Roles of the gut virome and mycobiome in faecal microbiota transplantation

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Summary

Faecal microbiota transplantation (FMT) is an innovative approach to treat diseases associated with a gut dysbiosis, by transferring a healthy stool microbiota to a diseased recipient. Beyond the bacteriome, the human gut also harbours diverse communities of viruses and fungi, collectively known as the virome and the mycobiome. The impact of these latter two microbiome components on success of FMT therapy has not been appreciated until very recently. We herein review the current literature on the effects of the gut virome and mycobiome in the FMT treatment of various diseases. We discuss both the beneficial effects and health concerns of the viral and fungal transfer during FMT. We particularly highlight the roles of bacteriophages (bacterial viruses) and *Candida* species (fungi) in FMT efficacy. We also summarise the intricate relationships between the gut virome, mycobiome, bacteriome, and host immunity, underlying FMT. Future efforts should be devoted to understanding the versatile roles as well as the therapeutic mechanisms of specific and/or combination of viral and fungal lineages in different diseases. Harnessing the gut virome, mycobiome, and bacteriome in combination and precision hold a promising prospect in future FMT- and microbiota-based therapies.

Introduction

A large number of diseases are characterized by compositional and functional changes in the gut microbiota. Faecal microbiota transplantation (FMT) is a gut microbiota restoration treatment, performed through oral, intra-intestinal or intra-colonic administration of donor faecal matter containing natural microbial consortia. It is well established that the bacterial microbiome (bacteriome) plays a prominent role in the pathogenesis of gastrointestinal tract (GI) diseases and affects the outcome of therapies.[1,2] Apart from bacteriome, the human gut contains diverse and largely under-explored communities of viruses and fungi.[3,4] Recent evidence suggests that the gut virome and mycobiome not only constitute a significant fraction of the total microbiome, but also work in synergy with the bacteriome to modulate host immunity and physiology.[5,6] Evidence on the roles of gut virome and mycobiome in FMT outcome is also accumulating.[7–9]

FMT has garnered substantial clinical and translational research interests.[10,11] It has broad applications across different diseases, both intra- and extra-intestinal diseases, including *Clostridioides difficile* infection (CDI), recurrent CDI (rCDI), inflammatory bowel diseases (IBD), graft versus host disease (GvHD), irritable bowel syndrome (IBS), obesity and diabetes.[8–10,12–14] While FMT was previously found comparable with the treatment of probiotics in efficacy when treating various diseases, it was recently demonstrated that autologous FMT, but not a multi-strain probiotic product, was effective in post-antibiotic restoration of microbiome in mice.[15,16] This is likely due to the fact that FMT transfers a complete and complex consortium of host-adapted microbial species, including bacteria, bacteriophages, fungi, as well as their metabolites, as opposed to a greatly simplified multi-strain bacteriotherapy. The efficacy of FMT has long been ascribed to the transfer of bacteria.[10,11] Compared to that, the roles of virome and mycobiome are relatively less studied and are yet to be fully emphasized. [2,6,17–19]

In this review, we aim to summarise relevant clinical, translational, and basic research evidence in the field to enhance the community's understanding of the roles of gut virome and mycobiome in FMT, to inform better clinical practice by incorporating the viral and fungal components of the gut microbiome into FMT regime, and in a broader context, to guide future development of microbiota-based therapies. We will discuss the roles of gut virome and mycobiome in FMT in conjunction with their functional importance, donor-recipient effect, as well as safety concerns, and highlight how they potentially impact treatment efficacy and host immunity during FMT.

Roles of gut virome in FMT

The human gut virome

The human gut virome is primarily comprised of prokaryotic viruses (mostly bacteriophages infecting bacteria, collectively known as 'phageome') and eukaryotic viruses (infecting humans), with bacteriophages of the order *Caudovirales* (tailed icosahedral viruses with dsDNA genomes) and the family *Microviridae* (small icosahedral viruses with ssDNA genomes) being most abundant (figure 1a).[3,20–22] Bacteriophages in the human GI tract have both temperate and lytic lifecycles, driving the bacteriome composition by prophage integration and lytic predation respectively.[3,23] Healthy human gut virome is dominated by temperate bacteriophages, while it shifts from temperate to lytic replication during host inflammation and stress.[3,24] Given the predominance of bacteriophages over eukaryotic viruses in the gut virome and its direct roles in regulating bacteriome composition and function, most gut virome research in humans has been focusing on the phageome. While a minority of eukaryotic viruses can cause serious infections, emerging data on a large diversity of gut-resident eukaryotic viruses show that they are somewhat capable of recapitulating the beneficial effects of commensal bacteria through different mechanisms involving host immunity.[25,26] Eukaryotic viruses can ameliorate gut inflammation in mice via viral RNA sensing by host Toll-like Receptors (TLRs)-3 and 7 and its downstream IFN-β secretion, and antiviral treatment led to more severe colitis in dextran

sulfate sodium (DSS)-treated mice.[27] A recent study in mice also shows that enteric eukaryotic viruses evoke broad and enduring host immune responses resembling those elicited by the commensal bacteria.[28] These data suggest that gut viruses play an important role in host immunity and homeostasis.

