Exploring the role of the microbiota: in defence against Clostridioides difficile and multidrug resistant Gram-negatives

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Chapter 9

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Chapter 9. Discussion

Summary of main findings

Part I: New insights in the epidemiology of *Clostridioides difficile* and multidrug resistant Gram negatives.

Infections with *C. difficile* have long been considered as a nosocomial acquired diarrheal disease, transmitted primarily from symptomatic patients. However, transmission of *C. difficile* spores to the hospital environment, health care workers and other patients is not only accommodated by infected patients, also asymptomatically colonized individuals shed these microorganisms [1]. Recognition of asymptomatically colonized patients is essential in reducing nosocomial transmission. In **Chapter 2** the performance of several diagnostic *C. difficile* tests in comparison to toxigenic culture as gold standard was evaluated in asymptomatically colonized patients at admission to three large hospitals in the Netherlands. In this study, 5.1% of the patients attending a tertiary-care hospital were positive with *C. difficile*, and 3.1% contained toxigenic *C. difficile*. In a setting of low endemicity of asymptomatically colonized individuals, all three assays (an enzyme-linked fluorescent assay (ELFA) targeting glutamate dehydrogenase, and two molecular tests targeting the toxin(s) of *C. difficile*; a commercial *artus* PCR which targets *TcdA* and *TcdB*, and an in-house PCR only targeting *TcdB*) can be applied as a first screening test to detect the presence of (toxin producing) *C. difficile*, as they display a very high negative predictive value. Similar to the diagnosis of patients with symptomatic *C. difficile* infection (CDI), the positive predictive values of the tests in a low endemicity setting were suboptimal. Discrepancy analysis demonstrated that the majority of the small number of false positive results could not be confirmed upon retesting. This illustrates that in a low prevalence setting a positive GDH or PCR result is not automatically based on an increased sensitivity of these assays as compared to the toxigenic culture as gold standard.

Nosocomial transmission by asymptomatic carriers is also recognized for MDRO. Prevention of this transmission in hospitals is pursued by screening selected patient groups for carriership of MDRO. The approach varies per country and per micro-

organism, as illustrated by the variety of national guidelines. In 2015, a novel plasmid mediated resistance mechanism of colistin, *mcr-1* was discovered in animals and humans in China [2]. Currently, ten *mcr* genes types (*mcr-1* to *mcr-*10) have been detected in Enterobacterales isolates [3]. Epidemiological data on the prevalence of faecal carriage of *mcr-1* in healthy individuals were not available shortly after the discovery of this novel resistance plasmid. To assess the risk of *mcr* introduction from asymptomatic carriers into our academic tertiary care hospital, the prevalence of *mcr* in faecal samples obtained from patients attending our hospital was investigated in **Chapter 3.** Two of the 576 (0.35 %) patients tested positive for *mcr-1*, whereas no *mcr-2* was found. This suggests that *mcr* spread from the community into the hospital environment was low in the Netherlands but could not be excluded. The finding of a phenotypically colistin susceptible, *mcr-1* plasmid containing *E. coli* underlined the importance of phenotypical confirmation after molecular screening.

In contrast to healthy young individuals in the community, nursing home residents have multiple risk factors for colonization and infection with *C. difficile* and MDRO [4-10], and are considered a reservoir for transmission [11-14]. Frequent contact between residents due to communal living, increased frequency of healthcare contact and presence of factors that facilitate MDRO spread such as incontinence provide additional opportunities for transmission. The data presented in **Chapter 4** show that a high abundance of *C. difficile* and MDRO risk factors was present in Irish and Dutch nursing home residents. Surprisingly, this did not result in a high prevalence of MDRO's; 9 % and 11% Extended Spectrum Beta Lactamase (ESBL)-producing *E. coli* in Ireland and the Netherlands respectively, and 0 % carbapenemase producing Enterobacterales (CPE), vancomycin resistant Enterococci (VRE) or *C. difficile* in both countries. Using core-genome multi locus sequence typing (cgMLST) small-scale spread of MDROs between residents of the same ward in the Netherlands was demonstrated. However, cross-transmission of MDRO's between three different wards in Ireland was observed by whole genome sequencing. The differences between Ireland and The Netherlands may reflect differences in nursing home infrastructure, specifically communal areas and multi-bedded resident rooms in the Irish nursing home, which were not present in the Netherlands.

In conclusion, though asymptomatic colonization of MDRO and *C. difficile* can become a nidus for nosocomial transmission, its prevalence is still low in the Netherlands.

Part II: The initiation of the Netherlands Donor Feces Bank to facilitate quality assured faecal microbiota transplantation

Faecal microbiota transplantation (FMT) has become an established treatment for rCDI and is extensively studied as new treatment option for many other indications. As result, stool banks focussing on standardisation, safety, quality assurance and cost effectivity become increasingly important [15-17]. Stool banks provide ready-to-use faecal suspensions to hospitals for treatment of patients. **Chapter 5** describes the establishment of the Netherlands Donor Feces Bank (NDFB). It addresses the difficulties encountered with donor recruitment and screening, preparation of the faecal suspension and transfer of the faecal microbiota suspension. It also provides treatment data and follow-up of patients treated with donor faeces of the NDFB. In comparison with the experiences of others stool banks, the NDFB has a high cure rate of rCDI at two months of nearly 90 % and a sustained cure rate of over 70 % after a mean follow-up of 42 weeks (**Chapter 6**). This high success rate is most likely achieved by the efforts of our multidisciplinary FMT-expert panel. This expert panel discusses the indication for FMT for patients for whom an FMT is requested and provides advice during treatment and follow-up of the patients. This strategy results in efficacious, appropriate and safe use of FMT for treatment of rCDI.

Healthy stool donors colonized with *Blastocystis* sp. are usually excluded from FMT donorship [21-26], resulting in considerable exclusion of donors (30-50 %). It is questionable whether this is justified as the entero-pathogenicity of *Blastocystis* sp. is debatable. The presumed pathogenicity is based on anecdotal case reports and retrospective reviews and a human challenge model has not been applied [7]. Recent literature shows a lower prevalence of intestinal carriage of *Blastocystis* sp. in patients with disorders associated with a reduced diversity of the gut microbiota, such as inflammatory bowel disease or hepatic encephalopathy [18-21]. Metagenomic studies reveal furthermore that *Blastocystis* sp. correlates with a more diverse and healthier microbiota [18, 22-27]. Through a combination of PCR and subtyping techniques of faecal samples of donors and patients (pre-FMT and post-FMT), the first human to human transmission by FMT of *Blastocystis* sp. ST1 and ST3 is described in **Chapter 7**. This transmission did not influence the success rate of the FMT to treat rCDI. More importantly, it did not result in gastrointestinal symptomatology of the recipients. This

study is an important step towards a possible exempt of *Blastocystis* sp. (ST1 and ST3) as donor exclusion criterion in FMT.

In **Chapter 8** the potential of FMT for eradication of MDRO was explored in a patient suffering of recurrent urinary tract infections with a carbapenemase producing *Pseudomonas aeruginosa* hampering planned kidney-pancreas transplantation. Antibiotic pre-treatment subsequently followed by FMT prevented recurrence of a urinary tract infection with this Verona integron-encoded metallo-β-lactamase (VIM) positive *P. aeruginosa* by eradication of intestinal colonization. Although the treatment was a clinical success, a partial microbiological failure was observed as intestinal colonization with an ESBL-producing *Escherichia coli* persisted. In contrast to the diminished microbiota of rCDI patients, microbiota analysis showed an intact microbiota diversity and composition at phylum level before FMT. This suggests co-colonization rather than replacement of indigenous strains and eradication of this MDRO *E. coli* requires perhaps other microbiota interventions.

With the increasing number of reports pointing towards potential beneficial effects of FMT in patients with a variety of gastrointestinal and extra-intestinal disorders, a growing demand of FMT can be expected in the near future. Initially, experimental studies will have to be performed in a controlled setting, both *in-vitro* and *in-vivo*. Once proven effective, a standardised screening and manufacturing procedure, quality control and careful and long-term monitoring of outcomes and adverse events requires stool banks and registries. The experience of this thesis and the NDFB may help the establishment, utilization, standardization and maturation of stool banks throughout Europe, and FMT as therapy.

General discussion

Targeting the pathogen alone is often not sufficient for diagnosis and treatment of CDI

Diagnosis of CDI

To prevent inappropriate use of FMT and increase the clinical benefit and cost-effectiveness, evaluation of FMT requests by a multidisciplinary team is extremely important. The NDFB FMT-expert team rejects a quarter of FMT requests, which is in the majority of cases (30/47, 64%) because the diarrhoea was attributed to another cause that coincided with *C. difficile* carriership (**Chapter 6**). In the past, *C. difficile* was difficult to isolate and cultivate from other anaerobic and facultative members of the gut microbiota. Currently, many molecular tests are available to demonstrate the presence of *C. difficile* and cultivating is not routinely applied anymore. However, the new diagnostic challenge is to distinguish colonization from infection. Due to the possibility of asymptomatic colonization and diarrhoea due to another cause, presence of the bacterium in the faeces does not consequently indicate disease. ESCMID and the ECDC recommend a two-stage algorithm with a screening test with-high sensitivity (nucleic acid amplification test (NAAT) or glutamate dehydrogenase enzyme immune assay (GDH EIA)) followed by a specific assay for free toxin detection in the stool (toxin EIA) [28]. The presence of free toxins in faeces is considered as the best proof for active *C. difficile* disease [29, 30].

New developments in diagnosis of CDI

In order to simplify this diagnostic approach and minimize multiple testing, alternatives have been explored. The NAATs have gained much popularity in recent years because of their ease of use and the ability for automation and standardization. We and others have found that the cycle quantification threshold (Cq) value can be a predictor of free toxin presence as measured with toxin EIA [31-34], clinical disease [35, 36] or poor outcome [33, 37, 38]. In addition, in a study reporting both PCR, as well as a predicted toxin result based on Cq-value, all patients with CDI related complications were predicted correctly. This strategy furthermore reduced the treatment of toxin-negative patients [39], and can augment a more timely diagnosis in the more severe CDI cases or clear carrier cases (for instance: Cq value < 25 = CDI,

Cq value > 33 = carrier). It is however, still not sufficiently specific to use PCR as stand-alone tests for CDI diagnosis, with a grey area of intermediate high Cq value of 25-33 in which clinical assessment, a toxin EIA or cell cytotoxicity neutralization assay (CCNA) is necessary. Attempts to enhance the sensitivity of tests able to detect free toxin in stool have been unsuccessful so far. An example is a next generation enzyme immune assay; the ultrasensitive single-molecule array (SIMOA) technology [40]. This ultrasensitive assay is capable of separately detecting and quantifying *C. difficile* toxins A and B down to picogram-per-millilitre levels. This more sensitive way of detecting free toxin in stool, increases the sensitivity to 85.2-100 % compared to CCNA [40-43]. This highly sensitive toxin assay may however suffer from the same specificity problem (with a reported specificity of 79.3 % [42]) as the highly sensitive molecular tests, which can lead to the erroneous identification of colonized individuals as diseased [44]. Although a decreased specificity was not reported in all studies [40, 41, 43, 45]. Pollock and colleagues showed that on individual patient level both PCR as SIMOA could not distinguish a patient with CDI from asymptomatic carriage [46]. Only on population level *C. difficile* toxin concentrations measured by SIMOA but not with NAAT, were significantly higher in CDI patients than in colonized individuals [46]. This is in agreement with a study showing that the rate of asymptomatic *C. difficile* carriage was similar to the symptomatic positivity rate [47]. These results suggest that part of the PCR and SIMOA positive samples in symptomatic patients are likely due to *C. difficile* colonization, and exclusive reliance on highly sensitive tests results in overdiagnosis, overtreatment and increased health care costs.

