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Advances in Fecal Microbiota Transplantation

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[abstract] **Intestinal microbiota has an important role in our health. The resident microbiota of the human intestine in an undisturbed state provides protection against bacterial infections. The microbiota is influenced by our diet and environment. Dysbiosis is associated with a range of gastrointestinal and non-gastrointestinal diseases including Clostridium difficile infection (CDI). Fecal microbiota transplant (FMT) is a process used when stool is taken from a healthy individual and instilled into a sick person to cure a certain disease. FMT is now an emerging treatment for a wide range of disorders. Recent clinical trials have shown that FMT has become an exciting avenue for the treatment for**

CDI and related illnesses. This review highlights advances in FMT for treating CDI in light of advances in genomics, animal models of CDI, increased understanding the microbiome, gut biochemistry and the relationship of gut with other body regions
[abstract]

[manuscript]

INTRODUCTION

The term FMT has replaced preceding name of 'bacteriotherapy' because fecal preparations contain organisms belonging to all three domains of life, including archaea, eukaryota and viruses (Bakken et al. 2011, Floch 2010). The importance of FMT has risen in the wake of CDI epidemic although it was practiced since early 4th century AD (Lessa et al. 2015). Application of FMT for the treatment of severe CDI has been found to have a success rate of over 90% (van Nood et al. 2013) and is also effective in preventing later recurrence (Aroniadis et al. 2016, Lagier, Delord, et al. 2015). In a long term follow-up of recurrent CDI (RCDI), FMT was found to have an efficacy of 91 % primary cure rate, 98 % secondary cure rate and that 97 % of patients expressed willingness to undergo another FMT in the future, and 53 % stated that they would choose FMT as a first-line treatment before antibiotics (Brandt et al. 2012). The first reports of the administration of human fecal suspension by mouth for patients with food poisoning or severe diarrhea dates back to the 4th century in China (Zhang et al. 2012). The use of a variety of stool products for treatment of diarrhea, fever, pain, vomiting and constipation have been reported as early as the 16th century (Zhang et al. 2012). In the 17th century, FMT was used in veterinary medicine and later termed 'transfaunation' (Borody et al. 2004). In 1958 the first use of fecal enemas for the treatment of pseudomembranous colitis in four human patients exhibited dramatic resolution 24-48h post-treatment (Eiseman et al. 1958).

A fecal suspension can be administered by nasogastric or nasoduodenal tube, colonoscope, enema, or capsule. The high success rate and safety in the short term reported for RCDI has elevated FMT as an emerging treatment for a wide range of disorders, including Parkinson's disease, fibromyalgia, chronic fatigue syndrome, myoclonus dystopia, multiple sclerosis, obesity, insulin resistance, metabolic syndrome, and autism (Choi and Cho 2016). The resident microflora can stay up to 70 days after FMT (Fuentes et al. 2014). The transplanted feces from a healthy donor can possibly preserve 1,000–1,150 functional bacteria species and can eventually reestablish a "healthy" functional microbiota in the recipient (Qin et al. 2010). A number of agents have been used for treatment and prevention of CDI in clinical trials (Table I). Availability of orally deliverable FMT products, such as capsules containing lyophilized fecal

microbiota, has simplified CDI treatment in both CDI and non-CDI diseases. FMT can also be used to intentionally eradicate colonization with antibiotic-resistant bacteria from the GI tract (Bilinski et al. 2016, Crum-Cianflone, Sullivan, and Ballon-Landa 2015, Lagier, Million, et al. 2015, Singh et al. 2014).

To characterize the microbiome from Intensive Care Unit (ICU), sequential microbiome sampling from ICU patients was performed, (Wischmeyer, McDonald, and Knight 2016). The fecal, oral, and skin samples from 115 mixed ICU patients across four centers in the United States and Canada were collected at two time points: within 48h of ICU admission, and ICU discharge or ICU day 10 and compared to the large control group. Critical illness showed a rapid and distinct change from a 'healthy' fecal and oral microbiome. Fecal ICU samples tended to have a lower relative abundance of Firmicutes and increased relative abundance of Proteobacteria. Organisms such as Faecalibacterium shown to confer anti-inflammatory benefits and which produce short-chain fatty acids that are vital to the gut were found to be depleted (Sokol et al. 2008). The study concluded that severe dysbiosis occurred in a broad, larger population of critically ill study participants. They proposed targeted microbial therapies using specific probiotics or targeted, multimicrobe 'stool pills' to restore a healthy microbiome and improve outcomes in critical illness (Wischmeyer, McDonald, and Knight 2016).

CLOSTRIDIUM DIFFICILE INFECTION

CDI has become one of the most prevalent hospital-acquired infections in recent years. *C. difficile* is known to cause severe disease and death (McDonald et al. 2005). The risk factors for CDI are previous hospitalization, underlying disease, advanced age (>65 years), use of antibiotics, impairment in humoral immunity, renal disease and hypoalbuminemia (Smits et al. 2016, Lutynski and Kuratowska 1977, Miller et al. 2013, Islam et al. 2014, Di Bella et al. 2015). Certain antibiotic treatments have also been associated with higher recurrence of CDI (Abou Chakra et al. 2014). In one study the expression of at least a subset of colonization factors by the bacterium such as cell surface protein Cwp84 and surface layer protein A (SlpA) was found to be stimulated in the presence of antibiotics ampicillin and clindamycin (Deneve et al. 2008).