Gut viruses overall constitute a more diverse genetic entity than the gut bacteria, with virus to microbe ratio (VMR) ranging from 1:1 to 10:1 in the gut.[29,30] Human gut virome is highly diverse and immensely affected by geography, ethnicity, diet, lifestyle and age (figure 1a, together accounting for ~30% of gut virome variations).[31,32] Gut virome dysbiosis has been implicated in the pathogenesis of a diversity of GI and extra-GI diseases, such as IBD[21,33], IBS[34], CDI[7,35], GvHD[12,36], obesity and diabetes[37,38], and FMT have demonstrated a varying degree of success in treating these diseases.[7,12,14,39–42] One commonly observed feature of the gut virome alterations in GI inflammation-related diseases, including in CDI and IBD, is expansion of *Caudovirales*, which was significantly decreased after FMT.[7,21,33,35,43]. In a DSS-induced colitis model of a mouse colony without colonisation of *E. coli*, cocktail of *Enterobacteriaceae* bacteriophages, belonging to *Caudovirales*, exacerbated intestinal inflammation and did not induce lysis of any endogenous microbes.[43] This study corroborated that *Enterobacteriaceae* phages alone were sufficient to elicit inflammatory responses without engagement of *Enterobacteriaceae* or its constituent LPS. These data highlight the importance of *Caudovirales* bacteriophages in gut homeostasis and inflammation and that *Caudovirales* can be manipulated by FMT.

Translational studies investigating FMT and virome

Transfer and engraftment of viruses, along with bacteria, during naïve FMT correlated with treatment outcome in difference diseases, including CDI, IBD, GvHD following hematopoietic stem cell transplant (HSCT).[7,12,44–47] In a seminal study conducted by Ott et al, the authors showed that sterile faecal filtrate (containing viruses, as well as various metabolites and polymers, but

not bacteria) was sufficient for successful remission in patients with CDI[48]. Following that, a number of studies further emphasised the importance of gut viruses, beyond bacteria, in FMT therapies.[7,14,48,49] In recent years, a handful of sterile faecal filtrate and faecal viral transplantation (FVT) studies have emerged investigating the role of gut virome in disease therapeutics, including obesity, type 2 diabetes (T2D), necrotizing enterocolitis (NEC), small intestinal bacterial growth, and post-antibiotic microbiome dysbiosis.[14,49–51]

FMT in CDI

The efficacy of FMT in treating CDI is 90%, which is the most promising compared to its applications in other diseases.[52–56] In our pilot FMT study on patients with CDI (n=9; FMT was conducted via nasoduodenal infusion of donor stool from a healthy household individual to each patient), we found that the gut virome of CDI was characterized by a significant elevation in *Caudovirales* bacteriophages and a reduced virome diversity compared to healthy individuals.[7] After FMT, patients showed substantial viral transmission from donor to recipient.[7] Patients who were cured from CDI exhibited much higher engraftment rates of *Caudovirales* taxa derived from the FMT donor, than those who were not responsive to FMT.[7] Recently, a study on rCDI (n=9) showed that after FMT, the coding genes repertoire of the patients' gut virome was more similar to that of the donors' compared to their pre-FMT profiles, including KO (Kyoto encyclopedia genes and genomes Orthology) terms associated with viral replication, iron transporters, ssDNA phage assembly, and antimicrobial peptide resistance, paralleling the changes in their host bacteria after FMT.[35] This data indicates a potential functionality change in the gut virome of recipient along with the compositional changes in viral taxa after FMT. Concordantly, several studies reported that recipient viromes resembled those of their donors and remained stable after FMT.[7,46,47] Among these studies, one with longer follow-up showed that patients with CDI (n=14) showed a post-FMT gut virome profile similar to that of their donors for up to 1 year after FMT.[47]

FMT in IBD

The efficacy of FMT in treating IBD varies from ulcerative colitis (UC) to Crohn's disease (CD). A systematic analysis documented 53 studies that the overall FMT efficacy is around 36% in UC, 50.5% in CD and 21.5% in patients with pouchitis.[57] In patients with IBD, the faecal virome displayed increased richness of *Caudovirales* bacteriophages along with a decreased bacterial diversity (n=174; patients were from Cambridge, UK; Chicago, Los Angeles, and Boston, in USA).[21] At the intestinal mucosal level, patients with UC (n=91; three China cohorts) showed that the rectal tissues consistently had an expansion of *Caudovirales* bacteriophages compared with healthy individuals. [33] Phages of *Escherichia* and *Enterobacteria*, belonging to the order *Caudovirales*, were experimentally demonstrated to aggravate intestinal inflammation and colitis as a consequence of overproduction of IFN-γ via TLR-9 signalling in the murine host [43]. These data together suggest that certain *Caudovirales* taxa play a crucial role in the disease course of IBD. However, the role of the phageome (particularly *Caudovirales* bacteriophages) in FMT therapies of IBD has not been thoroughly investigated to date, which warrants in-depth research.

An increased eukaryotic virome richness was also observed in faeces of patients with UC.[44] One pilot FMT study in UC patients (n=9) found that UC individuals who successfully responded to FMT contained a significantly lower eukaryotic virome richness (both before and after FMT) compared to non-responders.[44] This study indicates that a low baseline eukaryotic viral richness might be important for a successful FMT in UC, albeit the mechanism is lacking. Overall, the role of viral engraftment and virome alterations in FMT therapies targeting IBD is still in its infancy and represents an area of particular interest.