Personalized CDI diagnostics

A complicating factor in the discrimination between infection and carriership of *C. difficile* is that only the presence of free toxins in the faeces as measured with CCNA highly correlates with clinical *C. difficile* disease [29, 48]. The CCNA test however, is only performed in reference laboratories and is too cumbersome for routine diagnostics. In contrast, the routinely applied toxin EIAs lack sufficient sensitivity with a pooled sensitivity of 83 % [28]. In addition, due to frequent testing in a low prevalence setting, the high specificity of 98-99 % can result in a low positive predictive value (69-81% when the prevalence of CDI is 5 %) [28]. How can we then differentiate between colonization and infection? A personal view on personalised diagnostics is visualised in *Figure 1*. To enhance the discrimination, disease specific biomarkers can be included

in the test-algorithm, such as host inflammatory markers, stool metabolites and microbiota analysis in addition to multiplex testing for additional pathogens and virulence characteristics of *C. difficile* (e.g. RT027) [49]. The serum presence of the chemoattractant C-C motif ligand 5 (CCL5), which is expressed by many cells and actively recruits leucocytes to inflammatory sites, was associated with CDI as compared to patients with non-CDI diarrhoea [50]. Moreover, severe CDI patients had higher serum levels of TNF-α, procalcitonin, hepatocyte growth factor, IL-6 and/or IL-8 suggesting worse inflammation [50-54], and a serum based biomarker panel could inform about CDI diagnosis, treatment response and mortality [54]. Interestingly, CCL5, which marks acute intestinal inflammation and severe CDI, seemed also to be significantly associated with an increased survival [51]. Assessment of local host responses, with faeces as proxy, may be a more sensitive disease indicator than the systemic response. Faecal levels of biomarker as TNF-α, CXCL-5 messenger (m)RNA, IL-8 mRNA and IL-8 protein, lactoferrin, calprotectin and procalcitonin at initial presentation correlated with disease severity or persistent diarrhoea [49, 55-59], and were more sensitive than clinical severity scores or organism burden in identifying patients at risk for treatment failure [60].

Diagnostics in relation to FMT indication

Measurement of the humoral immunity against *C. difficile* can play a significant role in the detection of patients who will most likely benefit of immunity enhancing anti-*C. difficile* therapy with bezlotoxumab, since a higher risk of recurrence of CDI is associated with low serum concentrations of antibodies directed against the toxins TcdA and TcdB [61-63]. High serum endogenous IgG antibodies on day 1 against *C. difficile* toxin B but not toxin A, were associated with protection with rCDI after bezlotoxumab, although the effect was limited (25% versus 35% relapse) [64]. Furthermore, biomarkers can play a significant role in the evaluation of FMT-candidates, which has proven to be difficult especially in patients with inflammatory bowel disease (**Chapter 6**). The biomarker procalcitonin can assist in differentiating infection from colonization with *C. difficile* in ulcerative colitis (UC) patients, as serum procalcitonin was significantly elevated in UC patients responding to CDI treatment, in comparison to UC patients diagnosed with a UC flare in combination with *C. difficile* colonization [65]. Moreover, it could support the identification of patients potentially benefitting of repeat FMTs, as faecal calprotectin concentrations just prior FMT were higher in rCDI patients that needed multiple FMT treatments [66]. The composition of

the microbiota can also be used to predict the likelihood for CDI recurrence. Compared to non-recurrent CDI, patients with rCDI have a diminished bacterial diversity and species richness, and significant shifts of *Escherichia/Shigella* (Enterobacterales), *Veillonella,* Streptococci, *Parabacteroides* and *Lachnospiraceae* [67, 68]. Interestingly no particular taxon seemed to be associated with the severity of CDI, likely reflecting the dominant role of host-related factors [67]. Reconstitution of a healthy microbiota after FMT both defined by a high diversity or by an alteration in abundance of specific taxa or restoration of functions (e.g. bile acid conversion, short chain fatty acid production), showed to be an excellent predictor of clinical response after FMT [69, 70]. A prediction model based on 16S analysis of faeces at day seven post-FMT (with freeze dried capsules), which included the abundances of members of the families *Lachnospiraceae, Ruminococcaceae, Bacteroidaceae, Porphyromonadaceae* and *Enterobacteriaceae* showed an accuracy of 100 % and 97 % in predicting recurrences of respectively training and test data (n=89) [69]. In addition, a higher engraftment of donor strains (50 %) was observed in the responders [69]. Alternatively, bacterial fermentation products can be measured as read-out of a successful FMT, as a combination of urinary p-cresyl sulphate and the faecal concentrations of lithocholic acid seven days post-FMT could predict FMT success with high accuracy [71].

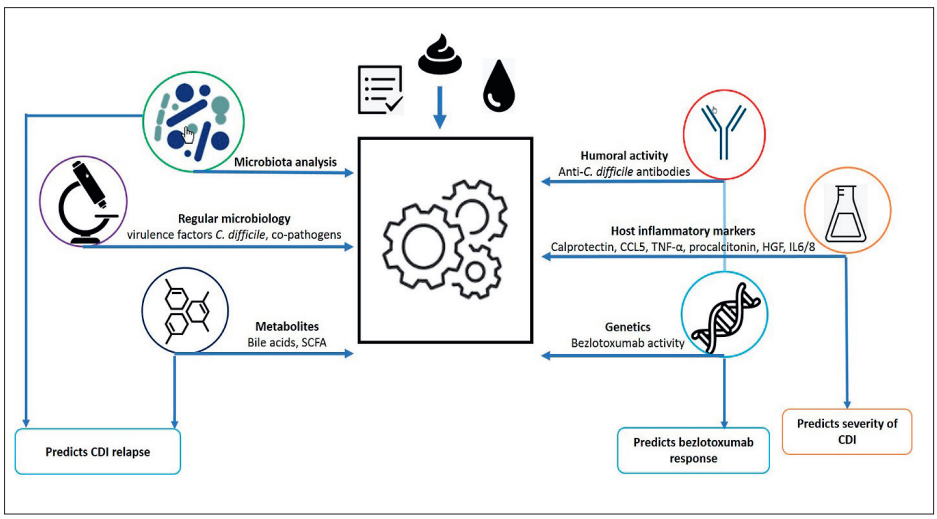

Figure 1. Personalised diagnostics for CDI

The future potential of personalised CDI diagnostics for enhanced discrimination between *C. difficile* colonisation and infection, and improved prediction of disease severity and treatment outcome.

Personalized treatment of rCDI

Personalized diagnostics can optimize treatment and subsequently improve the outcome of CDI. For a subset of patients, targeting the pathogen *C. difficile* alone is proven to be insufficient for sustained cure [72]. Depending on the above described diagnostic outcomes, the patient can be categorized as having different systems failing. Each system failing requires a different treatment approach. Therefore, I would envision the following treatment strategy *(Figure 2)* based on the assumptions mentioned below:

- √ A diverse and healthy microbiota is important for resilience against disease, not only to CDI, but also to other intestinal and extra-intestinal diseases. The aim of CDI therapy should therefore be to restore the patients' perturbed microbiota.

- √ Fidaxomicin replaces vancomycin, because of the severe impact of vancomycin on the indigenous microbiota [73]. Fidaxomicin is proven to be evenly effective in resolving CDI and preserves the microbiota, thereby resulting in fewer relapses after treatment [74-77]. In addition, although fidaxomicin is more costly than vancomycin or metronidazole, it was proven cost-effective due to averted mortality, utility loss, and costs of rehospitalisation and/or further treatments of rCDI [78]. Although fidaxomicin is superior in gain of quality-adjusted life years, the cost-effectiveness differs between studies in various countries [79, 80], and a definite conclusion is difficult to make. However, preliminary data from the new IDSA and ESCMID guidelines (2020-2021) show that fidaxomicin will probably become the first agent of choice for CDI treatment.

- √ Extended-pulsed fidaxomicin (taper therapy) is superior and more cost-effective than a regular scheme of fidaxomicin [81, 82]. However, the presumed decreased compliance of the patients to follow the more difficult treatment regime does not justify the extended-pulsed approach for a first CDI episode, but could be given for patients with a recurrence.

- √ FMT is both more effective and less costly than any other antimicrobial therapy for CDI [83-86]. Because of the unstandardized nature of this treatment and potential (low) risk for transfer of unrecognized pathogens or disease traits, treatment with anti-CDI antibiotics is preferred before FMT is administered. Consequently, FMT should be considered as treatment for the first relapse of CDI *(Figure 2)*.

- √ Bezlotoxumab is a human monoclonal antibody against *C. difficile* toxin B. Especially patients with a higher risk for rCDI (age ≥65 years, history of previous CDI, compromised immunity, IBD, renal disease, severe CDI or CDI with ribotype 027/078/244)may benefit of bezlotoxumab [87-89] to prevent relapses. When added to anti-*C. difficile* antibiotics it enhances the resilience for CDI relapses in general with ~40-50 % (relapse rate with vancomycin versus vancomycin + bezlotoxumab was 8 % vs 17 % and 16 % vs 26 %), but can be higher in particular patient groups with enhanced risk of rCDI [88, 90]. Its efficacy appears to be due to prevention rather than delayed onset, as sustained cure was observed of 69 patients cured at 12 weeks after treatment [91].

- √ The clinical relevance of the difference in absolute relapse reduction by bezlotoxumab (~9-10 %) versus fidaxomicin (~10-16 %) can be questioned. Bezlotoxumab is mainly studied in vancomycin and metronidazole treated patients, and only in small groups of patients using fidaxomicin [90, 92]. If both therapies are additive to each other remains therefore unknown although they certainly could be due to the different working mechanism [75, 77]. The combination of fidaxomicin with bezlotoxumab has been successfully applied in a few patients (n=10) with multiple rCDI for which FMT was contraindicated (personal communication prof. Maria Vehreschild and prof. Ed Kuijper).