C. difficile causes a spectrum of clinical diseases ranging from mild diarrhea to toxic megacolon, colonic perforation and death. Systemic complications in life threatening CDI include cardiopulmonary arrest (Johnson et al. 2001), acute respiratory distress syndrome (Jacob et al. 2004), multiple organ failure (Dobson, Hickey, and Trinder 2003), renal failure (Cunney et al. 1998) and liver damage (Sakurai et al. 2001). However, this bacterium might also be carried asymptotically in the gut, potentially leading to 'silent' onward transmission. The period from spore ingestion to symptom onset is variable but typically short. One study reported 82% of CDIs occurred within 4 weeks of a potential donor infection (Walker et al. 2012). At least four

events are integral to *C. difficile* pathogenesis before the development of symptomatic infection.

- i. Transmission of spores via the fecal-oral route.
- ii. Exposure to antibiotics (or immunosuppressant's) establishes susceptibility to infection through perturbation of the intestinal microbiota.
- iii. Transformation of spores into vegetative bacteria through interaction with small molecular germinants, such as bile acids.
- iv. Release of *C. difficile* toxins, which are essential for the disease manifestations.

It is important to diagnose symptomatic and asymptomatic carriers of *C. difficile*. The diagnosis of CDI includes presence of clinical symptoms and laboratory assays (Smits et al. 2016). The three types of assays are

- i) Testing for *C. difficile* products such as glutamate dehydrogenase (GDH), aromatic fatty acids, and the two major toxins (TcdA/TcdB)
- ii) Culture methods for detecting toxin producing *C. difficile*
- iii) Nucleic acid amplification for *C. difficile* genes such as 16sRNA, toxin genes or GDH gene

The first nucleic acid amplification test to receive Food and Drug Administration (FDA) approval was the BD GeneOhm™ Cdiff assay in 2009. Tests which detect toxins are specific to subjects with symptomatic CDI whereas tests which detect parts of the bacterium indicate asymptomatic carriers (Planche and Wilcox 2015). Cytotoxigenic culture can detect toxigenic *C. difficile* and gives a positive result more frequently because of colonization, which means that individuals can have the bacterium but no free toxin compared to the cytotoxin assay, which detects preformed toxin in feces (Planche et al. 2013, Planche and Wilcox 2015).

Rapid diagnosis of CDI is desirable to allow early isolation and treatment of patients, reducing potential patient-to-patient transmission and length of hospital stay for those affected. In addition, *C. difficile* strain typing can identify outbreaks within a hospital or the wider community.

CLOSTRIDIUM DIFFICILE STRAIN TYPING

There are several methods for typing *C. difficile* (Collins, Elliott, and Riley 2015, Rupnik 2010). The most active polymerase chain reaction (PCR) ribotyping program is in the United Kingdom available on request via the *C. difficile* Ribotyping Network (CDRN) (<https://www.gov.uk/guidance/clostridium-difficile-ribotyping-network-cdrn-guide-to-services>).

The variant *C. difficile* strains differ from the notoriously virulent reference strain (VPI 10463) in restriction sites and length of toxin producing genes *tcdA* and *tcdB*, and other pathogenicity locus (PaLoc) regions (Merrigan et al. 2010). These variant strains have been identified and can be defined by a series of overlapping PCRs spanning the PaLoc, allowing strains to be assigned to different toxinotypes I-XXXI (Rupnik 2010). PCR ribotyping is the most frequently (n=49, 89%) performed method to type isolates at the laboratories. Other methods include Restriction Enzyme Analysis (REA), Arbitrarily Primed PCR (AP-PCR), Pulsed-Field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism, Toxinotyping (PCR-RFLP), *SlpA* PCR-RFLP (S-layer precursor protein), Multi-Locus Sequence Typing (MLST, 7-12 genes), Multi-Locus Variable number of tandem repeat Analysis (MLVA), Tandem repeat sequence analysis. The *Clostridium* ribotype doesn't predict severity of the disease. The molecular typing methods are described in Table II.

PFGE types represent different ribotypes on PCR assay, according to an analysis that is performed on a random sample of the most prevalent NAP (North American PFGE) types: NAP1, 027; NAP4, 020; NAP6, 002; NAP7, 078; and NAP11, 106. The characteristics of NAP1 are positive for toxins A and B and *C. difficile* binary toxin with an 18-bp deletion in *tcdC*) but does not meet the 80% cutoff for relatedness on PFGE. Characteristics of NAP7 are positive for toxins A and B and *C. difficile* binary toxin with a 39-bp deletion in *tcdC*) but does not meet the 80% cutoff for relatedness. The strains in the unnamed category include 80 PFGE types that do not fall within NAP1 through NAP12.

Amongst these the virulence pattern of *C. difficile* ribotype BI/NAP1/027 is high because it is unable to down regulate toxin production, with consequent high levels of toxin synthesis. Additionally *in vitro* studies with ribotype BI/NAP1/027 strains have demonstrated 16- and 23-fold higher levels of TcdA and TcdB production, respectively as well as greater numbers of spores compared to other ribotypes. This increased toxin and spore production capability enables ribotype BI/NAP1/027 to compete and become the dominant strain within any environment to which it is introduced (Warny et al. 2005). A range of disease severity was found in mouse models infected with *C. difficile* clinical isolates (Lewis, Carter, and Pamer 2016). The most virulent strains were members of clade 2 (MLST1/ribotype 027) and clade 5 (MLST11, also classified as ribotype 078). These strains approached and/or surpassed the disease score of their reference strain, VPI10463 (Merrigan et al. 2010). Members of clade 4 (1 isolate) and clade 1 (16 isolates) had heterogeneous disease scores but consistently produced lower morbidity than VPI10463.