FMT in GvHD

Limited studies have found some effectiveness of FMT in treating GvHD, though the overall efficacy is lacking.[12,58] Patients with GvHD following HSCT manifested significant GI symptoms and showed a persistent dominance of eukaryotic viruses (anelloviruses, herpesviruses, papillomaviruses and polyomaviruses) in the gut.[36] Among them, picobirnaviruses were determined as a predictive marker for the development of severe GvHD.[36] In a single case study, a GvHD patient was treated with 4 episodes of FMT. [12] Following treatment, an increase in faecal virome diversity was observed, accompanied by expansion of *Caudovirales* bacteriophages and shrinkage in the eukaryotic Torque teno viruses.[12] Consistent with FMT effects on virome reported in CDI, the transfer of *Caudovirales* bacteriophages also underpins a positive treatment outcome in GvHD.[7,12] Future research with expanded sample sizes is needed to convincingly address the therapeutic effect of FMT and virome modulations in GvHD.[58,59]

Faecal viral transplantation (FVT) in other disease indications

As viruses are co-transferred alongside bacteria during naïve FMT, the role of viruses is fastidious to tease apart from that of bacteria in treating disease. Two pilot case series studies (n=5 and 4) explored the effect of sterile faecal filtrate in treating CDI, a refined FMT protocol where the bacteria were filtered out.[48,60] This study showed that sterile faecal filtrate restored normal stool habits and eliminated symptoms of CDI, suggesting a possible role of bacteriophages and viruses (as a prominent component of sterile faecal filtrate) in the therapeutic mechanism of FMT. However, caution should be taken when interpreting these studies considering that the biological effect of sterile faecal filtrate is not solely attributed to the viral microbiota *per se*, where the metabolites, digested micro-nutrients, extracellular active ingredients (such as viral envelope, bacterial cell wall components and antimicrobial peptides) are also present in sterile faecal filtrate.

Following that, more studies have employed a further refined protocol, FVT, to specifically investigate the effect of faecal viruses in treating diseases, where faecal virus-like particle (VLP) are isolated, purified, and enriched.[14,49–51] In a piglet model of preterm infants, Brunse et al. showed that orally administrated FVT successfully prevented the development of necrotising enterocolitis (NEC) in all preterm piglets that received FVT, whereas FMT did not perform better than the control arm, indicating that a gut virome transfer and modification might be critical in preventing the development of NEC.[50] Following antibiotic-mediated disruption of the bacteriome in human patients, rapid restoration of the depleted microbiota might confer significant health benefits.[49] Probiotics were shown to be not only ineffective in post-antibiotic restoration of the gut microbiome, but sometimes even leading to impaired microbiome recovery[15,16]. In contrast to that, a study investigated the effect of autologous FVT (harvested prior to antibiotics perturbation) in mice reported a better gut bacteriome recovery after antibiotic perturbation, compared to the control treatment.[49] More recently, a study reported the effect of FVT in treatment of type 2 diabetes and obesity in mice.[14] Obese mice treated with FVT showed improvement in glucose tolerance and reduced further weight gain caused by high-fat diet.[14] In these mice, FVT was found to impact not only the virome but also the bacteriome, due to the transfer of bacteriophages.[14] In addition, FVT can reduce high-fat diet-induced small intestinal bacterial overgrowth.[51] Taken together, these findings emphasise the critical role of virome transfer in successful FMT therapies of various human diseases.

Donor and recipient effect

In our prior FMT-CDI study, we found that donor-recipient pairs with a higher faecal viral richness of *Caudovirales* in donor than recipient was predictive of a better clinical outcome in CDI.[7] This result at the same time was coupled with changes in the bacteriome, where FMT-driven *Caudovirales* transfer was positively correlated to the bacterial richness and diversity in the responders.[7] Due to the co-transfer nature of virome and bacteriome during FMT, it precludes us separating the effect of virome and that of bacteriome in

influencing FMT outcome in this setting. Albeit, it highlights the importance of donor virome composition and significance of appropriate donor-recipient pairing for the efficacy of FMT. Consistently, another study found that donors with a high faecal bacteriophage α-diversity and a low bacteriophage abundance were associated with a successful FMT in CDI.[45]

By adopting a single-cell viral tagging (VT) approach, researchers investigated whether individual phages isolated from one subject's faeces could interact with bacteria isolated from a different subject, as a proxy for FMT.[61,62] They found that a high level of cross-reactivity between bacteriophages and bacteria from different human subjects, which however varied across donor-recipient pairs, highlighting a significant donor-recipient pairing effect on FMT/FVT outcomes.[62] Therefore, by modelling a bacteria–bacteriophage interaction network between the donor and recipient microbial communities, based on single-cell VT results, it may become possible to identify optimal donor-recipient pairs and to predict the outcomes of FMT.

Safety concerns of virome transfer in FMT

While data suggests that viral transfer during FMT is an important beneficial factor for the success of therapy, undesirable viral transmissions constitute a serious safety concern, especially when transplanting into an immunocompromised recipient. One report showed that norovirus was transmitted to 2 out of 13 CDI patients via FMT procedure, who later developed a post-FMT norovirus gastroenteritis.[63] This report claimed that viral contamination and transfer during the FMT procedure by a procedure-involved employee who had previously developed norovirus-like symptoms might be a cause to the observed post-FMT norovirus gastroenteritis.[63] A number of pathogenic eukaryotic viruses can potentially be present in the human gut, including papillomaviruses, herpesviruses, hepatitis viruses, bocaviruses, enteroviruses, rotaviruses, and sapoviruses.[64] To prevent potential transfer of pathogenic eukaryotic viruses during FMT, a thorough faecal virome screening of the donor should be performed to ensure the

safety of faecal transplant. Despite such concerns, FVT seems to be generally safer than FMT due to the removal of intact bacteria, in particular obligate and opportunistic bacterial pathogens, prior to transplantation. FVT can also avoid transfer of certain gut commensals with unwanted properties, which under certain conditions can interact with host physiology exacerbating disease. Overall, FMT in general has been found to be safe provided that the donor screening is done adequately as instructed in international guidelines. Most, if not all, unfortunate cases have been due to sloppy and highly unacceptable donor screening.