- √ A recent exploratory genome-wide association study revealed three genetic variants located in the extended major histocompatibility complex (MHC) that were associated with a two to three fold reduction of *C. difficile* relapses in bezlotoxumab treated patients [93]. Around 40 % of patients have these genetic variants. This suggest a host-driven, immunological mechanism in response to bezlotoxumab. If these alleles are confirmed in a validation study, a human genetic analysis can be used to personalise CDI treatment.

- √ Bezlotoxumab is considered less effective in patients with multiple recurrent CDI (≥2 episodes) but prospective studies are missing [90, 92]. The LUMC Center for Infectious Diseases (LU-CID) and NDFB are therefore currently designing a randomized controlled trial to assess the efficacy of vancomycin + bezlotoxumab compared to FMT for the treatment of multiple recurrent CDI.

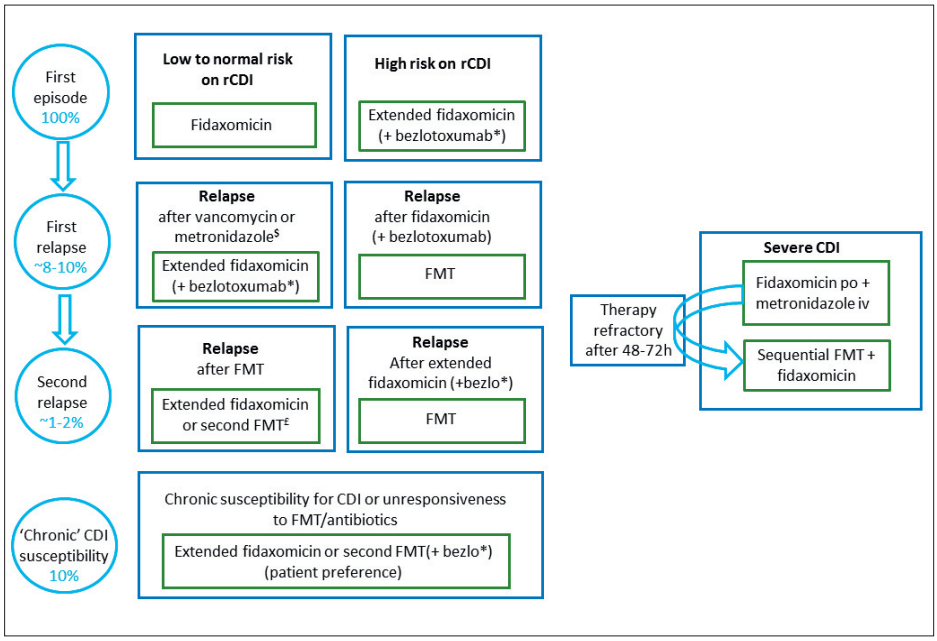

Figure 2. Personalised treatment for CDI

The future potential of personalised CDI treatment based on the in the text-mentioned assumptions. The blue circles on the left reflect the estimated percentage of patients that has the specific type of CDI (a first CDI episode is set at 100 %). The dark blue boxes give information on the indication, the green boxes inside about the proposed therapy.

* In case a genetic variant of the major histocompatibility complex (MHC) is present (~40 % of patients); this predicts a two to three-fold reduction in CDI relapses with bezlotoxumab use in comparison to patients without these genetic variants.

$ In case this personalized treatment is not followed, and metronidazole or vancomycin is given.

£ Based on shared decision making between patient and physician. Patients with 'Chronic CDI susceptibility' have suffered from multiple (recurrent) episodes in the past, but do not meet the criteria of recurrent CDI (CDI relapse within two months after prior episode) with the present episode.

Patients with high susceptibility for CDI have suffered from multiple (recurrent) episodes in the past, but do not meet the criteria of recurrent CDI (CDI relapse within two months after prior episode) with the present episode.

Mechanism of action of faecal microbiota transplantation

Mechanism of action of FMT to prevent relapses of *Clostridioides difficile* infection

The mechanism of FMT is likely multifactorial. Reinstatement of a robust and diverse, functional microbiota is an essential mechanism for resilience against CDI relapses [94]. FMT was found to restore both short chain fatty acid levels and bile acid metabolism (bile salt hydroxylation (BSH) as well as 7-α-dehydroxylation) [95-98]. In a mouse model, the bile acid converting *Clostridium scindens* (7-α-dehydroxylase) bacterium was inhibitory to *C. difficile* [99]. The co-administration of other bacterial species from *Lachnospiraceae* and *Porphyromonadaceae* families enhanced the protective potency [99]. An additional effect of 7-α-dehydroxylase producing bacteria, is the secretion of tryptophan derived antibiotics; 1-acetyl-β-carboline and turbomycin A [100]. These antibiotics inhibit the cell division of *C. difficile*, and the activity of tryptophan is enhanced by secondary bile acids [100]. However, reconstitution of the bile acid converting microorganisms with synthetized bacterial communities or live biotherapeutic products (LBPs) enhance the colonisation resistance to *C. difficile* but are not sufficient for complete prevention of acquiring CDI or sustained cure of CDI. To date, no synthetic bacterial community has achieved a success comparable to CDI antibiotics or FMT though recent interim analyses of some phase 3 studies are promising. In a proof of principle phase I study with a fractionated and encapsulated bacterial spores product (SER-109 with spores from approximately 50 bacterial species), following standard of care, most patients (29/30) achieved clinical resolution [101]. However, SER-109 failed to be of additional benefit compared to standard of care in a phase II study in rCDI patients (44 % relapse versus 53 % in placebo, n=89) [102]. Recently, SERES claimed via twitter that SER-109 did met their phase III primary endpoint, showing a statistically significant 30 % absolute reduction in rCDI compared to placebo (relapse rate of 11 % versus 41 % in placebo) [103]. A remarkable finding in both studies is the high rate of relapses that occurred in the control group. This could either be explained by study inclusion of patients with a high risk on rCDI or inclusion of patients colonised with *C. difficile*. Various studies with rational selected bacterial consortia or faeces microbiota derived products (VE303 of Vedanta, Finch, Rebiotix) are underway and show promising preliminary results.

The gut microbiota does not solely consist of bacteria but does contain various other microorganisms. Recently, it was shown that the presence of *Candida albicans* in the intestinal tract reduces the efficacy of FMT [104]. In contrast, healthy donors and patients responding to FMT displayed a high relative abundance of *Saccharomyces* and *Aspergillus* [104]. A recently completed pilot study of NDFB and CMAT (Center for Microbiome Analyses and Therapeutics) analysing the mycobiome of healthy individuals and patients either infected or colonised with *C. difficile* confirms this observation. A relatively high abundance of *C. albicans* in CDI patients, and more *Saccharomyces* and *Aspergillus* in non-CDI patients and healthy donors was observed (Zwittink, unpublished observation).

In patients with severe and therapy refractory CDI, administration of FMT results in fast but sometimes temporary improvement of clinical symptoms within hours [105-107]. The temporary improvement is sufficient to deescalate the clinical status, enabling a response to repeat FMTs [106, 107]. In this short period, stable engraftment of a functional microbiota is unlikely and inhibition of intestinal inflammation is likely to play a significant role [107]. The afunctional and unbalanced patient's inflammatory response could be reshaped by yet not fully understood mechanisms and compounds, as the microbiota impacts various immune pathways that aid in recovery from CDI colitis [108, 109]. An interesting target is interleukin-33 (IL-33), an important guardian of the gut barrier during *C. difficile* colitis that prevents CDI-associated mortality via activation of group 2 innate lymphoid cells [110]. Intestinal IL-33 expression is regulated by the microbiota, and FMT was proven to rescue the antibiotic-associated depletion of IL-33 [110]. Also regulatory T-cells play a critical role in the maintenance of immune homeostasis and seem an interesting immunological target [109]. FMT was shown to control inflammation and colitis via induction of regulatory T cells [111, 112]. Regulatory T cells are activated by many different pathways and different bacteria, for instance via commensals activating IL-10 and/or TGF-β which recruit the regulatory T cells to the intestine [111-114], via bacterial polysaccharide A that results in inhibition of IL-17 and thereby an increase in regulatory T-cells [113], or via short-chain fatty acids that promote the fitness and differentiation of regulatory T-cells [115, 116]. Whether FMT-directed immunosuppression aids also in the recovery of CDI colitis requires further investigation [109].

In addition to direct amelioration of severe CDI symptoms by immunomodulation through faecal suspension, a direct impact of the FMT can also be achieved by bacteriophages [117] or production of bacteriocins such as thuricin CD or nisin by the living bacterial fraction of the faecal suspension [118-120]. In addition, the toxin expression of *C. difficile* could be suppressed by carbohydrates present in the faecal suspension [94, 121], or human donor metabolites such as alpha-defensins [122]. The toxins could be rapidly neutralized, as bile acids reversibly bind to *TcdB*, causing a 'balled up' formation of the toxin which is no longer able to bind to the host's cell surface receptors [123].

In conclusion, it is likely that not a single bacterium or bacterial community contribute to colonisation resistance and prevention of CDI relapses by FMT. Multiple microbiota communities and networks (including bacteria, viruses, eukaryotes etc.) exist that enhance resilience or protection to rCDI. In addition, by faecal microbiota transplantation a complete functional ecosystem is transplanted. The effect of FMT is the result of a complex interplay of microbiota networks, immune modulation and metabolites that not only influence the colonisation resistance to *C. difficile* but also affect host inflammation and bacterial toxin production.

Mechanism of action of FMT for eradication of multidrug resistant organisms

Intestinal colonization of MDRO, and general decolonization strategies

Most infections with ESBL producing Enterobacterales are preceded by intestinal colonization [14, 124], and prevention and eradication of these MDRO from the gut is therefore of interest. Spontaneous intestinal clearance of an ESBL containing micro-organism varies per bacterial (sub)species and per ESBL enzyme. Duration of colonisation is on average longer in patients with comorbidity; 43 % remained ESBL positive after 1 year [125]). Contrary, healthy individuals in the general population had a mean duration of ESBL colonisation of 4.2 months [126] and 33 % remained MDRO positive for > 8 months [127]. Individuals who travelled had a median duration of ESBL colonisation of 30 days, whereas only 14.3 % and 11.3 % remained colonized at 6 and 12 months after return, respectively [128]. Spontaneous clearance of certain *E. coli* sequence types (ST) appears to be more difficult, as colonization of *E. coli* ST131 is

associated with a longer duration of carriage in a long-term care facility residents, with a half-life of 13 months versus 2- to 3- months for other STs [129]. Presence of the MDRO in the intestinal tract below the limit of detection in faeces can sometimes complicate study outcomes or interpretation. This phenomena is well known for detection of vancomycin resistant enterococci (VRE), as on average four to five rectal swabs, collected on separate days, are needed to detect >90 %-95 % of new VRE carriers [130, 131]. Especially in the first stages of colonisation, just after a transmission event, VRE detection using rectal or perianal swab can be less sensitive than faeces samples [132, 133]. Difficulties with detection of MDRO in the intestinal tract due to low levels is also observed for Gram negatives [134, 135]. A RCT that studied the decolonisation effects of a combination of colistin and neomycin versus placebo observed a significantly lower rectal carriage of ESBL in the non-absorbable antibiotic treated group at the end of treatment (32 % versus 77 %), but the effect was lost 7 days post-treatment [134]. A negative result may therefore reflect suppression of the MDRO below the detection limit or temporary suppression rather than decolonisation. An ESCMID guidance document could not find sufficient evidence for a successful therapeutic decolonisation therapy, not with orally non-absorbable antibiotics or any other therapeutic approach [136]. The current knowledge on this topic provided by randomized and observational studies suffers of much heterogeneity between tested populations, used decolonisation therapy, inconsistency in defining and reporting end points and small sample sizes [136], and both large, well-designed RCT as innovative strategies are desperately needed.