CLOSTRIDIUM DIFFICILE TOXINS

The 19.6 Kb chromosomal pathogenicity locus (PaLoc) of *C. difficile* not only carries the

toxin producing *tcdA* and *tcdB* genes (Hammond and Johnson 1995, Hundsberger et al. 1997) but also carries the *tcdR* gene encoding an alternative RNA polymerase sigma factor responsible for *tcdA* and *tcdB* expression (Mani and Dupuy 2001), the *tcdE* gene encoding a putative holing necessary for extracellular release of both toxins (Tan, Wee, and Song 2001) and the *tcdC* which negatively regulates TcdA and TcdB synthesis. These three genes regulate toxin expression and promote toxin release (Matamouros, England, and Dupuy 2007, Heinlen and Ballard 2010, Solomon 2013). The two protein exotoxins toxin A and toxin B are responsible for the symptoms of CDI. The severity of the diseases caused by *C. difficile* has been correlated to the levels of toxins that are produced during host infection. This observation has strengthened the idea that the regulation of toxin synthesis is an important part of *C. difficile* pathogenesis (Martin-Verstraete, Peltier, and Dupuy 2016). The disease severity is higher when infected by strains *C. difficile* BI/NAP1/027 which produce toxin A and toxin B in addition to the binary toxin (known as *C. difficile* transferase (CDT)). In addition *C. difficile* 027 ribotype has been associated with higher rates of recurrence at some institutions (Loo et al. 2005). The CDT toxin consists of two polypeptides: a binding component, CDTb, responsible for attachment of the toxin complex to the host cell surface and the active component, CDTa that displays an actin-specific ADP-ribosyltransferase activity (Papatheodorou et al. 2011). It has been shown that CDT depolymerizes the actin cytoskeleton and at low doses, enhances the adhesion of *C. difficile* cells to the gastrointestinal epithelium by inducing the formation of microtubule-based protrusions in the host cell membranes (Schwan et al. 2009). Therefore, CDT might potentialize the toxicity of TcdA and TcdB and lead to more severe disease, which would be consistent with the correlation between the presence of binary toxin and the severe outcomes of CDI leading to higher mortality rate (Gerding et al. 2014). Non-toxigenic strains do not produce toxin A or B (Heinlen and Ballard 2010). The creation of *C. difficile* strains with mutations in the genes encoding toxin A and B indicate that toxin B plays a major role in overall CDI pathogenesis (Di Bella et al. 2016).

TREATMENT OF CDI

During the last 15 years, CDI has become epidemic and continues to gain momentum. As the *C. difficile* epidemic continues to grow, the numbers of failed treatments and rates of relapses or recurrences also are increasing. Metronidazole and vancomycin are the first-line agents for *C. difficile* treatment; however, recent data suggest that metronidazole is losing its efficacy, and expert opinion is shifting toward the use of vancomycin as first-line therapy (Zar et al. 2007).

In 2011, the first drug from a new class of antibacterial, fidaxomicin (FDX), was approved by the U.S. Food and Drug Administration (FDA) for the treatment of *C. difficile* associated

diarrhea in adults ≥ 18 years of age (Optimer Pharmaceuticals, Difucid Package Insert, San Diego, CA, 2011 [www.accessdata.fda.gov/drugsatfda_docs/label/2011/201699s000lbl.pdf]). FDX inhibits the growth of *C. difficile*, and other susceptible organisms, by inhibiting the clinically validated target bacterial RNA polymerase (Coronelli et al. 1975). The mechanism of inhibition and the predicted binding site for FDX on RNA polymerase (RNAP) is distinct from that of the rifamycins (Srivastava et al. 2011). Fidaxomicin had a lower rate of recurrences compared with vancomycin in 2 studies, but its role in the therapy of RCDI has not been established. The only currently available immunologic approach to treat CDI is administration of pooled intravenous immunoglobulin (IVIG). However, clinical trials assessing the efficacy of IVIG have been less than ideal in treating RCDI (O'Horo and Safdar 2009). In another study no statistical significance in mortality rate was found between IVIG versus non IVIG group treated for severe CDI (Shahani and Koirala 2015).

The FDA also regulates the FMT process. The FDA issued guidance in 2013 to exercise enforcement discretion regarding the investigational new drug (IND) requirements for using of FMT to treat *C. difficile* infection not responding to standard therapies (<https://www.advancingbio.org/Portals/0/Documents/FDA%20Guidelines%202013.pdf>). In the United States there are two not-for-profit stool banks - OpenBiome, in Medford, Massachusetts, and AdvancingBio, in Mather, California. OpenBiome was founded in 2012 and is the older of the two, serving some 500 hospitals with ready-to-use fecal transplants. AdvancingBio, which opened in February 2015, has about 15 hospital customers. OpenBiome already has submitted a drug master file to the FDA whereas AdvancingBio proposes to submit a drug master file to the FDA. This document describes how a company manufactures, processes, packages, and stores its products.

FMT PROCEDURE AND FOLLOW-UP STUDIES

The current protocol for FMT involves fairly intense screening followed by simple techniques (Bakken et al. 2011, Kelly et al. 2015). The FMT recipient ceases antibiotics 2-3 days prior to FMT and an FMT donor is selected. A bowel preparation is administered to all patients on the day before FMT regardless of route. A donor not only completes a questionnaire but is also screened for different pathogens. The screening of bacterial pathogens includes *C. difficile*, *Listeria monocytogenes*, *Vibrio cholera*, *Helicobacter pylori*, *Treponema pallidum*, the parasites include *Giardia* and *Cryptosporidium* and viruses include rotavirus, hepatitis A/B/C, Creutzfeldt-Jakob, and human immunodeficiency virus (Paramsothy et al. 2015, Vestal 2016). Donors can be excluded if they have had recent antibiotics or tattoos, or a history of gastrointestinal disease. Although donor stool is used within 8 hours of passage, frozen stool samples have been

administered 1-8 weeks after passage with similar success rates (Costello et al. 2015, Hamilton et al. 2012). Stool is then collected and prepared for transplant (e.g., diluted and homogenized before filtration through gauze pads to remove large particulate matter). A recent study has demonstrated that frozen/thawed stool works as well as fresh stool (Costello et al. 2015, Hamilton et al. 2012). The routes for FMT until 1989 was retention enema (Bakken et al. 2011), however, alternative methods have been used subsequently, including nasogastric tube in 1991 (Aas, Gessert, and Bakken 2003), colonoscopy (2000), (Persky and Brandt 2000) and self-administered enemas (Silverman, Davis, and Pillai 2010).

