J (2020) [127]	RCT in mice	Normal chow (Nc) vs high-fat, high-sucrose diet (HFHS) plus exercise-or sedentary life	MetS	The HFHS diet led to glucose intolerance and obesity in the donors, whereas exercise training (ET) restrained adiposity and improved glucose tolerance.
Pérez-Matute P (2020) [128]	RCT in mice	-randomization to control groups vs caloric restriction, then randomization to donor FMT or autologous FMT	MetS	Autologous FMT potentiated the effects of a moderate CR on weight loss and adiposity in the short term, with a significant increase in bacterial richness/diversity.
Rinott E (2021) [129]	RCT	Three different groups: -healthy dietary guidelines; -Isocaloric mediterranean diet -Green-mediterranean diet (rich in polyphenols) After diet regimen, patients were randomized to receive autologous FMT or placebo	Visceral obesity or dyslipidemia	A-FMT significantly attenuated weight regain in the green Mediterranean group but not in the dietary guidelines or Mediterranean diet groups
Sarbagili Shabat C (2022) [130]	Blinded RCT	Standard FMT vs UCED + FMT with donor dietary pre-conditioning vs UCED alone	Active UC	UCED alone more effective than other options in inducing remission
Kedia S (2022) [123]	RCT	-FMT plus anti-inflammatory diet vs standard of care	UC	FMT-AID superior to standard of care in inducing clinical response and remission, and deep remission, at 8 weeks. Anti-inflammatory diet was superior to SMT in maintaining deep remission until 48 weeks (6/24 (25%) vs 0/27, p=0.007).
Jang Y (2022) [132]	Prospective study (murine model)	Mice were fed ad libitum with diets based on the purified AIN-76-A diet supplied by Daehan Biolink Co., Ltd. (Chungbuk, Korea). The AIN 76-A diet was modified to study	Emphysema	AID and high-fiber diet was effective in reduce inflammation

		the effect of different diets. The high-protein diet (40% protein) was modified with the increment of casein, high-fat diet (40% fat) with corn oil, high-fiber diet (20% fiber) with 20% cellulose (Study 2), and high-fiber diet (20% fiber) with 10% cellulose and 10% pectin		
Koopen AM (2021) [131]	RCT	Mediterranean diet for 2 weeks then randomization to -lean donor FMT vs autologous FMT	MetS	Consumption of the Mediterranean diet resulted in a reduction in body weight, HOMA-IR, and lipid levels. However, no large synergistic effects of combining the diet with lean donor FMT were seen on the gut microbiota diversity after 6 weeks
Mocanu V (2021) [41]	RCT	Low-fermentable vs high-fermentable fibers before FMT	Severe obesity and MetS	The post-FMT supplementation of low-fermentable fibers was more effective than high-fermentable fibers in promoting microbial engraftment and improving insulin sensitivity

AID: Anti-inflammatory diet. CB: *Clostridium butyricum*; CDAD: *Clostridioides difficile*-associated diarrhoea; CD: Crohn's disease; CDI: *Clostridioides difficile* infection; FMT: Fecal microbiota transplantation; GI: Gastrointestinal; HF: high-fermentable; IBD: Inflammatory bowel disease; IBS: irritable bowel syndrome; IBS-D: Irritable bowel syndrome with predominance of diarrhea; MetS: Metabolic syndrome; NA: Not available; PEG: Polyethylene glycol; LF: Low-fermentable (LF); SMT: standard medical therapy; RCT: randomized clinical trial. UCED: UC Exclusion Diet.

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