Colonisation resistance against (multi drug resistant) Enterobacterales

Modifying the (failing) indigenous intestinal microbiota to prevent or treat gut colonisation with MDRO is an interesting therapeutic intervention, although specific targets are unknown. A healthy indigenous microbiota does not contain an abundance of Enterobacterales. This family of facultative anaerobic Gram-negative bacteria harbours many species capable of MDR carriership. Colonisation resistance against MDRO or Enterobacterales in general, is accomplished by a complex interplay between different species and functions of the host's microbiota. In our recently submitted study on microbiota-associated risk factor for asymptomatic MDRO colonisation study in nursing home residents, several taxa belonging to *Dorea, Atopobiaceae* and *Lachnospiraceae* of the ND3007 group were consistently more

abundant in faeces samples of nursing home residents who were never colonised with an MDRO during a six month time period (submitted, Genome Medicine, Ducarmon et al). At a functional level, many species of the Firmicutes and Bacteroidetes, are capable of metabolizing food fibres to Short Chain Fatty Acids (SCFA's) [137]. Of these SCFAs, butyrate is essential in maintaining host health by providing energy for colonocytes, contributing to the acidification of the lumen, modulating the immune system (maturation and expansion of colonic regulatory T-cells) and affecting diverse metabolic routes in the body (e.g. in liver and brain) [114,116,138-140]. During homeostasis, butyrate-producing bacteria limit the availability of oxygen and nitrate in the colonic lumen through the intracellular butyrate sensor peroxisome proliferator-activated receptor (PPAR-γ) [141]. PPAR-γ represses the gene encoding inducible nitric oxide synthase (*Nos2*), resulting in a lowered nitrate production. Consequently, the bioavailability of electron acceptors is limited, which is normally used for anaerobic respiration and drives an expansion of facultative anaerobes [141-143]. Microbiota induced PPAR-γ signalling also directs the colonocytes towards oxidative phosphorylation and β-oxidation of short and long chain fatty acids, resulting in high epithelial oxygen consumption [141,143]. The consequent epithelial hypoxia helps to maintain a microbial community dominated by obligate anaerobic bacteria [143] or eukaryotes [144]. A depletion of butyrate-producing Clostridia was shown to drive an aerobic luminal expansion of *Salmonella* species [145]. Furthermore, SCFAs at an acidic pH were able to inhibit the replication of *E. coli* and *Salmonella* sp. [146-149], and provide subsequent resistance against colonisation and infection of *Salmonella* following streptomycin treatment [150]. The gut microbiota of nursing home residents carrying an ESBL producing Enterobacterales was indeed characterised by a lower abundance of SCFA producing bacteria [151]. In addition to the above described mechanisms of the healthy microbiota in combat against a perturbed expansion of Enterobacterales, the healthy indigenous microbiota is also capable to inhibit acquisition of antibiotic resistance and horizontal gene transfer [152]. In an *in vitro* model containing a human gut microcosm, the microbiota not only suppressed growth and colonisation of a newly introduced *E. coli* strain, but also prevented it from evolving antibiotic resistant upon exposure to ampicillin. The invading *E. coli* only acquired resistance in the absence of the resident microbial community, even though highly effective β-lactam resistance plasmids were present in the resident microbial communities [152]. In addition, inflammatory responses in the gut can generate transient blooms of Entero-

bacterales in which conjugative transfer occurs at unprecedented rates, as shown by the high rate of conjugative horizontal gene transfer of a resistance plasmid of *Salmonella enterica serovar Typhimurium* to *E. coli* in a mouse colitis model [153].

Innovative prevention of multidrug resistant gut colonisation, and decolonisation strategies

Prevention

An important key to reduce spread of antimicrobial resistance is the prevention of MDRO colonisation in the gut. Restricting the use of (broad spectrum) antibiotics reduces the selection, colonisation and outgrowth of MDRO [154]. This mechanism is well recognized and better known as antibiotic stewardship. Antibiotic stewardship programs are designed to restrict antibiotic overuse and misuse by educating physicians on antibiotic selection, dosage, route of administration and duration of therapy. These programmes have proven their effectiveness and significantly reduce the incidence of colonisation and infections with MDROs and *C. difficile* [155-157]. Accordingly, the improved rational use of antibiotics also reduces sepsis [158] and the overall mortality rates [159]. It seems plausible that at least some of these effects are mediated by the preservation of a diverse and healthy microbiota. Antibiotic stewardship programmes should therefore incorporate consideration for the impact of antimicrobial therapy onto the commensal microbiota [160]. Disruption of the healthy microbiota is in line with use of broad-spectrum antibiotics, and CDI can be considered as surrogate indicator for a disturbed microbiota. However, other infectious complications or diseases associated with a perturbed microbiota may also arise on the short or long term. For example, the use of metronidazole was correlated with intestinal enterococcal domination and subsequent bacteraemia in hematopoietic stem cell transplant patients [161]. In addition, early administration of broad-spectrum antibiotics in allogenic stem cell transplantation patients resulted not only in a decreased abundance of Clostridiales (especially cluster XIVa), but also in a significant higher transplant related mortality [162]. A lower diversity of the intestinal microbiota at the time of neutrophil engraftment was associated with a higher mortality [163]. The early administration of antibiotic therapy active against commensal organisms warrants the use of commensal sparing antibiotics and rapid restoration of the microbiota after cessation of antibiotic therapy. In patients with

rCDI, it was shown that FMT as microbiota restoring therapy (and not anti-CDI antibiotics) lowered the chance on developing a blood stream infection, and subsequent mortality [164].

Therapy

Once the indigenous microbiota failed to provide colonisation resistance against an MDRO and the gut has become colonised, targeting the microbiota could support decolonisation. This decolonisation strategy demonstrated its potential when it was observed that patients receiving an FMT for multiple recurrent CDI, had a significant reduction in the number and diversity of antimicrobial resistance genes after FMT [165-167]. Moreover, not only resistance genes in the microbiota as determined with metagenomics, but also resistance of clinically relevant MDRO Gram negatives decreased after FMT. Data from the NDFB indicate that 50 % of pre-FMT MDRO colonised rCDI patients (ESBL producing or fluoroquinolone and aminoglycoside resistant Enterobacterales), lost the MDRO within three weeks after FMT (preliminary results NDFB and LU-CID, K.E Vendrik and E.M Terveer). This observation adds to various case-reports of patients colonized with ESBL or carbapenemase producing Enterobacterales (CRE) treated and often successfully decolonized by FMT [168-182]. **Chapter 8** describes such a case-report. We experienced that infusion of a healthy donor microbiota into the gut of a patient with normal microbiota diversity did not result in eradication of the ESBL-producing *E. coli*. Possibly specific microbial strains are required, or an improved donor engraftment by antibiotic pre-treatment (further described in paragraph "Optimal donor selection for FMT"). Seven larger case series display mixed results, but varied in study design, patient characteristics and outcome measurement. Of the patients colonised with ESBL, 20 % was decolonised one month after a single FMT, 40 % after two FMTs (n = 15) [171]. Haematological patients (n=25) colonised with either ESBL or carbapenemase producing Enterobacterales (CPE) were decolonised in 60 % of cases one month after FMT [168]. Of note, patients that received antibiotics within seven days after FMT achieved significantly less decolonisation (36 % versus 93 %). Of patients colonised with CRE, decolonisation rates varied from 33 %, 50 % to 80 %, two weeks to four months after FMT [169, 170, 180]. A recent retrospective analysis of CRE and/or VRE colonised patients (n=35) treated with FMT showed that 69 % was decolonised after one year. In addition, microbiota analysis prior FMT could be used to predict the patients response on FMT (or spontaneous

decolonisation), as a higher initial level of Verrucomicrobia and Proteobacteria and lower species richness was observed in the non-responders [183]. These case series should be interpreted with caution as they suffer from publication bias, and also have different study designs with varying pre-treatments, number and application routes of FMT and different follow-up periods. Besides, definitions of gut MDRO colonisation differed and various microbiological tests to detect MDRO in faeces were used. Only one RCT was performed in which 39 adults colonized with ESBL- or carbapenemase-producing Enterobacterales were randomized to either no intervention or a 5-day course of non-absorbable antibiotics followed by FMT. Unfortunately, no statistically significant advantage of FMT was found, though the trial suffered from inclusion of insufficient number of patients [184,185]. Similarly, only small differences in the microbiota composition were observed in the patients after treatment with FMT. Relative to baseline, post-FMT microbiota was significantly enriched in *Bifidobacterium* species and *Collinsella aerofaciens* [186].

The eradication potential of various microbiota modifying agents can be studied via transplantation of a complete ecosystem of a healthy donor. However, in the long run, application of this 'black box' therapy is undesired, and several treatment components merit further research. These are for example "live biotherapeutic products (LBPs)", bacteriocins or other microbial metabolites or bacteriolytic phages. Bacteriophages are highly specific for one bacterial (sub) species, providing a desirable asset in the refine modification of a host microbiota. A number of animal studies showed that bacteriophages can be used as treatment for infections caused by MDRO [187], and also demonstrated potential as eradication strategy for colonisation of MDR Gram negatives (MDR Pseudomonas and MDR uropathogens) of the gut in nematodes and mice respectively [188,189]. The stability of bacteriophages during intestinal passage, their impact on the non-targeted human microbiota, potential side effects and the achievable effect size and duration merit further research [185,187]. An interesting treatment approach is adding bacteriophages to sub-lethal dosages of a non-absorbable antibiotic leading to synergy [190]. The 'phage-antibiotic synergy' is considered an enhanced phage production and accelerated lysis of infected cells, in response to the filamentation of bacterial cells upon exposure to the antibiotic [190]. Other interesting components of FMT acting against MDRO are bacteriocins or (in combination with) live biotherapeutic products. A lantibiotic-producing commensal of the gastro-

