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

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Chapter 1

Introduction

Chapter 1. Introduction

Antibiotics provided humanity resilience to the majority of bacterial infections. It thereby altered the natural course of most infectious diseases and saved millions of lives. One could argue that antibiotics are the most significant development in modern medicine. An important trade-off is however, the emergence of antimicrobial resistance, and a diminished and perturbed microbiota, resulting in an increased susceptibility for *Clostridioides difficile* infections and Western (lifestyle associated) diseases [1-3]. The World Health Organization (WHO) has declared antimicrobial resistance one of the greatest challenge to global public health today, compromising the treatment of common bacterial infections [4]. More specifically, the Centers for Disease Control and Prevention (CDC) has outlined and prioritized the threats posed by specific multidrug Resistant Organisms (MDRO) of which drug resistant *Neisseria gonorrhoeae*, *Candida auris*, carbapenem resistant Enterobacterales and *C. difficile* were the most urgent [5].

To understand the role of the microbiota in defence against various infectious and non-infectious diseases, we need to define and measure the human microbiota by well accepted and standardized techniques, including methods to determine the function of the microbiota. This thesis focusses on the significance and possible interventions of the gut microbiota of patients colonized with antibiotic resistant bacteria or patients suffering from multiple recurrent *Clostridioides difficile* infection. This introduction summarizes the basic knowledge on the human microbiota of healthy and diseased individuals, the worldwide problem of increased antibiotic resistance and the threat of antibiotic-associated *C. difficile* infections.

The microbiota

The human gut microbiota

The human body houses a diverse microbial ecosystem, consisting of bacteria, archaea, fungi, viruses and parasites, together called the 'microbiota'. Because bacteria reach the highest density, the bacterial fraction of the microbiota is most studied. It is estimated that the ratio of bacterial to human cells in an adult body is 1:1 [6], and the collective bacterial genome contains 450-fold more genes than the human genome [7]. Each body site (e.g. gut, skin, vagina, stomach, oral cavity) has a different microbiota composition. With 10^{10}-10^{11} bacterial cells per gram feces, from approximately 1000 different species, the colon contains the largest number of bacteria [8]. The bacterial part of the gut microbiota is largely composed of two groups at the phylum level, the obligate anaerobic Bacteroidetes and Firmicutes [9]. In addition, the gut microbiota comprises of members of the Actinobacteria, Verrucomicrobia, Fusobacteria and Proteobacteria phyla [9]. It is important to realize that classification to phylum level gives a rather simplistic view of an extremely complex ecosystem *(Figure 1)*. Humans belong at phylum level to the Chordates, just like a Komodo dragon and a dolphin for example. Subsequently, the microbiota can be further subdivided at different levels; Phylum, Class, Order, Family, Genus and (sub) species *(Figure 1)*.

In general, it is believed that colonization, competition and engraftment of the human microbiota starts during labour. However, the "sterile womb" paradigm is currently challenged by several studies reporting bacterial communities in the intrauterine environment by next generation sequencing [10]. Others have found that viable bacterial colonization is highly limited in the foetal intestine or placenta, however can contain potential pathogens [11,12]. Still, the consensus is that current scientific evidence does not support the existence of a foetal microbiota [13], and that the finding of bacteria in the intrauterine environment is probably explained by contamination (e.g. of the kit, background DNA) [14,15]. Irrespective of the precise starting point of colonisation, birth gives microbiota development a boost. The human microbiota is acquired both vertically (via vaginal birth from mother) and horizontally (from the environment). Consequently, birth mode (vaginal or caesarean section) has significant effects on the microbiota development and composition [16]. In addition, early life microbiota succession is