In one study subjects with relapsing CDI were treated by FMT using frozen encapsulated inoculum from unrelated donors and had a positive outcome (Youngster et al. 2014). In this study twenty patients were enrolled with at least 3 episodes of mild to moderate CDI and failure of a 6- to 8-week taper with vancomycin or at least 2 episodes of severe CDI requiring hospitalization (Youngster et al. 2014). In another study on two subjects, a mixture of 33 bacteria was effective in two patients with CDI (Petrof et al. 2013). In a study in Denmark, rectal bacteriotherapy with a mixture of 12 bacteria resolved CDI in 64% of 55 subjects in 30 days suggesting that rectal bacteriotherapy is a viable alternative to FMT in patients with relapsing *C. difficile*-associated diarrhea (Tvede, Tinggaard, and Helms 2015).

Long term follow-up of subjects who received FMT suggest that both bacterial and viral genomes need to be sequenced. Bacterial and viral microbiota in the feces at various time in a long term follow-up for 4.5 years in a patient who recovered CDI post FMT was done and compared with the stool donor (Broecker et al. 2016). DNA sequencing to characterize bacteria and double-stranded DNA (dsDNA) viruses including phages were carried out in the feces. Until 7 months post-FMT the patient's microbial communities showed little overall similarity to the donor, but after 4.5 years, the patient's bacteria attained donor-like compositions at phylum, class, and order levels with similar bacterial diversity. Unexpectedly they also identified viruses such as Caudovirales phages and sequences related to giant algae-infecting *Chlorella* viruses suggesting that virome analysis should be included in gut microbiota studies (Broecker et al. 2016). In another study three pediatric ulcerative colitis patients received FMT from a single healthy human donor and transfer of multiple viral lineages between human individuals through FMT was identified after a course of 22 to 30 FMT treatments (Chehoud et al. 2016).

In an unblinded randomized control trial of FMT on 43 patients, because of widely different response rates in the control and FMT arms the study was stopped early (van Nood et al. 2013). The study compared FMT administered via nasoduodenal tube to oral vancomycin for 14 days or vancomycin administered for 14 days plus gastrointestinal lavage in patients with 1–9 prior CDI recurrences. The FMT group received 4 days of vancomycin followed by bowel lavage

before nasoduodenal FMT. 13 of 16 (81%) patients in the FMT arm sustained resolution of diarrhea after the first fecal infusion compared with 4 of 13 (31%) patients who were treated with vancomycin and 3 of 13 (23%) who were treated with vancomycin plus bowel lavage ($P = 0.008$ and 0.003 , respectively). Repeat FMT in patients who failed their first FMT resulted in success in 2 of 3 patients, raising FMT response rate to 94% (van Nood et al. 2013).

In another trial the authors compared vancomycin alone with vancomycin+FMT to treat RCDI (Cammarota et al. 2015). In the FMT+vancomycin group, eighteen of the twenty patients (90%) exhibited resolution of *C. difficile*-associated diarrhea. In FMT group, five of the seven patients with pseudomembranous colitis reported a resolution of diarrhea, whereas in the vancomycin group resolution of CDI occurred in 5 of the 19 (26%) patients ($P < 0.0001$). Future studies should be targeted on a larger population size with diverse disease conditions and longer term follow-up time to evaluate the safety and efficacy of FMT.

BIOCHEMISTRY OF GUT MICROBIOTA

Before *C. difficile* can colonize a susceptible host, its highly resistant, metabolically dormant spore form must germinate in response to specific bile salts in the gastrointestinal tract (Kevorkian, Shirley, and Shen 2016, Olguin-Araneda et al. 2015, Lewis, Carter, and Pamer 2016). Spore germination begins when germinant receptors bind specific germination-inducing small molecules known as germinants (Koenigsnecht et al. 2015). The relative concentrations of bile acids are especially important in *C. difficile* infections. The primary bile acid taurocholic acid induces germination of metabolically latent spores (Sorg and Sonenshein 2008), while secondary bile acids such as lithocholate serve as potent inhibitors for spore germination (Francis, Allen, and Sorg 2013, Theriot et al. 2014, Lewis, Carter, and Pamer 2016). Francis et al identified the germination-specific protease, CspC, as the *C. difficile* bile acid germinant receptor and showed that bile acid-mediated germination is important for establishing *C. difficile* disease in a hamster model of infection (Francis et al. 2013). Only a select group of bacteria produce the enzymes necessary to dehydroxylate primary bile acids, and they are sensitive to killing by many commonly prescribed antibiotics (Ridlon, Kang, and Hylemon 2006, Weingarden et al. 2016).

Pseudoproteases play a critical role in regulating the signaling pathway during *C. difficile* spore germination (Kevorkian, Shirley, and Shen 2016). Glycine can also act as a germinant through an uncharacterized mechanism (Sorg and Sonenshein 2008). Interestingly, the gut microbiota of a healthy gut possesses 7 α -dehydroxylation activity which allows them to convert primary bile acids to secondary bile acids (Stellwag and Hylemon 1978, Sorg and Sonenshein 2008, Ridlon, Kang, and Hylemon 2006). This correlates with high levels of primary bile acids and low levels of secondary bile acids observed in rCDI patients pre-FMT with levels post-FMT

resembling that from a healthy gut (Weingarden et al. 2014).