intestinal tract, *Blautia producta*, reduced and prevented VRE colonization in man and mice [191]. Another interesting candidate demanding further research is *Lactobacillus*. *Lactobacillus* species in the faecal microbiota of hospitalised patients were associated with resistance to MDRO acquisition during admission [192]. Additionally, mice treated with a combination of 10^{10} CFU *L. plantarum* and *L. acidophilus* were able to eradicate MDR enteroaggregative *E. coli* from the gut [193]. However, up till now probiotics, synthetic bacterial communities or live biotherapeutic products have failed to eradicate intestinal carriage of Gram negative MDROs in human randomized controlled trials. Amongst them, an attempt to eradicate MDROs in ICU patients with *Lactobacillus rhamnosus* [194], a trial to eradicate MDR *E. coli* in long-term care facility residents using *E. coli* Nissle 1917 as probiotic [195], and a combination of *L. bulgaricus*-*L. rhamnosus*-fructo-oligosaccharides failed to eradicate MDR Gram-negative bacilli in hospitalized patients [196]. A live biotherapeutic product of eight living bacterial strains could not eradicate ESBL Enterobacterales in outpatients [197]. The concept of super donors have recently drawn attention in the development of live biotherapeutic products from these donors [198]. The super donors have specific microbial traits and are thereby significantly more capable in treating a specific disease than other donors. Studying these super donors might be crucial in understanding MDRO colonisation resistance and subsequent cure. The concept of super donors for MDRO eradication is currently investigated in a clinical trial by us and others (Davido of the Hôpital Universitaire Raymond-Poincaré in Paris in collaboration with NDFB and Vedanta). For these trials, an FMT donor is selected based on the capability of MDRO clearance in 80 % of the FMT-treated and MDRO colonised mice [199]. Lastly, immunological approaches can be explored as option for MDRO eradication. Vaccination of pregnant cows with the inactivated cells of the globally disseminated and MDR *E. coli* sequence type 131 resulted in highly specific anti *E. coli* 'hyperimmune bovine colostrum'. This colostrum was able to disrupt the intestinal colonization of the ST131 *E. coli* in mice [200].

In conclusion, many therapeutic options are currently explored, but demonstrate high heterogeneity in set-up as well as outcome, and need harmonization. Furthermore, it appears that the concept of FMT for treatment of rCDI is not applicable for decolonization of MDROs. Metagenomic studies could provide answers on the effect of the decolonizing agents on the microbiota composition and dynamics, and should guide the design of future research [136, 186]. Robust and well-designed multicentred

trials to assess the above described innovative therapeutic approaches, with assessment of the optimal pre-treatment, for a larger panel of clinically relevant MDRO's are needed in the future. These studies should also include sufficient long-term follow-up on microbiological and clinical outcomes which assess both adverse events as well as clinical relevance of the decolonization (e.g. reduced MDRO infections and subsequent readmission, fewer long-term complications). Additionally, more fundamental research should be performed using in vitro gut microbiota models to study specific donors, specific microbiota species and networks essential for colonisation resistance for several clinically relevant MDRO's.

Optimal donor selection for FMT

Donors and super donors

A perturbed microbiota has been observed in a large variety of disorders, and FMT as microbiota modulating therapy, is increasingly used in trials not only for intestinal but also for extra-intestinal diseases. The presumed mechanism of action of FMT for other diseases is however likely different than for rCDI and could vary per treatment indication. Though it is generally considered that FMT restores the functionality of a perturbed gut microbiota by engraftment of donor strains, the precise mechanism is probably more complex than a simple replacement of bacterial species. Bacterial networks, metabolites, archaea, viruses, fungi and other eukaryotic microorganisms also influence the composition and function of the microbiota. Corresponding with the variation in gut microbiota composition between healthy individuals (e.g. donors), variability exists in the faecal suspensions used for FMT treatment. Donors of faecal suspensions with a significantly higher success-rate are referred to as super donors. Super donors for rCDI treatment do not appear to exist as no donor related factors attributing to the FMT success could be identified by us (**Chapter 6**) and others [198, 201, 202]. Patients with multiple, recurrent CDI have a perturbed and diminished microbiota diversity [203]. Replenishing this severely reduced diversity with any healthy donor microbiota results in prompt resolution. For other diseases, such as ulcerative colitis super donors do seem to exist [204], but the evidence is sparse. Studying these super donors might be crucial in understanding complex disease pathology and subsequent cure [198]. The question is how to find these super donors?

With respect to donor selection, it is very unlikely that one super donor can cure all microbiota related illnesses [205]. Like our society, a healthy microbiota is diverse. However, this encompasses not only a diverse community within the host, but also between the microbiota's of different hosts. In diseases with a perturbed microbiota, the specific failing network should be restored, and optimal donors could vary per microbiota related disease. For instance a donor for FMT to boost the immune response in patients that show progressive cancer while on checkpoint inhibitors [206, 207] could be very different than the donor needed to abolish the overactive immune system in patients that suffer of grade III/IV toxicity during checkpoint inhibitors [208, 209].

Replenish the beneficial bacteria

Different strategies exist for rational donor selection, depending on the specific disease intended to cure with FMT. A patient can have a decreased load of beneficial bacteria which can be replenished by healthy donor strains *(Figure 3)*. Replenishment is based on supplementation of unique taxonomic or functional deficiencies present in the diseased microbiota [210]. A very successful open-label trial among patients with cirrhosis with recurrent hepatic encephalopathy randomized to receive either standard of care or FMT (with antibiotic pre-treatment), performed rational donor selection. Using microbiome data of hepatic encephalopathy patients and healthy controls a machine learning technique was performed to identify a single donor with the highest relative abundance of *Lachospiraceae* and *Ruminococceae*. FMTs derived from the faeces of this donor significantly reduced hospitalizations, improved the cognition and perturbed microbiota over more than 12 months [211, 212]. If this effect was indeed due to the selected 'super-donor' is questioned [213], since the relative abundance of *Lachospiraceae* and *Ruminococceae* was not significantly different before and after FMT in the FMT-treated patients [213]. A metaproteomic and metabolomic analysis added to the metagenomic data should provide more insights in the functional changes of the group of *Lachospiraceae* and *Ruminococceae*.

An example for which taxonomic selection would be rational is Ulcerative Colitis (UC). The short chain fatty acid; butyrate is important in alleviating inflammatory bowel diseases (IBD) such as UC [214]. A meta-analysis showed a consistent lack of butyrate producing Clostridiales in patients with IBD [215]. A rational super donor would

be a donor with an overabundance of these gut bacteria. This super donor effect was indeed observed in an RCT with FMT for treatment of UC, using five faecal donors. The majority (78 %) of patients who achieved remission received faecal suspensions prepared from one single donor. The other donors were not more efficacious than placebo **[204]**. The super donor contained the highest load of butyrate producing bacteria. Though, in a study combining microbiota data of three RCT's for UC, an abundance of butyrate producing bacteria of the donor was not associated with patient response **[210]**. In addition, host factors are also important in the response to FMT treatment for UC. Younger age, moderate disease severity and endoscopic mayo scores predicted achievement of clinical remission of FMT in patients with active UC **[216]**. This reflects the multifactorial aetiology and treatment of this disease and the challenges of donor selection in the real world. A critical note is that abundance or shortness of a certain group of bacteria in correlation with a specific disease may be an oversimplification. The mucosa associated microbiome and host immune factors may play a more prominent role.

Replacement of the undesired bacteria

A disease could also be mediated by the presence or overabundance of one or more harmful bacteria for which competitor donor strains can be selected *(Figure 3)*. The most straightforward competitors are bacteria that directly inhibit the undesired strain (direct competition), for instance bacteria that produce bacteriocins. An example is the lantibiotic-producing commensal, *Blautia producta*, which demonstrated a reduction and prevention of VRE colonization in man and mice **[191]**. Competitors of undesired bacteria can be identified by another mechanism of colonisation resistance; competitive exclusion. Bacteria occupying the same nutritional or environmental niche can be selected from literature. For instance, *Bacteroides thetaiotaomicron* is a direct competitor of food (carbohydrates) for *Citrobacter rodentium*, a gastro-enteritis pathogen in mice **[217]**. Selection of a donor-mouse containing (high rates of) *B. thetaiotaomicron* to treat a *C. rodentium infection* would therefore make sense. A second illustrative example involves a subgroup of non-alcoholic fatty liver disease (NAFLD) patients, who suffer of auto brewery or gut fermentation syndrome. After a carbohydrate-rich meal, the microbiota of these patients is capable of ethanol production, resulting in an impaired mitochondrial function and subsequent liver injury **[218-220].** Though the pathogenesis of this disease is still unknown, several members of the

microbiota like *Candida* species, *Saccharomyces*, *Enterococcus faecium* or *Klebsiella pneumoniae* have been identified as potential ethanol hyperproducers [221]. Yuan and colleagues show that a high-alcohol-producing *K. pneumoniae* (HiAlc-Kpn-strain) was present in 60 % of individuals with NAFLD in a Chinese cohort, only 6 % of the healthy controls was colonised with this *Klebsiella* [218]. Transfer of the intestinal microbiota from a NASH patient containing HiAlc-Kpn-strain, as well as the HiAlc-Kpn-strain alone into mice, introduced detectable blood alcohol and steatohepatitis. Selective removal of the HiAlc-Kpn-strain (using a bacteriophage) before FMT prevented NAFLD in the recipient mice [218]. Removing this pathology-causing bacterium could thus lead to clinical improvement [218]. Unfortunately, our laboratory could not reproduce these findings of a hyper ethanol producing *K. pneumoniae* in faeces samples of a suspected patient with an auto-brewery syndrome and the Chinese researchers did not provide their strains for further analysis. In patients with auto-brewery syndrome, not only replenishment with healthy microbes but also replacement of the detrimental bacterium is needed. In patients with metabolic syndrome it was already shown this is

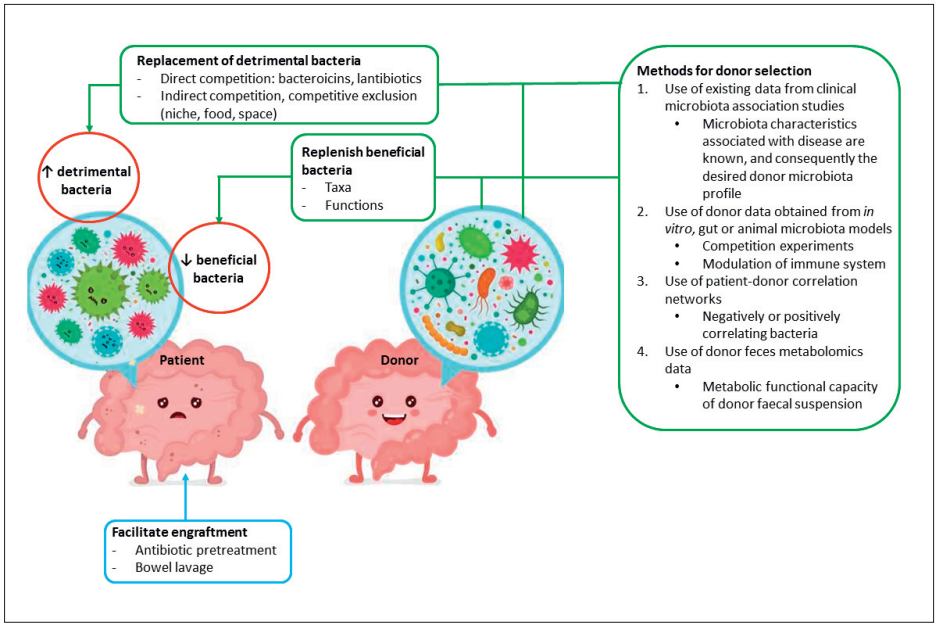

Figure 3. Strategies and methods for optimal donor selection and patient pre-treatment

not always straightforward, as donor strains rather co-colonize than replace similar patient strains [222]. Faecal donors should be screened and excluded when containing (an overabundance of) the undesired bacteria, in addition to positive selection for bacteria known to out-compete the harmful taxa [210].