influenced by, gestational age and breastfeeding. At two to three years of age, microbiota composition becomes more stable and adult-like. [17,18] The adult-like gut microbiota is functionally more complex and is structured to metabolize plant-derived polysaccharides from the diet [19]. The primary determinant of the microbiota composition is body site or habitat [9,20,21]. Within habitats, the interpersonal variability is high, whereas individuals exhibit only minor temporal variability [21]. Although the microbiota is constantly exposed to environmental stressors, its composition and function in an individual are relatively stable against most perturbations [22]. This resistance to change is described as resilience; the property of a microbial community that defines how fast, and to what extent, it will recover its initial functional or taxonomical composition following a catastrophic perturbation [22]. Still, minor changes in environmental factors such as diet, medicine use, season, travel or house-hold contact can affect the microbiota [23-25]. A recent study showed for instance that a large percentage of non-antibiotic drugs can inhibit the growth of certain bacteria, or even complete bacterial classes [24]. In fact, 24% of human drugs, amongst all therapeutic classes, inhibited bacterial growth of at least one bacterial strain [24]. Surprisingly, the chemically diverse antipsychotics were overrepresented as microbiota effectors. One could even speculate that regular use of pharmaceuticals nowadays may contribute to the decrease in microbiota diversity of the modernized human populations [26,27].

Kingdom	Bacteria						Animalia	
Phylum	Firmicutes	Bacteroidetes	Actinobacteria	Verrucomicrobia	Fusobacteria	Proteobacteria	Chordata	Chordata
Class	Clostridia	Bacteroidia	Actinobacteria	Verrucomicrobiae	Fusobacteria	Gammaproteobacteria	Mammalia	Reptilia
Order	Clostridiales	Bacteroidales	Bifidobacterales	Verrucomicrobiales	Fusobacterales	Enterobacterales	Primates	Squamata
Family	Ruminococcacae	Bacteroidaceae	Bifidobacteriaceae	Akkermansiaceae	Fusobacteriaceae	Enterobacteriaceae	Hominidae	Varanidae
Genus	Faecalibacterium	Bacteroides	Bifidobacterium	Akkermansia	Fusobacterium	Escherichia	Homo	Varanus
Species	prausnitzii	thetaiotaomicron	breve	muciniphila	necrophorum	coli	sapiens	komodoensis

Figure 1. Taxonomic classification of the most abundant bacterial phyla present in the gut.

Subsequently, phyla can be further subdivided at different levels; Class, Order, Family, Genus and (sub) species. From each of the most dominant phyla present in

the gut, an example of a bacterium from phylum to species level is depicted. For comparison also humans and a Komodo dragon, belonging to the same phylum, are displayed in the Animalia kingdom.

Techniques to study the microbiota

The microbiota can be studied by several techniques. In the past, researchers depended highly on culturing techniques. However, many bacteria are very difficult to culture. With recent innovations in the field of sequence technology and analysis, scientists are now able to determine and analyse these difficult to culture bacteria. In the 2010-2020 decade, sequencing of a small part (for instance the V4 region of about 250 nucleotides) of the 16S ribosomal RNA gene, was most commonly used. The 16S rRNA gene consists of around 1500 nucleotides and contains regions conserved among all bacteria and archaea, interspersed with nine regions (V1 to V9) that are highly variable among bacterial phylotypes. Phylotypes are defined as a group of 16S sequences having 97-99 % sequence identity, and usually equals taxonomically to genus or sometimes species level. Because only a small part of the complete genome is sequenced, one can determine the composition and relative abundance of bacterial taxa present in a sample in a relatively fast and inexpensive way. Samples are generally compared using alpha-diversity (within-sample diversity; one value per sample, an example is a Shannon-diversity index) and beta-diversity (between-sample diversity; pair-wise values for all sample combinations, an example is Unifrac-distance or Bray-Curtis-dissimilarity). Bacterial abundance and composition gives insight in 'who is present', which does not necessarily describe the functionality ("what are they doing"). To analyse the functional potential, metagenomic shotgun sequencing is more suitable **[28]**. This technique shotgun sequences the total DNA of the microbiota. Therefore the complete genomic make-up of the microbiota (which is called, the 'metagenome' or more frequently the 'microbiome') is assessed, and one can not only determine the composition, but also predict the potential functions of the microbiota. In *Figure 2*, a general overview of pipelines of 16S and metagenomic shotgun sequencing is depicted.