Weingarden *et al*, 2016 analyzed spore germination of 10 clinical *C. difficile* isolates exposed to combinations of bile acids present in patient feces before and after FMT (Weingarden et al. 2016). They found that the concentration of bile acids found in patients' feces prior to FMT induced germination of *C. difficile*; however, bile acids at concentrations found in patients after FMT did not induce germination and inhibited vegetative growth of all *C. difficile* strains. In addition upon sequencing they found a correspondence of variation in germination responses across isolates with mutations in CspC the germinant receptor of *C. difficile*. Their results suggest the idea that intra-colonic bile acids play a key mechanistic role in the success of FMT. Future studies should aim at novel therapeutic alternatives for treatment of R-CDI by targeting manipulation of bile acid composition in the colon (Weingarden et al. 2016, Smits et al. 2016).

It was observed in mouse models that antibiotic administration not only caused dysbiosis but also increased free mucosal sialic acid (a carbohydrate energy source for *C. difficile*) which inadvertently caused an expansion of *C. difficile* in the gut (Ng et al. 2013). These results came from experiments involving colonization of gnotobiotic mice with a sialidase-deficient mutant of *Bacteroides thetaiotaomicron*, a model gut symbiont, that reduced free sialic acid levels resulting in *C. difficile* down regulating its sialic acid catabolic pathway and exhibiting impaired expansion (Ng et al. 2013).

GENOMICS OF CDI

The efforts of the The Human Microbiome Project and the European based MetaHit project followed with a large scale multicenter effort have comprehensively characterized the human microorganisms found on and in our bodies to determine their various roles (Human Microbiome Project 2012, Human Microbiome Jumpstart Reference Strains et al. 2010). The complete genome of the *Clostridium difficile* type strain DSM 1296^T was sequenced using a combination of single-molecule real-time (SMRT) and Illumina sequencing technology (Riedel et al. 2015). It revealed the presence of one chromosome and two extrachromosomal elements, the bacteriophage phiCDIF1296T and a putative plasmid-like structure harboring genes of another bacteriophage (Riedel et al. 2015). The chromosome of *C. difficile* DSM 1296^T has a size of 4,109,692 bp and contains 3,596 predicted coding sequences, 35 rRNAs, and 90 tRNAs. In addition to the genes in the paLoc locus, a Wood-Ljungdahl pathway cluster, genes coding for carbon monoxide dehydrogenase, a ferredoxin:NAD⁻ oxidoreductase (RNF) complex, formate dehydrogenases, and hydrogenases; mobile elements such as transposons and prophages were also identified (Riedel et al. 2015).

16S rRNA-encoding gene sequence analysis was used to compare the fecal microbiota

of patients with RCDI and non-RCDI (Seekatz et al. 2016). Distinct differences in microbiota diversity of patients that did or did not develop RCDI was identified by 16s rRNA sequencing (Seekatz et al. 2016). Their results implied that patients with a more dynamic fecal microbiota were less likely to develop recurrence (Seekatz et al. 2016). Using shotgun metagenomics data Li et al, (2016) quantified and described the extent of changes to population structure of gut microbiome after FMT at species and strain level (Li et al. 2016). They observed extensive coexistence of donor and recipient strains of the same species with considerable amount of strain replacement over a 3-month observational period. This raises the possibility of using well characterized and/or customized strains to modulate the microbiome for example outcompeting undesirable strains (de Vos 2013).

Metagenomic shotgun sequencing (MGS) revealed significant association between gut microbiome and various intrinsic, environmental, dietary and medication parameters and disease phenotype with a high replication rate between MGS and 16s rRNA gene sequencing data from same individuals (Zhernakova et al. 2016). The study showed a relationship between the microbiome and 126 exogenous and intrinsic host factors, including 31 intrinsic factors, 12 diseases, 19 drug groups, 4 smoking categories, and 60 dietary factors. The study associated 110 factors to 125 species and observed that fecal chromogranin (CgA), a protein secreted by enteroendocrine cells was exclusively associated with 61 microbial species whose abundance collectively accounted for 53% of microbial composition. Hence, CgA showed a high potential as a biomarker for gut health (Zhernakova et al. 2016).

In another study, the structure, function and diversity of the healthy human microbiome was analyzed from a total of 4,788 specimens from 242 screened and phenotyped adults (129 males, 113 females) representing the majority of the target Human Microbiome Project (HMP) cohort of 300 individuals (2012). Microbiome samples were collected from up to 18 body sites at one or two time points from the 242 individuals clinically screened for absence of disease. Samples were subjected to 16S rRNA gene pyrosequencing (454 Life Sciences), and a subset were shotgun sequenced for metagenomics using the Illumina GAIIx platform. The large sample size and consistent sampling of many sites from the same individuals allowed for an understanding of the relationships among microbes, and between the microbiome and clinical parameters which may ultimately be critical for understanding microbiome-based disorders.