Proposed methods for optimal donor selection

Option 1: Use of existing data from clinical microbiota association studies

Several approaches can be employed to select optimal donors *(Figure 3)*. De first and most simple technique is selection of donors with a known desired microbiota composition. There are two requirements; First, the microbiota of the donors must be profiled. Metagenomic analysis is preferred, as this provides insight on functional and strain-level associations [223]. Second, the microbiota characteristics associated with disease must be known from epidemiological studies or/and animal experiments.

The NDFB is currently designing a granted FMT pilot study for Parkinson's disease and considers rational donor selection. In this trial the safety and feasibility of FMT in Parkinson's disease patients is assessed. Parkinson's disease is a neurodegenerative disease characterized by neuron degeneration in the central, enteric and peripheral autonomous nervous system. Several mechanisms by which FMT could modulate Parkinson's disease exist. An important factor in the aetiology of Parkinson's is the aggregation of the protein alpha-synuclein [224]. The hypothesis is that under influence of the microbiota, a neurotropic substance, possibly alpha-synuclein, is formed in the gut and transported to the enteric nervous system and brain, via the vagus nerve [225, 226]. Key microorganisms or functions are not yet defined, although the microbiota of Parkinson's patients is in general more pro-inflammatory oriented, with LPS-producing Proteobacteria, and contains less anti-inflammatory butyrate-producing bacteria [227, 228]. The frequently observed obstipation in these patients could however bias the microbiota interpretation, and one could question whether rational donor selection to alter the natural course of disease is appropriate at the moment. Alternatively, the microbiota also seems to play a role in the bioavailability of the primary therapy of Parkinson's disease; levodopa [229, 230]. Bacterial decarboxylases (*tdc* gene) are identified that restrict local (intestinal) and blood levels of levodopa by

conversion to dopamine, which cannot pass the blood-brain barrier [230]. Rational donor selection could therefore be performed with the hypothesis; "FMT from a donor with low amounts of *tdc* genes present in the microbiota will reverse the levodopa resistance by replacement and/or out-competition of *tdc* containing patient strains." The patient will then again respond to levodopa therapy and patients will experience less side-effects (e.g. dyskinesia) due to the stabilised levodopa bioavailability and drug dosing [229]. If this hypothesis holds, the replacement of bacteria carrying *tdc* genes will be most likely based on similar bacteria without the *tdc* gene. If such an effect is found, the next question is whether this replacement will be permanent under continuous exposure to levodopa, or if the patient needs maintenance or sequential FMTs.

Option 2: Use of donor data obtained from in vitro, gut or animal microbiota models

A second strategy involves data obtained from *in vitro* or *in vivo* microbiota models. In close collaboration with Vedanta Biosciences, *in vitro* experiments in mice were performed with faeces of NDFB donors. Antibiotic-pre-treated mice were densely colonized with either a carbapenemase-producing *Klebsiella pneumoniae* or vancomycin resistant *Enterococcus faecium*. The mice were subsequently treated with faecal microbiota from various NDFB donors. Consistent with clinical findings, variability in FMT-mediated decolonization of resistant bacteria was observed. An FMT donor enriched in the microbiota capable to eradicate both MDRO's best, as determined by complete MDRO clearance in 80 % of treated animals [199], was selected for a clinical MDRO eradication trial in kidney transplant patients (trial currently performed, in collaboration with NDFB). Modulation of the immune response by FMT can also be tested in a mouse model. The microbiota plays an important role in the development, training and maintaining of the immune system [231], and the microbiota seems involved in many diseases with an imbalance of pro- and anti-inflammatory responses [232]. Regulatory T-cells are important for the maintenance of intestinal self-tolerance and will likely be important for therapeutically manipulation of IBD [233]. Again, in close collaboration with Vedanta Biosciences, mice sensitive for IBD were colonized with faeces of NDFB donors. The donors which could enhance the regulatory T-cells in the IBD mice the most were selected for an RCT with FMT for UC patients (trial currently ongoing).

Option 3: Use of patient-donor microbiota correlation networks

Another donor selection strategy can be deployed if the microbiota characteristics for a particular disease are not available from human epidemiological studies or animal experiments, but microbiome data of an individual patient is available. The existing microbiome data can be mined to find bacteria that consistently show a negative association with a pathogen or other undesired bacteria. An important assumption with donor selection based on these bacterial networks is that the bacteria that negatively correlate are competitors of the undesired bacterium, rather than that they are both consequence of the underlying disease. The preferred donor should have high abundances of these putative competitors [234]. We studied the microbiota composition of patients with *C. difficile* infection and compared the data with asymptomatically *C. difficile* carriers and healthy controls. The aim was to find special groups of bacteria responsible for progression of a carrier to a diseased state. It was found that the presence of *Eubacterium hallii* and *Fusicatenibacter* may indicate resistance against *C. difficile* colonization and infection, while *Veillonella* may indicate susceptibility [235]. A second example is the role of the microbiota in atopic disease. By mining microbiome data of healthy three months old babies staying either healthy of becoming atopic at the age of 1 year (e.g. asthma, atopic dermatitis, food allergy) Boutin and colleagues showed that this approach can also lead to a potential drug [236]. A machine learning approach revealed a consortium of commensals of the infant gut as candidates for a live biotherapeutic product that could be tested in the future for its potential to prevent the onset or progression of a variety of atopic diseases [236]. A nine-component bacterial community consisting of the following genera was proposed; *Blautia, Coprococcus* (Anaerostipes/Eubacterium_E), *Dorea* (Tyzzerella), *Faecalibacterium*, *Lachnospira*, *Oscillospira* (*Intestinimonas/Flavinifractor*), *Parabacteroides*, *Roseburia* and *Ruminococcus*, and follow-up studies are planned. Although promising, one has to realise with these microbiota association studies that the functional capacity of bacterial genera, species and even strains can be vastly disparate. The cultivation and functional testing *in vitro* and *in vivo* (mouse and human) will be critical for the actual development of a proposed biotherapeutic product

Option 4: Use of donor faeces metabolomics data

A fourth strategy involves measuring metabolomics. One could rationally select a donor based on molecules (butyrate (SCFA) or secondary bile acids) present in

donor faeces that serve as proxy for a 'healthy' metabolic output of the donor microbiota as ecosystem. In patients suffering of multiple recurrent *C. difficile* infections, the metabolomics of bile acids in the faeces clearly differs from healthy donors. In the faeces of rCDI patients, secondary bile acids were absent, whereas primary acids were abundant [237]. FMT promptly normalized the faecal bile acid composition to the healthy donor situation (low primary, high secondary bile acids) [95, 237]. Restoring a disturbance of the capacity of 7α-dehydroxylation of bile acids of part of the microbiota, is not only important in the course of (r)CDI. Many other diseases are influenced by a disturbed bile acid metabolism like liver diseases including cirrhosis, and could therefore be subject of targeted therapy with FMT of a selected optimal donor [211, 238]. A complicating factor is the difficulty of the read-out of the metabolic activity, which is complicated by several individual and environmental factors that influence the absorbance or conversion of the metabolite of interest. For instance the level of bile acids rises after meal [239]. To overcome this bias, the capacity of bile acid conversion of the faecal slurry can alternatively be measured. Ideally the mechanisms of action of the metabolites within complex ecosystems, like the human gut microbiota, must be further explored with a multi-omics approach. The reported integrated use of compositional (metagenomics) and functional (metabolomics and metaproteomics) approaches should preferably be validated with an *in vitro* model to assess the effects of human donor faecal microbiota transplantation to the bile acid pathway. This enables a greater understanding of how variation in the gut microbiota influences host bile acid signatures, their associated functions and their implications for health [240].

Optimizing the patient for FMT to facilitate engraftment

An important step in FMT is to optimize the patient's microbiota to facilitate engraftment of donor strains *(Figure 3)*. This can be performed with bowel lavage to reduce the patient's bacterial load, although its effect has never been compared to placebo [241]. In addition, the undesired strains could be diminished by a (semi-) targeted antibiotic pre-treatment, for example with polymyxin/neomycin for eradication of Gram negative MRDO's as described in **Chapter 8**. Although both in our case-report as well as in a RCT, this combination was unable to eradicate MDROs significantly [173, 184]. Intriguingly, in patients with rCDI, FMT together with vanco-

mycin pre-treatment results in a significant engraftment of donor strains as well as a decline in the number and diversity of antibiotic resistance genes [165, 242]. This decline does not necessarily mean eradication of MDRO's, and the reduction could reflect solely the normalisation of the overabundance of Gammaproteobacteria after FMT in rCDI patients irrespective of vancomycin. On the other hand, the decline in antibiotic resistance genes could also be the result of enhanced engraftment of donor strains capable to compete with the patients' MDRO. Therefore, in contrast to targeting the pathogen with pre-treatment, a more revolutionary idea is to target the indigenous microbiota and create a niche for the donor strains to colonize and compete. Vancomycin is a broad spectrum antibiotic and oral administration results in non-absorbable high intestinal concentrations causing a dramatic decrease of Firmicutes and to a lesser extent Bacteroidetes, the two most important phyla of the indigenous microbiota [73]. Preliminary data on engraftment of live biotherapeutic products in healthy volunteers show that prolonged engraftment is only successful when volunteers are pre-treated with vancomycin (preliminary data of VE303, Vedanta). Additionally, the spore-based microbiome therapeutic SER-287 reported that pre-treatment with vancomycin resulted in a significantly higher engraftment and clinical response in patients with mild-to-moderate ulcerative colitis [243]. Clinical remission was achieved at 8 weeks in 0 %, 13.3 %, 17.7 % and 40 % of patients receiving placebo/placebo, placebo/SER-287 weekly, vancomycin/SER-287 weekly and vancomycin/SER-287 daily respectively [243]. The superiority of vancomycin as pre-treatment enhancing engraftment could however not be confirmed in mice that received donor mice faeces through oral gavage [244]. Surprisingly pre-treatment with polymyxin B resulted in the highest rate of viable donor bacteria in the recipient mice [244]. Of all tested antibiotics, vancomycin, metronidazole and cefotaxime resulted in impaired engraftment efficiency [244]. Lastly, in a proof of principle study on the concept of antibiotic pre-treatment targeting the recipient microbiota, amoxicillin-metronidazole-fosfomycin in combination with FMT (n=27) alleviated the intestinal perturbed microbiota caused by a loss of Bacteroidetes in UC patients better than FMT alone (n=4) [245]. The optimal pre-treatment for bacterial engraftment is currently unknown, and likely varies for the underlying disease and possibly even differs between the specific microbiota modulating therapies (e.g. FMT or LBP).