While with 16S analysis only the 16S rRNA containing bacteria can be studied, metagenomics is not limited to sequencing bacteria, the microbiota including viruses, parasites and fungi, can be analysed. Importantly, analysing the results of sequencing

is challenging and relies on specialized and skilled bioinformatic experts. In addition, metagenomic sequencing is expensive, and one approach to lower the costs is minimisation of sequence depth or coverage. Coverage is the number of unique reads that include a given nucleotide (copy number) in the reconstructed sequence [29]. (Ultra) deep sequencing refers to the general concept of aiming for high copy number, which allows for detection of lowly abundant species or sequence variants in mixed populations. To reduce the amount of data and lower the costs, the sequence depth can be decreased. This is referred to as shallow sequencing [30]. A complete overview of all techniques studying the microbiota with all advantages and limitations is shown in Table 1.

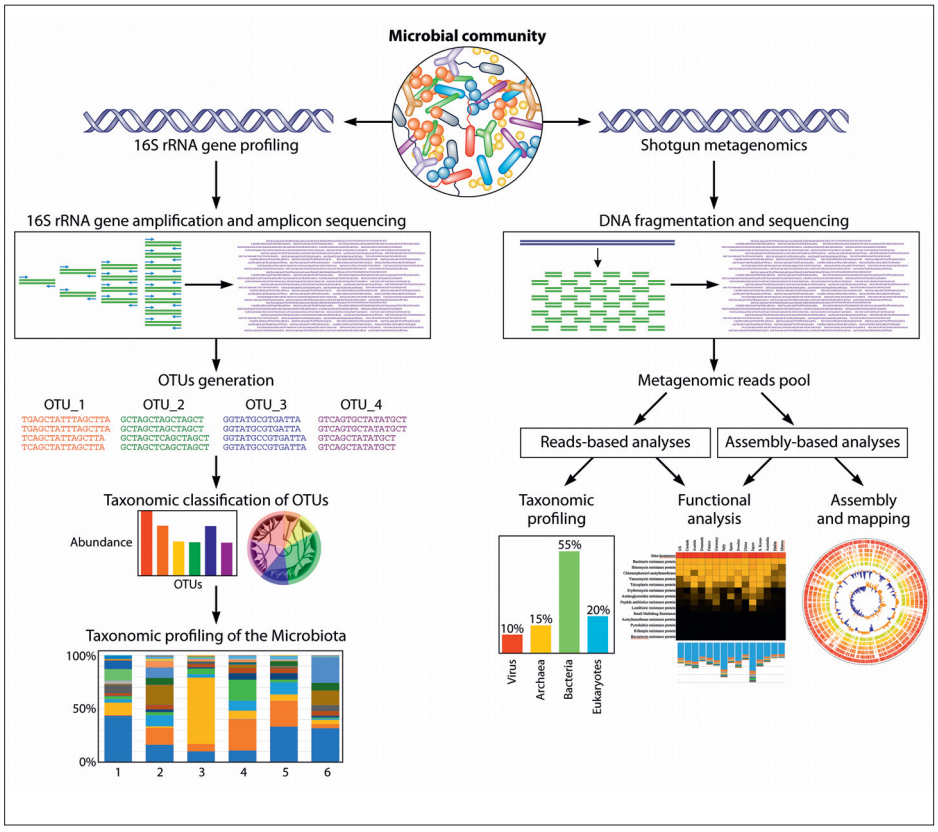

Figure 2. 16S rRNA sequencing and metagenomics pipeline.

Adopted from Milani and co-workers [31].

General overview of the bioinformatic pipelines for the 16S rRNA gene (amplicon) sequencing on the left and shotgun metagenomics on the right. First microbial DNA is extracted and subsequently sequenced. With 16S rRNA analysis, only (part of) the 16S rRNA is sequenced. Highly similar sequences are grouped into Operational Taxonomic Units (OTUs), which for identification can be compared to a 16S database. The microbiota community can be described in terms of which OTUs are present, their relative abundance, and/or their phylogenetic relationships. On the right, metagenomic shotgun sequencing of the microbiota is depicted, in which the total extracted and fragmented DNA is shotgun sequenced. The resulting DNA sequences are either pieced together using assembly algorithms or reference databases, or analysed in an unassembled manner to monitor whole-community functional capabilities. The phylogenetic origins of microorganisms and their functions can be determined by comparison with previously annotated genes in a database.