Bacteriophages targeting gut bacteria can have cascade effects on bystander bacteria, as demonstrated in gnotobiotic mice.[65,66] Therefore, which gut bacteria are going to be affected by phage predation during FMT, to what extent and in what direction, present an unpredictable and daunting challenge. Equally complex and unpredictable is the subsequent effects of microbiome manipulations on the overall health of the human host. In addition, horizontal gene transfer (HGT) from one bacterial strain to another, mediated by bacteriophages (phage transduction) can be a significant contributor to dissemination of antibiotic resistance genes, virulence genes and other unwanted genetic material.[67] . A number of bacteriophages encode toxins (such as diphtheria toxin, Shiga toxin and erythrogenic toxin) that increase survival and virulence of their bacterial host during lysogenic conversion.[68,69] FMT may transfer the bacteriophages carrying these toxin genes to confer a virulent phenotype to the co-resident bacteriome in the gut of the recipient, posing another health concern.

Administering rats with a bacteriophage cocktail led to an increased intestinal permeability, weight loss, and decreased activity.[70] This study demonstrates that increased intestinal permeability may be induced by bacteriophages that affect the microbiota. Recently, a temperate filamentous bacteriophage was found to integrate into the genome of a multidrug-resistant *Pseudomonas aeruginosa*, resulting in inappropriate antiviral immune responses and

impaired clearance of bacterial infection in the host.[71] These evidence suggest that akin to the transfer of certain eukaryotic viruses, the transfer of unwanted bacteriophages may also confer undesired health concerns to the human host.

Mechanisms of virome function underlying FMT

The mechanisms of viral action contributing to FMT therapies include tripartite mutualistic interactions between bacteriophages/eukaryotic viruses, bacteria, and the mammalian host (figure 1b).[6]

Functions of bacteriophages colonisation

Bacteriophage transfer engraftment can modulate not only the taxonomic composition but also the functional capacity of the gut bacteriome.[65,66,72] Faecal multi-omics profiling of mice receiving a transplant of a defined consortium of bacteriophages revealed shifts in both the microbiome and gut metabolome after bacteriophage colonisation.[66] The result showed that a large diversity of metabolites was altered after bacteriophage transplantation, including nearly all KEGG pathways (amino acids, peptides, carbohydrates, lipids, nucleotides, cofactors, vitamins, and xenobiotics).[66] A recent study also showed that Bacteroides phage BV01 altered the genome-wide transcriptome profiles and bile salt hydrolase activity in its bacterial host, leading to an altered profile of bile acids.[72] In return, bile acids are well known to regulate both host microbiome (including *C. difficile*) and host physiology.[73,74] Such changes in gut bacteriome composition and functionality induced by bacteriophage colonisation are important for host health.

Accumulating data suggest that bacteriophages can modulate the immune system both directly and indirectly. Indeed, bacteriophages can colonise the intestinal mucus layer, directly binding to mucin glycoproteins via their capsids, and provide the mammalian host with a defence mechanism against the bacteria trying to breach through the intestinal barrier.[75] Certain

bacteriophages, such as phage 536_P1, directly promote the production of antiviral cytokines, such as IFN-γ and IL-12, as well as chemokines, even in the absence of their host bacteria.[76] Bacteriophages can interact with the host immune system in various ways, by inducing the innate defenses against bacterial colonisation, stimulating production of inflammatory cytokines, and activating dendritic cells (DCs) and innate lymphoid cells (ILCs) to produce IFNs (figure 1b).[77]

Functions of eukaryotic viruses colonisation

In addition, colonisation with eukaryotic viruses in the gut is critical for the maintenance of gut microbial homeostasis and host immunity. Recognition of the bulk of enteric viruses via surface receptors TLR3 or TLR7 induces production of IFN-β, which protects the host from developing inflammation.[27] The RIG-I receptor for viral RNA recognition in the cytosol signals IL-15 production to maintain homeostasis of intraepithelial lymphocytes.[78] Another prime example of protective effects of intestinal eukaryotic virome was demonstrated in murine norovirus (MNV)-colonised mice.[79] The viral protein NS1/2 from MNV evoked a host protective response with increased production of IFN-I and IL-22, which conferred a critical protection against *Citrobacter rodentium* infection and promoted proliferation of the intestinal epithelial cells.[79] A recent comprehensive murine study profiled the immune responses to a panel of eukaryotic viruses and found a widespread capacity for asymptomatic intestinal colonisation and durable alterations that the both strain-specific and common to multiple viruses.[28] Most enteric viruses promoted T cell differentiation, Th1 polarization, and production of IL-22, a cytokine central to the dialog between host and microbiome at epithelial barriers.[28,80] Such enteric viruses could transcriptionally upregulate anti-bacterial peptides, though to a lesser extent than a consortium of 15 bacterial strains in germ-free (GF) mice.[28] In addition, astrovirus supplementation in immunocompromised mice can protect the mice from enteric pathogens via IFN-λ, transferable by cohousing and faecal transplantation.[81] These data together suggest that gut eukaryotic viruses also tune host homeostasis by orchestrating both the host immunity and the co-resident microbiome.

Taken together, these studies indicate that colonisation by gut viruses, both prokaryotic and eukaryotic, is essential to calibrate host immunity and physiology. Transfer and engraftment of gut viruses via FMT can have broad cascading effects on the mammalian host, including modulation of host microbiome, metabolome, and immunity.