The relation between *Blastocystis* species and a healthy microbiota

Blastocystis is a genus of a common unicellular obligate anaerobic intestinal parasite in humans and animals that belongs to the stramenopiles, 1 of the 8 major phylogenetic groups of eukaryotes. It is a diverse genus comprising 17 characterized lineages: the so-called subtypes (ST1 – ST17), of which 9 have been reported to occur in the human gastrointestinal tract [246, 247]. *Blastocystis* sp. carriage is very common but varies globally, from 0.5% in Japan to 100% in Senegal and 30–50% in Europe [18, 248-250]. An interesting finding in our NDFB patient cohort (**Chapter 7**) was that none of the rCDI patients carried *Blastocystis* species [251]. Low *Blastocystis* sp. colonization rates in diseased patients were previously also reported in IBD patients [18-20]. IBD and rCDI patients have a very disturbed microbiota in common. It is unknown if the association between a disturbed microbiota and low *Blastocystis* sp. colonization results from the inability of *Blastocystis* to survive in a disturbed environment. Homeostasis of the microbiota is associated with butyrate-producing bacteria, resulting in oxygen consumption by the colonocytes (for more information, see section "Colonisation resistance against (multidrug resistant) Enterobacterales", this thesis) [141, 143]. The subsequent epithelial hypoxia helps to maintain a microbial community dominated by obligate anaerobic bacteria [143], or oxygen sensitive eukaryotes like *Blastocystis* species [144]. The result of antibiotic related depletion of butyrate-producing bacteria can be observed in some perturbed microbiotas, such as rCDI and IBD, where a shift occurs from obligate anaerobic bacteria belonging to the phyla Firmicutes and Bacteriodetes to the facultative (an)aerobes of the phylum Proteobacteria [242, 252]. The presence of *Blastocystis* in half of the patients after transfer of healthy donor faeces (FMT), could reflect reestablishment of a healthy microbiota after FMT. The second theory encompasses a top-down control of the microbiota by *Blastocystis*, the parasite itself influences the composition of the microbiota by predation or ecosystem management and thereby creates a more diverse microbiota [27]. Evidence of the predatory capacity of *Blastocystis* on bacteria is shown by the capability of bacterial engulfment [253] and the low frequency of the ameboid form in axenic cultures. In this case, the transfer of *Blastocystis* sp. by FMT could enhance the microbial diversity of the patient more than non-*Blastocystis* containing faecal suspensions would.

In general, the concept is increasing that *Blastocystis* is a marker for a healthy microbiota [18, 22-27]. We showed that FMT containing *Blastocystis* ST1 or ST3 did not result in an altered treatment efficiency or gastrointestinal symptomatology (**Chapter 7**). Therefore, *Blastocystis* ST1 and ST3 should be deleted as donor exclusion criterion, although screening and long-term follow-up of the patients is preferred. Additionally, FMT trials for rCDI and other indications should allow *Blastocystis* positive donors and test whether this leads to a higher efficiency to cure disease with FMT.

Quality assurance of faecal suspensions

With the emergence of FMT as new treatment approach, stool banks are needed to provide ready-to-use donor faecal suspensions that are produced in a standardized way [254]. A donor faecal suspension is however not a standardized drug that is produced in a factory, but a highly diverse and donor-specific microbiota in its broadest sense, also known as substance of human origin (SoHo; blood, tissues, cells and organs) [255]. This implies that faecal suspensions and subsequently stool banks require (inter)national guidance of quality and safety measures, comparable of other SoHo therapies [255]. Significant advantages of centralized donor screening and production of donor faecal suspensions are the possibilities to provide quality assurance, standardisation of manufacturing and appropriate monitoring of unexpected adverse events. The current FMT product manufacturing protocols are for a large part based on expert opinion [256, 257], and optimized for treatment of *C. difficile*. The FDA recently published "Regulatory considerations for FMT products", in which it is stated that the stability and viability testing should be considered for FMT products used for clinical trials [258]. In Europe, the Guide to the quality and safety of Tissues and Cells for human application (Tissue Guide) of the European Council includes a chapter about FMT, that is currently revised and may serve as a reference for quality assurance of FMT in Europe.

Viability of anaerobic bacteria during processing and manufacturing

Faecal suspensions for FMT are most often produced in ambient air (aerobic preparation). A recent *in vitro* study applied Propidium Mono Azide (PMA) to measure the viability of bacteria after aerobic and anaerobic processing. PMA is a fluorescent dye which selectively enters cells with a compromised cell membrane. Upon exposure

to light, PMA covalently binds to DNA in these cells or naked DNA, thus leaving only viable cells available for PCR amplification [259]. An optimization of this method to apply on stool samples has been developed [260]. The study of Papanicolas and colleagues showed that aerobic processing decimated the yield of delicate obligate anaerobic bacteria like *Faecalibacterium* sp., *Eubacterium rectale*, *E. halli*, *Subdoligranulum* sp., Anaerostipes, *Megamonas*, *Bifidobacterium* and *Roseburia* up to 12-fold [259, 261]. Other taxa were found to be more oxygen resistant such as *Bacteroides*, *Parabacteroides*, *Barnsiellaceae* or *Rikenallaceae* [261]. In the case of rCDI, this aerobic degradation has little or no impact on clinical efficacy as studies using anaerobically produced FMT suspensions do not report a significant increase in rCDI cure rate (cure rate of 80 %) [262-264]. For other indications such as IBD or hepatic encephalopathy, where the therapeutic component is poorly understood, variation of the number of living anaerobic bacteria could theoretically have significant effect on the clinical outcome. In the ulcerative colitis RCT of Moayyedi and colleagues a super donor with high levels of butyrate-producing bacteria was found [204]. These butyrate producing bacteria often belong to the Firmicutes, a phylum disproportionally affected by oxygenic stress [261, 265]. Manufacturing faecal suspensions in ambient air resulted in a more than 2.5-fold reduction in relative abundance of butyrate-producing bacteria. Consequently this impacted the level of the gene encoding a terminal enzyme in the dominant pathway of butyrate biosynthesis (butyryl-CoA:acetate CoA-transferase gene), and subsequent post-fermentation of butyrate levels was reduced with approximately 50 % [259]. In contrast to oxygen exposure, lag time (time between defecation and processing) and freeze-thaw steps didn't seem to alter the living microbiota much, both in absolute amount as well as the composition [259, 261, 266]. Altogether the loss of butyrate-producing, and obligate anaerobes combined with the relative overabundance of oxygen tolerant bacteria could potentially transform a healthy donor microbiota into faecal suspensions containing a microbiota profile more closely resembling those of the patients.

Viability of anaerobic bacteria during storage

Two RCTs and one meta-analysis showed non-inferiority and comparable cure rates for the treatment of rCDI with fresh or frozen faecal suspensions (stored at -80°C for up to 30 days) [267-269]. Use of a frozen faecal suspension allows storage at -80°C for a longer period of time until the donor has been retested prior to actual use of the

donor faecal suspension. This lowers the risk of transferring transmissible diseases by bypassing the window of detection phase of some transmissible infections (e.g. HIV, Hepatitis C). Having well-screened donor faecal suspensions in storage will allow a more rapid and safe transplantation when needed, bypassing the logistical difficulties of preparing a fresh FMT suspension. In addition, it allows extended screening and selection of preferred donors and specific faecal suspensions that are required for FMT for non-CDI indications. To prepare frozen suspensions, a cryoprotectant should be added prior to freezing. In general, the cryoprotectant glycerol is used in a final concentration of 10 to 15 %. Cryopreservation is a process of preservation of the biological and structural functions of tissues or cells when cooling to sub-zero temperatures [270]. Viability of six representative groups of faecal bacteria after six months of storage at -80°C in normal saline with 10 % glycerol did not differ from baseline, whereas viability was reduced in suspensions stored with saline alone. Especially, the aerobes, total coliforms and lactobacilli were significantly reduced by >1 log in the faecal suspension stored without glycerol [263]. In addition, the authors conclude that the protective effect of glycerol outweighs the presumed detrimental osmotic effect of glycerol on living cells. Long-term storage should be at -80°C or lower to prevent sample degradation. High cure rates have been reported with frozen FMT suspension stored up to two years -80°C [16, 262, 263, 266, 271-275]. In fact, both the NDFB and OpenBiome concluded that storage duration did not impact the clinical effectiveness of FMT for rCDI patients (**Chapter 6**, [276]). Whether a shelf-life of two years is also applicable for other diseases remains to be investigated, as *in vitro* studies suggest long-term storage does seem to impact some bacteria more than others. To test to what extent donor microbiota communities are affected by the manufacturing and storage procedures at the NDFB, a culturing pilot study was performed by the NDFB in close collaboration with Vedanta Biosciences. Donor faeces was collected and divided in two aliquots, one placed in an anaerobic chamber, the other processed aerobically and frozen within 30 minutes. Both aliquots were serial diluted and inoculated onto eight different selective and non-selective media. PCR and Sanger sequencing was performed on 1288 picked colonies. A general 10-fold loss in cultivability of anaerobic bacteria was found during processing in combination with storage at -80°C and subsequent thawing. Bacillus species, and *Anaerostipes hadrus* were identified [277]. In a study that subjected fresh and frozen faecal microbiota suspensions to stress conditions that bacteria may undergo after transplantation in the

human gut with FMT, the results showed that the abundance of Bacteroidetes decreased with longer storage times [278], in particular when stored beyond 15 months of storage at -80°C (with glycerol). In contrast, Firmicutes showed good resistance to a harsh DNA extraction protocol, including Proteinase K treatment (solubilizes solid human tissues, disrupts biofilms), DNAse treatment next to a chaotrophic agent (guanidine hydrochloride; disrupts human cells and also has affinity for Gram negative bacteria). More specifically, Operational Taxonomic Units (OTUs) of butyrate producing bacterial species, showed relatively little changes of relative abundance when frozen samples were compared to fresh samples [278]. The question remains if this pre-treatment of the faecal suspensions represents the *in vivo* conditions after transplantation. However, an *in vivo* mice experiment showed similar results. In this study, the viability was assessed using 16S rRNA analysis after PMA pre-treatment in fresh faeces compared to faeces stored at -20°C. The viability of frozen faeces was comparable with fresh faeces [279], but after transplantation in mice, some bacterial taxa were attenuated in enteric colonization ability when stored frozen. Bacteroidetes, next to Actinobacteria and Deferribacteres showed less resilience or colonization ability after freezing at -20°C for more than 1 month [279]. A second mice study used a complementing technique to test the viability of transplanted microbiota by labelling the gut microbiota *in vivo* of donor mice with a fluorescent marker. After FMT of the fluorescent donor microbiota, the recipient mice received a second fluorescent marker with another colour. The viable (metabolic active) portion of the donor microbiota incorporates both markers and can readily be distinguished from dead donor bacteria. 16S rRNA analysis indicated that several bacterial genera were enriched, including Gammaproteobacteria, *Clostridium XlVb* and *Butyricicoccus*. Although FMT in this study is probably less efficient because the donor microbiota was administered by gavage and therefore did not bypass the acid stomach, the viability of donor *Clostridium XlVb* and *Butyricicoccus* strains is encouraging, since these are generally considered to be beneficial to the host [138].