Considerable progress has been made in recent years to describe the structure and function of the intestinal microbiota that belong to the major phyla of the *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* (Rajilic-Stojanovic, Smidt, and de Vos 2007). Significant attention has been given to culture-independent and high-throughput approaches that generated important baseline information on the intestinal

microbiota composition, the description of a reference metagenome of 3.3 Mb, and its structuring into clusters, termed enterotypes (Qin et al. 2010, Arumugam et al. 2011, Human Microbiome Project 2012). In a prospective study, changes in 16srRNA and bacterial and fungal microbiota sequencing from stool were able to distinguish *C. difficile* infection from other forms of diarrhea in 12 out of 24 enrolled patients who had CDI (Sangster et al. 2016). An increased numbers of *Akkermansia muciniphila* in CDI patients was observed which is known to degrade mucin thereby providing a selective advantage toward CDI. Of the fungal elements, *Penicillium* was predominant in CDI; these organisms produce antibacterial chemicals which may resist recovery of healthy microbiota. The most frequent CDI microbial community networks involved *Peptostreptococcaceae* and *Enterococcus*, with decreased population density of *Bacteroides* (Sangster et al. 2016).

A comparative genomic analysis of five Australian toxin-negative isolates of *C. difficile* that lack *tcdA*, *tcdB* and both binary toxin genes *cdtA* and *cdtB* that were recovered from humans and farm animals with symptoms of gastrointestinal disease was accomplished by 16s rRNA PCR, whole genome next generation sequencing (Roy Chowdhury et al. 2016). It revealed that five *C. difficile* isolates cluster closely with virulent toxigenic strains of *C. difficile* belonging to the same sequence type (ST) and have virulence gene profiles akin to those in toxigenic strains (Roy Chowdhury et al. 2016). They used the genome of the epidemic *C. difficile* CD630 strain in analysis with the test *C. difficile* genomes to identify genes that have been correlated with pathogenicity (Roy Chowdhury et al. 2016).

ANIMAL MODELS

Animal models provide a good alternative to study the host factors, microbiome and colonization during FMT. Although the microbiota composition at the phylum level generally appeared to be similar between humans and other animals, at the species and strain level there is considerable divergence, likely due to underlying differences in host anatomy/physiology, and dietary regimes (Nguyen et al. 2015). However, recent work has shown that it may be possible to mitigate this issue somewhat as a significant proportion of human-associated bacterial species appear to be able to successfully colonize the intestines of animal models following FMT (Ellekilde et al. 2014). In another study it was found that not only the obese microbiome had an increased capacity to harvest energy from the diet, this trait was transmissible: colonization of germ-free mice with an 'obese microbiota' resulted in a significantly greater increase in total body fat than colonization with a 'lean microbiota' (Turnbaugh et al. 2006). The obese (ob/ob) phenotype had been shown to be transmissible and is adopted in germ-free mice, infused with intestinal microbiota from conventionally raised, genetically obese mice [58].

FMT has also been shown to influence behavior as observed from experiments where GF

BALB/c mice, a strain with known deficits in sociality and diligent risk assessment of the environment received stool from explorative NIH Swiss mice and vice versa (Jacome et al. 2011, Brinks et al. 2007). The BALB/c mouse recipients exhibited more explorative behavior in the weeks following FMT. On the other hand, when NIH Swiss mice were colonized with the BALB/c microbiota, they displayed greater hesitancy (Bercik et al. 2011).

The role of antibiotics as a risk factor for CDI was also explored in mice. Mice treated with antibiotics were more susceptible to *C. difficile* infections due to gut dysbiosis (Theriot et al. 2014). Antibiotic treated mice had substantial changes in the gut microbial community and metabolome which made them susceptible to CDI. They had a decrease in the levels of secondary bile acids such as glucose, free fatty acids and dipeptides, while the primary bile acids and sugar alcohols increased. *In vitro* and *ex vivo* analyses demonstrated that *C. difficile* can exploit specific metabolites that became more abundant in the mouse gut after antibiotics, including the primary bile acid taurocholate for germination, and carbon sources such as mannitol, fructose, sorbitol, raffinose and stachyose for growth (Theriot et al. 2014).

Bacterial consortia transplantation (BCT) for targeted restoration of the intestinal ecosystem is considered a relatively safe and simple procedure and has recently been found to have effects comparable to FMT in reestablishment of mucosal barrier function in mice with intestinal dysbiosis (Li et al. 2015). To establish the dysbiosis model, male BALB/c mice were treated with ceftriaxone intra-gastrically for 7 days. After that, FMT and BCT were performed on ceftriaxone-treated mice for 3 consecutive days to rebuild the intestinal ecosystem. The effects of BCT were comparable to that of FMT, especially in normalizing the intestinal levels of oligomeric mucus/gel-forming (Muc 2), secretory immunoglobulin A (SIgA), and defensins (Li et al. 2015). Muc2 is the major component of the mucus layer in the small and large intestines. An increase in intestinal mucus production in mice causes dysbiosis. This is one of the causes of antibiotic-associated diarrhea because hypersecretion of glycoproteins by the intestinal mucosa is observed during acute infection (Hasnain, Thornton, and Grecis 2011). Muc2 helps the disassociation of pathogenic and normal microbiota from the intestinal mucosa to prevent infectious colitis (Bergstrom et al. 2010). Muc2-deficient mice developed spontaneous colitis (Wenzel et al. 2014). Intestinal mucus provides a large matrix for a rich array of antimicrobial molecules such as SIgA and defensins. They are essential components of the innate immune system and contribute greatly to intestinal barrier function. SIgA is the most abundant immunoglobulin found in intestinal mucus (Corthesy and Spertini 1999).

Using mouse model of CDI, the interactions between *C. difficile* cells and other bacteria and with host mucosa during CDI was investigated (Semenyuk et al. 2015). The GI tracts of infected mice were sectioned at various days post infection and probed with 16S rRNA

fluorescent *in situ* hybridization (FISH) probes targeting most bacteria as well as *C. difficile* specifically. By using FISH and 16SrRNA gene sequence analysis they drew four major conclusions about CDI in the mouse: (i) during infection, *C. difficile* is found in communities in the cecum and colon, starting at day 1 post infection.; (ii) these communities are associated with the loose, outer layer of the mucus; (iii) *C. difficile* is a minority member of these communities; and (iv) the communities contain bacteria of several families of *Bacteroidetes* and *Firmicutes*.