Roles of gut mycobiome in FMT

The human gut mycobiome

The human GI tract is also home to a large diversity of fungi, collectively known as the gut mycobiome. Fungi constitute a minor component of the gut microbiota, making up approximately 0.1% of the microorganisms shown by metagenomics sequencing.[4,82,83] Studies have shown that the gut harbours >50 genera of fungi with *Candida*, *Saccharomyces*, *Cladosporium* and *Malassezia* being the most abundant genera (figure 2a).[4,82,83] However, the gut fungi are highly under-explored relative to the gut bacteria, and hence are underrepresented in current mycobiome databases, hindering gut mycobiome profiling and functional characterisation.[84] Gut resident fungi have both mutualistic and antagonistic relationships with the gut bacteria, together shaping the host immunity.[85] Intestinal fungi have been shown to be causally implicated in microbiome assembly and immune development.[86] Accumulating evidence points to that the gut mycobiota can strongly influence the host immune system and this interaction is linked to bacteria activities.[87,88] Recent observations of dysbiosis in gut mycobiome across various diseases highlight a critical role of gut fungi in disease pathogenesis[89–91], suggesting that targeting gut mycobiome may represent a promising therapeutic modality.

Translational studies investigating FMT and mycobiome

Emerging studies report that FMT involves transfer of fungi, which may affect treatment outcomes.[8,9] Our FMT-fungi study in CDI (n=16) showed that donor

fungi were substantially transferred to recipient after FMT, and cure after FMT was associated with increased colonisation of donor-derived fungal taxa in recipients.[8] After FMT, responders displayed a high relative abundance of *Saccharomyces* and *Aspergillus*, whereas non-responders and patients treated with antibiotics displayed a dominant presence of *Candida* in faeces.[8] CDI patients who responded to the treatment showed a reduction in the abundance of *C. albicans* after FMT, while those who did not respond to FMT showed a still high abundance of *C. albicans* in faeces.[8] In favor of this finding in humans, we observed that *C. albicans* also negated FMT efficacy in a mouse model of CDI.[8] In a separate experiment, we showed that antifungal treatment to eliminate *C. albicans* in recipient mice before FMT re-established FMT efficacy using the same donor stool without presence of *C. albicans*.[8] These evidence together highlight a causal relationship between gut fungal dysbiosis and FMT outcome in CDI.

Interestingly, a recent FMT study conducted in patients with UC (n=24) observed a different association between faecal *C. albicans* levels and treatment outcome: UC patients with higher *C. albicans* abundance pre-FMT were much likely responsive to FMT. [9] On the other hand, in agreement with the finding in CDI, FMT resulted in a reduction of *C. albicans* in UC patients.[9] Decreased *Candida* abundance post-FMT was indicative of ameliorated UC severity. [9] These findings suggest that FMT reduces *C. albicans* abundance, and a decreased *C. albicans* abundance after FMT is associated with disease amelioration in both CDI and UC. The discordant findings between CDI and UC that a high faecal abundance of *C. albicans* in recipients pre-FMT may lead to discrepant treatment outcomes after FMT underscores that gut fungi in recipient baseline may affect FMT efficacy in a disease-dependent manner. Similarly, patients with CD had higher fungal burden and *Candida* colonisation in the gut, however CD patients were less responsive to FMT compared to UC.[10,92–94] Different immunophysiology and immunopathophysiology mechanisms, though calibrated by the same fungi, in different disease settings may underlie this discordance in FMT efficacy between diseases.

Overall, FMT treatment is able to reduce *C. albicans* levels in recipient and produce a positive treatment outcome in CDI and IBD. This effect is also evidenced in different mouse models, demonstrating that FMT prevented *C. albicans* colonisation.[8,95,96] In a pilot study, a patient with UC was cleared of recurrent *C. glabrata* infection after FMT [97], suggesting FMT may be able to deliver a possible beneficial effect on patients who are complicated with fungal infection. Another clinical study showed that FMT was effective to reduce the likelihood of developing blood stream fungal infection in rCDI patients compared to antibiotics treatment.[98] These studies further support that FMT is capable of modulating recipient fungi, including *Candida* species.

Another intestinal disorder IBS is also characterised by a significant alteration in the gut mycobiome, featured by predominance of two fungal species *C. albicans* and *Saccharomyces cerevisiae* compared to healthy individuals.[99] Interestingly, IBS-related visceral hypersensitivity was transferable between rats by FMT, suggesting a causal role of gut microbiome in IBS.[99] To date, whether FMT is effective in IBS is still controversial in clinical practices. One randomized clinical trial (n=90) conducted in a cohort of IBS patients in northern Norway showed that FMT relieved IBS symptoms compared to the placebo arm, whereas another randomized clinical trial (n=48) conducted in three US centres showed that FMT was ineffective in reducing IBS symptoms compared with placebo.[100] Whether gut fungi play a role in such heterogenous clinical efficacy of FMT in IBS warrants further investigation.

FMT is a promising agent for treating infectious diseases and gastrointestinal disorders, it has been utilised for treating severe colitis in GvHD patients following allogenic HSCT.[12] The antifungal drug fluconazole has been shown to prevent *C. albicans* colonisation and to reduce the likelihood of developing GvHD after HSCT, suggesting that modulation of gut mycobiota may be associated with the pathogenesis of GvHD post HSCT.[101] More recently, our observational FMT study on a single GvHD case reported some changes in

the gut mycobiome following a successful treatment of 4-dose FMT.[12] The serial FMT treatments altered the fungal composition and diversity in the faeces of this GvHD patient, which was more similar to the donor's faecal fungal profile after FMT compared to his pre-FMT profile.[12] However, as this pilot study was exploratory, an expanded sample size is needed in future to address any biologically meaningful changes in recipient's gut mycobiome by FMT underlying cure of GvHD.

The role of gut mycobiome in FMT is elusive and there is a significant lack of human and animal studies centered on fungal transfer in FMT treating different diseases. The keystone fungal species as well as their functions in the gut and FMT remain to be discovered in different disease settings.