Proposed quality control for viability and stability of faecal suspension microbiota

Some of the studies show a substantial donor variation in the viable component of faecal suspensions affected during manufacturing and storage [138, 259, 278, 279]. This could be explained by individual variation in microbiota composition, resulting in

a different vulnerability of the microbiota to stressors as oxygen exposure or freezing. The composition and function of a healthy individual's microbiota is in general stable, and resilient to most perturbations (low intra-donor variability) **[280]**. However, minor changes in environmental factors such as diet, medicine use, season, travel or household contact can have large effects on the microbiota **[281-283]**. The potential differences in intra- and inter-donor stability and viability during processing indicate the need for viability assays performed as quality control. Promising as relatively quick and less expensive screening tool for viability of the microbiota of a faecal suspension is a combination of staining (for instance a classical Live/Dead stain (based on fluorescein diacetate (FDA) and propidium iodide (PI), which stain viable cells and dead cells, respectively) and flow cytometry. It has the potential of facilitating the analysis of complex ecosystems through visualizing the changes in the dynamics of bacterial communities **[284]**. This can be combined with periodic deeper microbiota assessment with a subset of different methodologies to provide more detailed information. Analysing the microbiota of sequential faecal samples with a combination of culturomics, 16S analysis and flow cytometry showed that the various methods were additive to each other **[205]**. In addition, culturomics showed the relevance of using sequential samples as many bacteria were found irregularly as the faecal microbiota may, to some extent, change daily **[205, 285]**. In the end, investigating the functional microbiota, for instance by means of the pool of genes is the most important, as the functional traits of the microbiota should be maintained, and are not necessarily provided by the same organisms **[286]**. Further research should focus on the best strategy for quality control of faecal suspensions for FMT treatment. Most likely this will involve multiple of the above-mentioned techniques once every 6 months to 1 year, in addition to a more frequent performed basic microbiology viability and stability check which encompasses culturing of several indicator anaerobes. Careful clinical follow-up is the ultimate quality control and should be organised by stool banks to establish the safety of their protocols.

Future of stool banking

Stool banks were initiated to implement safe and cost effective FMT. Gradually, stool banks became expertise centres with experts in the fields of microbiology, gastroenterology, infectious diseases, biobanking, data science, microbiome research

and pharmacy. This may result in multidisciplinary trials addressing the effects of microbiota modulating therapies in a wide range of disorders. Stool banks also enable fundamental research addressing both pathogenesis, functional microbiota networks, and mechanism of action to develop new treatment concepts.

An interesting new application of the experience and expertise of stool banks is the banking of faeces for auto-transplantation. In case of an expected and undesired major change of the gut microbiota such as patients undergoing a stem cell transplantation, a stool bank can facilitate with storage of pre-event faeces. A second interesting application is the banking of faeces of patients that respond well to anti-cancer therapy. The NDFB will participate in an RCT phase Ib trial in metastatic melanoma patients refractory to immune checkpoint inhibitors (ICI) receiving either an FMT of an ICI responder or non-responder patient (prof. J Haanen, Oncology LUMC and AvL). The NDFB will in this case not provide faecal suspensions of healthy donors but collaborates with the knowledge on microbiota modulating therapies (e.g. patient-donor selection and screening, manufacture of faecal suspensions, biobanking an preservation of the microbiota, selection and pre-treatment of patient, FMT treatment itself and follow-up of the patients. The rationale for this trial is that pre-clinical data indicates that the gut microbiota controls the immune response and subsequent response to ICI. The use of antibiotics within the first 3 months prior to initiation of ICI has been demonstrated to negatively influence the treatment response **[206, 287, 288]**. Tumour bearing mice demonstrate that FMT of responder-patients can improve the anti-tumour immune response, and when combined with ICI can improve outcome **[206, 207]**.

Banking faecal microbiota suspensions for FMT is a new research field and is constantly evolving and developing. Already in the beginning, FMT was recognised to have great potential to cure microbiota related diseases. The strength of this treatment is the transplantation of a complete ecosystem. Nevertheless, the weakness of this therapy also lies in transplanting a complete, but uncontrolled, unstandardized and not fully understood ecosystem. An undesired pathogen or disease trait could be co-transplanted. To limit risks, standardisation of working processes of stool banks was established and standard operating procedures were formulated addressing; the recruitment, selection and screening of donors, processing and manufacturing of the

donor faeces and storage and distribution of frozen faecal suspensions, together with selection, treatment and follow-up of patients both on institutional and national as international level (**Chapter 5**) [241, 257]. Recommendations are regularly updated and adapted to new situations, such as the recent new advices to screen donors for the presence of enteropathogenic *E. coli*, MDROs and SARS-CoV-2 [289, 290]. The risk of infectious complications after FMT depends in part on appropriate donor screening. This may even be more important for severely immunocompromised patients, as suggested by the cases where transfer of MDRO by FMT in neutropenic patients resulted in sepsis and death [291]. Following these cases and the subsequent FDA warning, the NDFB evaluated their screening protocol, with periodic screening every three months and targeted rescreening after foreign country visits. Although 25 % of active donors became MDRO positive at some point during their donation activities, the current NDFB screening protocol did not result in approval of MDRO-positive faecal suspensions for FMT treatment (K.E.W. Vendrik et al., Lancet Infectious diseases, in press). However, although the residual risk of transmission of MDROs appears acceptable for most patients, this risk appears not acceptable for severely immunocompromised patients based on the above-mentioned cases. Therefore, the NDFB performs direct screening of suspensions used for immunocompromised patients [292]. These studies are a step towards a more evidence-based way of donor screening, and stool banking. Setting up a national or even international registry both for donor and patient follow-up data would lift FMT as quality-assured treatment strategy to the next level.

Future of microbiota modulating therapies

In recent years FMT has been implemented worldwide as effective rescue therapy for patients with multiple recurrent CDI, with cure rates of approximately 85 % [83, 293-295]. Transplanting faecal microbiota of a healthy donor with the aim to restore a patient's perturbed microbiota appears also promising for several other disorders, such as ulcerative colitis, hepatic encephalopathy and a subset of inflammatory bowel syndrome patients [211, 296-299]. Furthermore, many of the previously discussed indications are interesting and merit further research. It is illustrative that while writing this discussion, more new indications and applications pop-up as potential target for microbiota modulating therapy, such as FMT

for immune modulation of patients suffering of severe COVID-19 [300], or patients with systemic sclerosis [301] and patients with alcohol use disorder [302].

Faecal suspensions for FMT contain a highly diverse microbial ecosystem. Because of the unstandardized nature of this treatment, and potential risk of transfer of unrecognized pathogens or disease traits, a more controlled and standardised treatment is desired in the future. Most newly developed microbiota modulating therapies involve synthetic bacteria or bacterial communities (live biotherapeutic products). In the future well-regulated and characterized live biotherapeutic products are preferred over probiotics which are not regulated and can be sold without quality check as food additive. Most probiotic companies do not characterize the microorganisms or assess the presence of AMR and virulence genes. Recently the scientific community was startled by the finding that the probiotic *E.coli* Nissle 1917 contained colibactin encoded by a pks island [303]. Colibactin is tumorigenic in murine models and more prevalent in patients with colorectal cancer compared to healthy controls [304]. In the past, an unexpected increased mortality caused by the probiotic Ecologic 641 (a mixture of *Lactobacillus*, *Lactococcus* and Bifidobacteriae) was reported in patients with acute pancreatitis participating in the PROPATRIA study [305]. This was explained by the finding that the disrupted intestinal barrier of the patients with concomitant organ failure, in combination with the probiotic strains, resulted in increased bacterial translocation and enterocyte damage, with subsequent mortality in 16 % versus 6 % in the placebo group [306]. This illustrates that selecting an unbalanced mix of several "beneficial" strains, without complete understanding of the function, effects and their interaction with and within the host is not without risks. In a landmark paper on treatment and prevention of antibiotic induced perturbation of the microbiota, it was shown that a commercially available 11-species probiotic markedly delayed indigenous gut mucosal reconstitution after antibiotic exposure [307]. Compared to spontaneous post-antibiotic recovery, the microbiome reconstitution (both in composition as well as in transcriptome) was not only delayed, but also remained incomplete by the end of the intervention period (day 28) or five months after probiotics cessation. An auto-FMT induced rapid and near-complete recovery within eight days [307].

Many host-microbiota interactions pertaining with human health and disease are mediated by metabolites. These metabolites can be secreted, degraded or modified

by the gut microbiota or the host, or given as encapsulated therapy. By bypassing the transfer of live bacteria (e.g. in a bacterial mix or FMT), but instead provide metabolites, some of the caveats of current microbiota modulating therapy can be overcome, such as transfer of opportunistic pathogens or unwanted effects on unrelated conditions, or the individual variation in colonization resistance and engraftment of donor strains **[308]**. Microbiota associated metabolites of interest are short- or long-chain fatty acids, bile acids, vitamins or polysaccharides. This therapy aims at impacting their downstream signalling pathways when relevant to pathogenesis of disease. Microbial molecules of therapeutic potential are not limited to secreted metabolites, but may also include cellular components, such as membrane proteins **[117]** or even sterilised bacteria **[309, 310]**.

During the establishment of the NDFB in 2015, it was believed that other microbiota modulating therapies (for example live biotherapeutic products) would have replaced FMT for rCDI within five to ten years. To date, not a single microbiota drug has shown significant and relevant treatment outcomes for rCDI **[101]**. Rebuilding a well characterised synthetic microbiota community with the capability of resilience to *C. difficile* infection and relapse is much more difficult than previously thought. Once such strategies are of proven benefit in the future, this may result in effective and safe new drugs to cure and prevent rCDI and replace FMT as treatment approach. At present, transplanting a healthy faecal microbiota with the aim to restore a patient's perturbed microbiota remains the standard therapy for patients with multiple recurrent CDI, and is promising and performed in research setting for many other diseases. For development of more sophisticated precision microbiota therapeutics, FMT will pave the way by providing mechanistic insights in the effects of the transplanted microbiota on a specific disease. In the future, preferably an arsenal of several precision microbiota therapeutics would stand to our disposal which should be administered on a tailored basis as a personalised microbiota modification treatment.

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