Animal models have provided novel insights into the successes of FMT and how FMT can influence behavior, metabolism, microbiome and metabolome.

FUTURE DIRECTIONS

As each individual's microbiome is unique, blinded matches of donor FMT to recipient are likely to be met with limited success in treating many complex diseases; besides the host factors cannot be changed and extraneous factors cannot be controlled. Another relevant point to consider is the metabolome which includes both the host and the microbial derived metabolites (McHardy et al. 2013, Theriot et al. 2014). It is of significance to point that specific microbiota-mediated metabolite profiles can be associated with the predisposition to metabolic impairments, such as impaired glucose homeostasis and non-alcoholic fatty liver disease (NAFLD) (Dumas et al. 2006). It has also been postulated that the gut microbiota by means of the associated metabolome may influence the host's long-term physiology via modulating its epigenome (Mischke and Plosch 2013). There is evidence that susceptibility to CDI following antibiotic administration is associated with distinct shifts in gastrointestinal microbiome and metabolome (Theriot et al. 2014). Future research should be focused on these factors influencing the microbiota and the metabolome and how these influence CDI and FMT.

A drawback maybe that PaLoc can be horizontally transferred to non-pathogenic strains characterized by the lack of *tcdA* and *tcdB*, converting them in pathogenic strains producer (Braun et al. 1996, Brouwer et al. 2013). Although PaLoc possesses some characteristics of a mobile genetic element, it does not appear to be intrinsically mobile and is located at the same site in all toxigenic *C. difficile* strains (Braun et al. 1996). Genetic engineering *C. difficile* to generate *TcdA*^{-/-} and *TcdB*^{-/-} mutant strains or strains overexpressing *TcdC* being a negative regulator of *TcdA* and *TcdB* should be considered (Heinlen and Ballard 2010). Hence long term effects of FMT are unknown. Some of the other drawbacks of FMT are cases of peripheral neuropathy, microscopic colitis, contact dermatitis, Sjögren's disease, idiopathic thrombocytopenic purpura, rheumatoid arthritis, weight gain, bacteremia, and ulcerative colitis flare (Brandt et al. 2012, De Leon, Watson, and Kelly 2013, Quera et al. 2014, Alang and Kelly 2015). The most frequent adverse events caused by FMT are fever, abdominal pain, diarrhea, increase of C reactive protein which are transient and self-limiting, even if long-term

immunological or infectious effects have not yet been evaluated due to short-term follow-up of patients. FMT is somewhat less effective in clearing RCDI from patients with Inflammatory Bowel Disease (IBD), compared with patients without IBD, based on an analysis of 272 patients, regardless of immunosuppressive therapy (Khoruts et al. 2016).

Fischer et al., 2016 found that severe and severe-complicated indications, inpatient status during FMT, and the number of previous CDI-related hospitalizations were strongly associated with early failure of a single FMT for CDI on the basis of a multivariable logistic regression model (Fischer et al. 2016). In the univariate analysis some of the variables associated with early FMT failure were the use of non-CDI antibiotics within 8 weeks of FMT, a history of CDI-related hospitalization, a number of CDI-related hospitalizations, severe or severe-complicated CDI, pseudomembranous colitis, serum albumin concentration, and inpatient FMT (Fischer et al. 2016).

In another study effectiveness of FMT was evaluated on gastroenterological diseases based on 45 studies; 34 on CDI, 7 on IBD, 1 on -metabolic syndrome, 1 on constipation, 1 on pouchitis and 1 on irritable bowel syndrome (IBS) (Rossen et al. 2015). The study found that FMT is highly effective in CDI, and holds promise in ulcerative colitis. As for Crohn's Disease, chronic constipation, pouchitis and IBS data was too limited to draw conclusions. In CDI, 90% resolution of diarrhea in 33 case series ($n = 867$) was found, and 94% resolution of diarrhea after repeated FMT in a randomized controlled trial (RCT) ($n = 16$). In ulcerative colitis remission rates of 0% to 68% were found ($n = 106$). In Crohn's disease (CD) ($n = 6$), no benefit was observed. In IBS, 70% improvement of symptoms was found ($n = 13$). Reversal of symptoms was observed in 100% of constipation ($n = 3$) cases. In pouchitis, none of the patients ($n = 8$) achieved remission. One RCT showed significant improvement of insulin sensitivity in metabolic syndrome ($n = 10$) (Rossen et al. 2015).

FMT can also be used to decolonize the gut from multi drug-resistant (MDR) bacterial infections (Manges, Steiner, and Wright 2016). Observations from eight case reports illustrated the potential effectiveness and safety of FMT for MDR bacterial decolonization. FMT therapy involved the replacement of a patient's existing dysfunctional microbiota, containing MDR opportunistic pathogens, with a healthy microbiota, characterized by high levels of beneficial microorganisms, exhibiting lower levels of bacterial drug resistance.

A link between antibiotic exposure and altered brain function is well evidenced by the psychiatric side-effects of antibiotics, which range from anxiety and panic to major depression, psychosis and delirium (Sternbach and State 1997). The gut microbiome has been shown to influence mental illnesses (Rogers et al. 2016). The delicate balance between the human microbiome and the development of psychopathologies is significant given the ease with which

the microbiome can be altered by external factors, such as diet (Gohir et al. 2015), exposure to antimicrobials (Russell et al. 2012, Ma et al. 2014), or disrupted sleep patterns (Thaiss et al. 2014). Future studies should be targeted to i) identify links between dysbiosis and other human diseases, ii) develop sensitive diagnostic assays for detecting *C. difficile* in body fluids and iii) identify biomarkers which can detect susceptibility prior to CDI.