Donor and recipient effect

As aforementioned, our FMT-CDI study in both humans and mice found that existence of *C. albicans* in donor and high abundance existence of *C. albicans* in CDI recipient pre-FMT were both detrimental to FMT outcome.[8] Similarly, two filamentous fungi *Penicillium brocae* and *Aspergillus penicillioides*, present in either donor or recipient pre-FMT, also nullified the treatment efficacy of FMT in CDI mice, highlighting a generic deleterious role of overrepresentation of certain fungi in donor or recipient in FMT.[8] Antibiotics are always the primary treatment for patients with CDI, which may lead to a fungal bloom and a high likelihood of developing fungal infections in patients, particularly expansion of *Candida* species.[102] These data underscores the importance of donor selection (exclusion of invasive *Candida* carriers) and appropriate recipient preparation based on their faecal fungal profiles, to enhance FMT efficacy in CDI. In contrast, a high faecal abundance of *C. albicans* in UC patients pre-FMT was associated with a favourable FMT outcome.[9] Taken together, it suggests that the gut mycobiome composition of donor and recipient should be considered separately in different disease settings when employing FMT.

Safety concerns of mycobiome transfer in FMT

Analogous to gut bacteria, a lot of gut fungi are opportunistic species which can mount detrimental immune responses in the host under non-homeostatic conditions, including *C. albicans.* Therefore, to avoid transfer of fungal pathogens and opportunistic fungal pathogens, thorough donor screening based on the faecal fungal profile is necessary during FMT practice. To date, there has no report of death or infection caused by transmission of life-threatening fungi from FMT, but screening for fungal candidates in the donor and susceptible recipients should also be cautious. Fungal infection is often seen in immunocompromised patients with CDI, IBD and GvHD, and the responsible candidates are *Candida* species that contribute to the majority of infections.[97,98,101] Most common fungal infections in CDI are caused by *C. albicans*, *C. parapsilosis*, and *C. tropicalis* in the blood stream due to prolonged use of antibiotics.[98] These fungi constitute a health concern to the host, and may nullify FMT efficacy and invoke undesired immune responses after FMT.

Other fungi, such as *Malassezia restricta* and *Histoplasma capsulatum* should also be checked in donor screening.[103,104] *M. restricta* is a skin commensal that preferentially colonises in CD patients with CARD9 polymorphism, whereas *H. capsulatum* preferentially infects IBD patients with immunocompromised condition. [103,104] CARD9 and dectin-1 are well known fungal recognition receptors, and polymorphisms in these genetic loci are associated with increasing susceptibility to fungal infections.[105–107] It is known that antibiotics can alter the gut bacterial-fungal community structure and that antibiotics treatment was found to offer longer-lasting impact on gut fungi other than bacteria.[108] Hence, fungal profiling in the donor, the genetically susceptible or immunosuppressive recipient, as well as those with a drug or antibiotics history, are needed to avoid potential fungal bloom or infection following FMT. [105–107,109]

Mechanisms of protective immunity elicited by the gut fungi

The commensal fungi in the gut can evoke protective immunity in the host and impact gut microbiome assembly. [6,88,105,110,111] The mechanisms of mycobiome action underlying FMT treatment of diseases hence also involve a tripartite interaction between fungi, bacteria, and the mammalian host (figure 2b).

It is shown in gnotobiotic mice that commensal fungi can functionally recapitulate the protective benefits of intestinal commensal bacteria, by mitigating tissue injury and extra-intestinal infection as well as calibrating the activation of protective CD8+ T cells.[112] Elimination of the gut fungi by oral antifungals in mice worsened the outcome of colitis and allergic airway disease[91], where both the host immune profile and the gut bacteriome composition were disrupted, suggesting that colonisation of gut commensal fungi plays an import role in host immune and microbiome homeostasis. Fungi can stimulate host cells through a variety of microbial pattern recognition receptors (PRRs). [6,105,110,113–117] Surface receptors, such as dectin-1, dectin-2, mincle and CX3CR1 receptors on mononuclear phagocytes (MNPs) can recognize β-glucans in the fungal cell wall.[6,105,110,113–117] Fungal sensing by CARD9 signalling induces release of IL-18 for reconstitution of intestinal epithelial cells (IECs).[6,105,110,113,118] IL-22 elicited by fungal sensing restricts inflammasome from triggering immune pathology.[119] Fungal-derived molecules and metabolites, such as mannans and glucans, are also critical components to calibrate host immunity by changing the cytokine profile.[120,121] These mechanistic studies underscore the sophisticated molecular pathways underlying gut fungi regulating host immunity.

Another aspect of mycobiome function on the host is its interactions with gut bacteria and other co-resident fungi. In steady state, bacteria and fungi keep each other in check in the gut. One study demonstrated that *C. albicans* affects the recolonisation of the cecum by the microbiota in mice treated with antibiotics.[122] The presence of *C. albicans* in the gut increased colonisation

by the bacterium *Enterococcus faecalis* and reduced colonisation of probiotic *Lactobacillus* strains.[122] A follow-up study revealed that antibiotic-treated *C. albicans*-colonised mice had reduced expression of specific immune genes, hinting at dual role of *C. albicans* on host immunity and bacteriome assembly.[123] Several studies have shown that *Saccharomyces boulardii* can suppress *C. difficile* by the production of a protease to degrade toxins A and B of *C. difficile*.[124,125] These studies imply that colonisation of *Saccharomyces* species by FMT may favour a positive outcome in CDI. Moreover, *S. boulardii* has a protective effect against various other bacterial gastrointestinal pathogens, including *Helicobacter pylori*, *Vibrio cholerae*, *Salmonella enterica serovar Typhimurium*, *Shigella flexneri*, and *Escherichia coli*.[85] Both *E. coli* and *S. Typhimurium* bind to the surface of *S. boulardii*, potentially preventing adhesion to intestinal epithelial cells and thus allowing quicker excretion through faecal matter.[126,127] *S. boulardii* was found to suppress colonisations of both *C. albicans* and Adherent-invasive *Escherichia coli* (AIEC) and to alleviate colitis in mice.[128–130] Overall, FMT is able to cause a collection of fungi colonised in recipient, where they individually or together with gut bacteria regulate host immnunophysiology influencing FMT outcome.

Conclusions

A large body of studies demonstrated efficacy of FMT in re-shaping the microbial composition and function in the recipients in a number of human diseases, though resolution of symptoms and cure can only be consistently achieved in CDI and inconsistently achieved in other diseases, such as IBS, IBD and obesity. Successful FMT is not only ascribed to the restoration of healthy gut bacteriome, but also involves modulation of the virome and mycobiome. The intricate relationships between the bacteriome, the virome and the mycobiome on one hand, and the human host on the other, underpin clinical and microbiological effects, and overall efficacy of FMT. Enhanced understanding of gut virome and mycobiome will guide and facilitate future precision FMT-based therapies of various human diseases. Well-designed

clinical trials based on targeted bacterial-, viral/phage- and fungal-transfer will be required, so are preclinical mechanistic studies investigating functions of the individual components of the microbiome. Of particular interest are different lineages and combinations of *Caudovirales* phages, as well as fungi from the genera *Candida* and *Saccharomyces*, due to their possible contributions to FMT efficacy. Precision FMT should be adopted in future, including FVT and/or defined consortia of phages-fungi-bacteria combinations, in a personalised, disease-specific manner. We look forward with optimism to the future of precision FMT, as it transfers a holistic, tailor-made, and well donor-recipient paired microbiome for disease treatment.

Search strategy and selection criteria

A literature search was performed on Pubmed, Google Scholar and Web of Science using the keywords “Faecal microbiota transplantation”, “FMT”, “Faecal viral transfer”, “FVT”, “Fungal microbiota”, “Gut virus”, “Virome”, “Gut fungi” and “Mycobiome” to select relevant clinical and animal studies.

Contributors:

TZ conceived the manuscript. TZ and SL wrote the manuscript. ANS, PL and XJW provided significant intellectual contribution and edited the manuscript. TZ, PL and XJW supervised this study. HP and XWB provided critical comments.

Declaration of interests:

The authors declare no conflict of interest.

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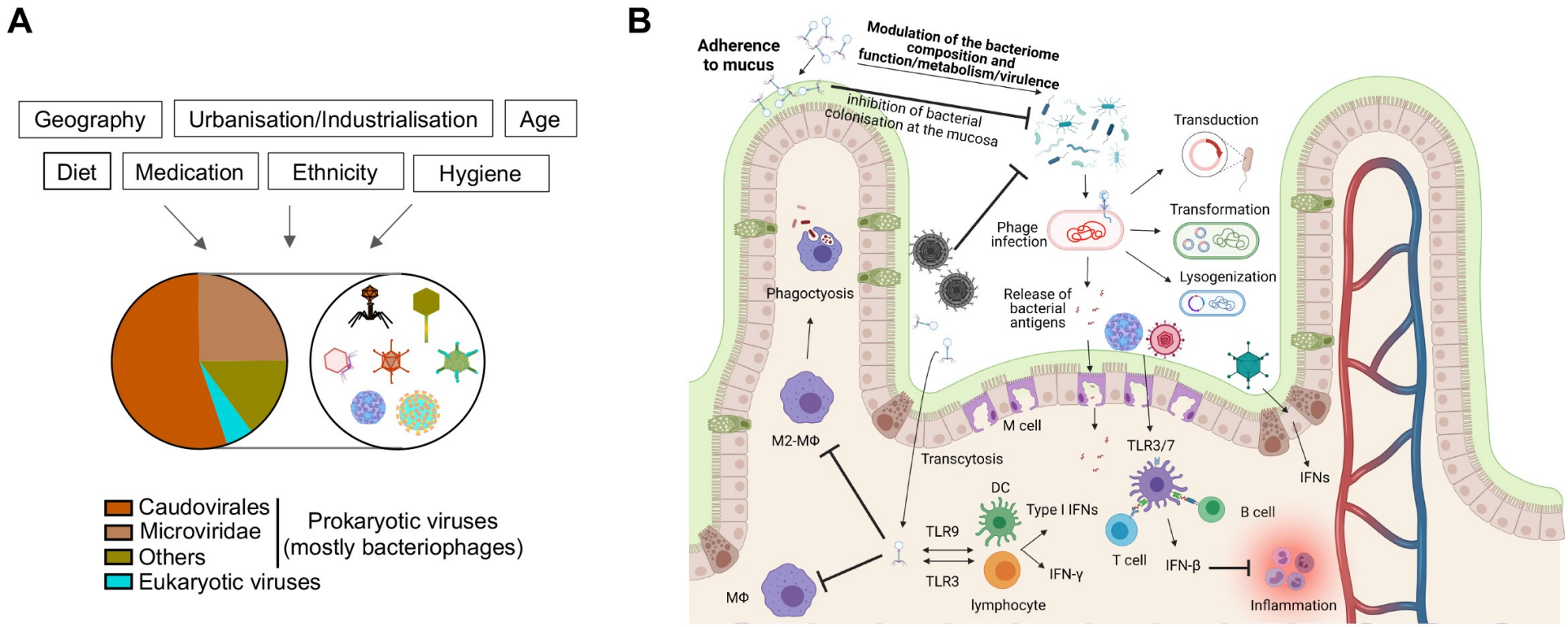

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1050 Figure 1. Composition and function of the gut virome. A. The composition of human gut virome and factors influencing its composition. B.