Manges *et al* proposed that ideally, whole stool FMT could be replaced by a clearly defined and regulated complex mixture of functional micro-biota organisms (defined microbiota transplant) (Manges, Steiner, and Wright 2016). Future work may involve use of FMT for not only curing CDI but a whole array of human diseases based on the discovery of the relationship between the gut-brain axis (Carabotti et al. 2015) and brain-gut-bone marrow axis (Santisteban et al. 2016) and others.

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Table I. Selected agents for the treatment and prevention of CDI in clinical trials(Wiep Klaas Smits 2016).

Agent (manufacturer)	Indication	Notes	Clinical trial identifier
Phase III			
Actoxumab and bezlotoxumab alone or in combination (Merck)	Prevention of recurrent CDI	Antitoxin A (MK-3415) and antitoxin B (MK-6072) monoclonal antibodies given intravenously as adjuncts to standard treatment	NCT01241552, NCT01513239
Surotomycin (Merck)	Treatment	Cyclic lipopeptide antibiotic related to daptomycin but administered orally	NCT01598311, NCT01597505
Cadazolid (Actelion)	Treatment	Hybrid antibiotic molecule, consisting of fluoroquinolone and oxazolidinone moieties, for oral administration	NCT01987895, NCT01983683
Cdiffense (Sanofi Pasteur)	Prevention	Vaccine containing toxoids of TcdA and TcdB from <i>C. difficile</i>	NCT01887912
Phase II			
IC84 vaccine (Valneva)	Prevention	Vaccine consisting of recombinant protein of the two truncated toxins TcdA and TcdB from <i>C. difficile</i>	NCT02316470
LFF571 (Novartis)	Treatment of moderate CDI	Semi-synthetic thiopeptide	NCT01232595

SER-109 (Seres Therapeutics)	Treatment of recurrent CDI	Oral microbiome therapeutic (mixture of bacterial spores) granted orphan drug designation by the FDA	NCT02437500
SMT19969 (Summit Pharmaceuticals)	Treatment	Oral non-absorbable antibiotic with a narrow spectrum of activity and high selectivity for <i>C. difficile</i>	NCT02092935
<i>C. difficile</i> vaccine (Pfizer)	Prevention	Bivalent toxin vaccine	NCT02561195, NCT02117570
SYN-004 (Synthetic Biologics)	Prevention	Class A β -lactamase designed to protect gut microbiota from the action of systemically administered β -lactam antibiotics that might otherwise predispose for CDI	NCT02563106
VP20621 (Shire)	Prevention of recurrent CDI	Orally administered non-toxigenic <i>C. difficile</i>	NCT01259726

Phase I

PolyCAb (Micropharm)	Treatment of severe CDI	Polyclonal antibodies against <i>C. difficile</i> given intravenously	Not available
CRS3123/REP3123 (National Institute of Allergy and Infectious Diseases)	Treatment	Methionyl-tRNA synthetase inhibitor oral antibiotic	NCT02106338, NCT01551004

CDI, *Clostridium difficile* infection; Tcd, *C. difficile* toxin.

Table II. Summary table of *C. difficile* typing methods applied currently (Huber et al. 2013).

Typing method	Technique applied	Benefit(s)	Challenge(s)
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Restriction endonuclease analysis (REA)	Whole-genome restriction and detection by agarose gel electrophoresis	High discriminatory power and stability	Method is technically demanding; data are difficult to exchange between laboratories
Pulsed-field gel electrophoresis (PFGE)	Whole-genome restriction and detection by pulsed-field gel electrophoresis	High discriminatory power	The method is laborious; results cannot easily be compared between laboratories
Multilocus sequence typing (MLST)	PCR amplification and sequencing of parts of housekeeping genes	Stability and transferability of data	Costs are high
Repetitive-element PCR typing (Diversilab typing)	Repetitive-element PCR system	High discriminatory power	Interlaboratory reproducibility needs to be assessed
Toxinotyping	PCR amplification followed by restriction enzyme digestion of 10 regions of the pathogenicity locus	Highly reproducible while giving a clear view of the toxin status, excluding binary toxin	Less resolution power than other typing methods
PCR ribotyping	PCR amplification of the 16S - 23S intergenic spacer region		
Agarose-based PCR ribotyping	PCR amplification followed by agarose gel electrophoresis	A globally used reference library has been established	Data are not easily interchangeable between laboratories
Sequencer-based PCR ribotyping	PCR amplification followed by sequencer-based capillary separation	Highly accurate, reproducible, and interchangeable digital data are obtained	No standardization of ribotype nomenclature
Qiaxcel-based PCR ribotyping	PCR amplification followed by Qiaxcel-based capillary separation	Accurate and reproducible digital data are obtained at low cost	Not widely used in laboratories; normalization across capillaries may be inaccurate
Multilocus variable-number tandem-repeat analysis	Multiple singleplex PCR amplifications followed by sequencer-based capillary separation	Digital data with high resolution power and the possibility to determine phylogenetic relationships are obtained	Relatively costly and labor-intensive compared with PCR ribotyping
Surface-layer protein A-encoding gene (<i>slpA</i>) typing	PCR amplification of the variable region of the <i>slpA</i> gene, followed by DNA sequencing	Relevance to vaccine development	Method is not widely used
Whole-	Whole-genome	Accurate and reproducible	Informatics expertise

genome sequencing sequencing by methods such as Sanger, Roche 454, and Illumina sequencing digital data with very high discriminatory power are necessary are obtained

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