1051 Functions of the gut virome on the host.

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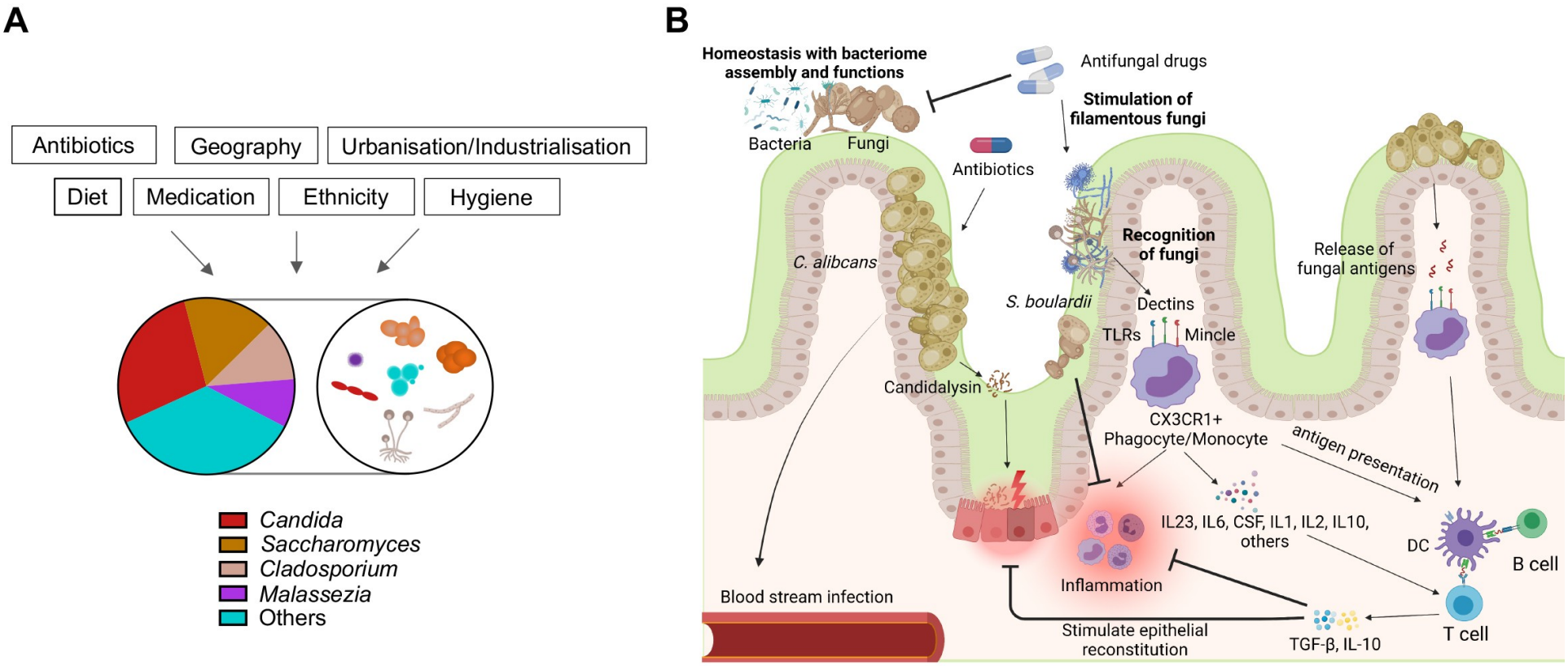

Figure 2. Composition and function of the gut mycobiome. A. The composition of human gut mycobiome and factors influencing its composition. B. Functions of the gut mycobiome on the host.

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Table 1. Summary of human studies investigating FMT and gut virome/mycobiome

Indication	Number of study subjects	Type	Methodology	Treatment outcome in association with gut virome/mycobiome alterations	Reference
CDI	N=44	Randomized Controlled Trial	Faecal virome and bacteriome were profiled in association with treatment outcome	*Caudovirales* derived from the donors with increased richness displayed responsiveness	7
	N=55	Randomized Controlled Trial	Faecal mycobiome and bacteriome were profiled in association with treatment outcome	High abundances of *Candida albicans* in donor or recipients led to failure of FMT; FMT decreased *Candida albicans* abundance	8
	N=9	Pilot-study	Analysis in the metagenome, prophage- and CRISPR-based bacteria-phage association and gene functions of the bacteriome and virome after FMT treatment	Successful FMT resulted in functional restoration in the bacteriome and virome resembling donors' profiles. A negative correlation between *Microviridae* and *Proteobacteria* was found before and after FMT	35
	N=1	Case-study	Longitudinal investigations up to 42 months on patient's bacteriome and virome after a successful FMT	Bacteriome and virome in the patient resembled the donor's profile long-term; low phage abundance is associated with a 'healthy' virome profile	47
	N=14	Pilot-study	Investigations up to 12 months on the viral transfer from 3 donors to 14 patients	A successful FMT was associated with durable virome alterations up to 12 months in recipients	46
IBD	N=39	Randomized Controlled Trial	Faecal mycobiome was profiled in association with treatment outcome	FMT responders showed lower faecal *Candida albicans* abundances and anti-*Candida* antibody levels after FMT	9
	N=9	Pilot-study	Analysis in the UC faecal virome and its association with induction of clinical and endoscopic remission	FMT responders showed lower baseline eukaryotic virome richness	44
GvHD	N=1	Case-study	Faecal virome, mycobiome and bacteriome were profiled longitudinally in one patient treated with 4-dose FMT	Decreased faecal fungal diversity after serial FMTs, while virome maintained stable after FMT; relative abundance of Torque teno viruses was decreased, whereas *Caudovirales* bacteriophages was increased in faeces after FMT	12

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