A famous project trying to unravel the composition and function of the microbiota is the Human Microbiome Project *(Figure 3)*. This project illustrates the value of the use of a combination of different techniques; the difference between 'who is present' is studies with 16S and 'what can they do' with metagenomics. The combination of both techniques revealed that healthy individuals can have a very different microbiota composition, while the relevant functions of those microbiota compositions for their host remained very similar between these individuals **[32]**.

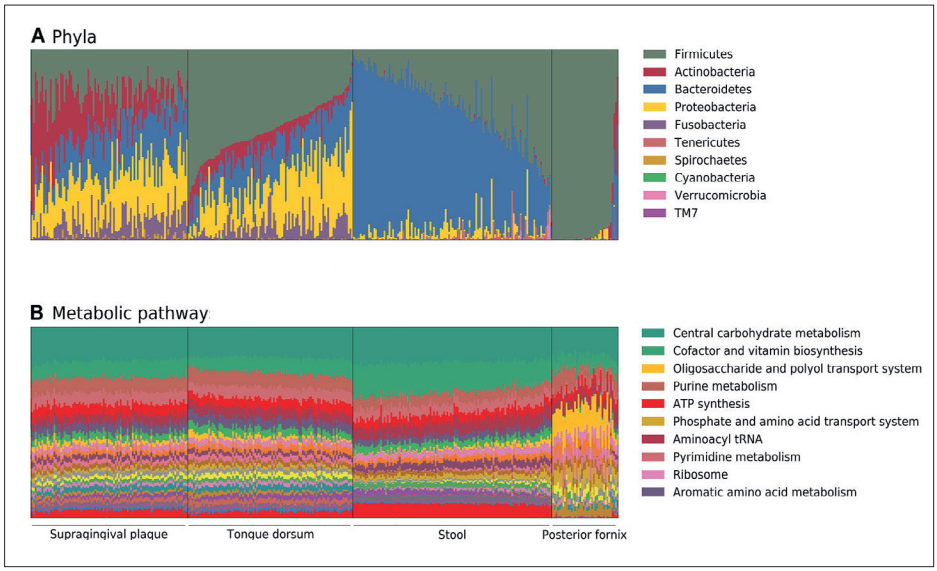

Figure 3. Carriage of microbial taxa in the gut varies while predicted metabolic pathways remain stable within a healthy population.

Adopted from the Human Microbiome Project [32].

Vertical bars represent microbiome samples by body habitat in seven locations with both shotgun and 16S data; bars indicate relative abundances coloured by microbial phyla from binned OTUs (a) and metabolic modules (b). Legend indicated most abundant phyla or pathways by average within one or more body habitats. OTU: operational taxonomical unit.

Measuring the functional capacity of the microbiota with metagenomics does not necessarily provide insight in what the bacteria are actually doing. The following – omics techniques each unravel different layers of information of the microbiota as complex ecosystem *(Figure 4)*. The direct measurement of transcripts (mRNA) with (meta)transcriptomics or proteins with (meta)proteomics is at present increasingly used as complementary technique to metagenomics **[33]**. The combination of metagenomics with metatranscriptomics enables the identification of gene repression or induction under specific conditions, and can additionally distinguish metabolically active from inert or dead micro-organisms **[34]**. Not all transcripts are however translated into proteins. Therefore metaproteomics, in which the expressed proteins are measured with high resolution mass spectrometry, should provide more insight into gut microbial functionality as compared to metatranscriptomics. In addition, it determines proteins derived from the microbiota and their host, which is important when studying microbiota-host interactions. In the past this technique suffered from low measurement depth and lack of efficient bioinformatic tools **[35]**. The availability of new metaproteomic data processing tools has enabled better characterization of the proteome. The fecal metabolome is often regarded as an endpoint read-out of biological processes originated from the gut microbiota and their host. To measure these metabolites, nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry can be applied, known as metabolomics. Identified metabolites in faeces can include those derived from microbiota (e.g. Short Chain Fatty Acids, lipopolysaccharides) or the host (e.g. anti-microbial peptides (AMPs)). Integrating multi-omics data provides a comprehensive overview of microbiota composition, function and metabolomic activity, in relation to its host. Due to the increased complexity and diversity of multi-omics data, efficient bioinformatic tools, advanced statistical methods and machine-learning approaches are needed, which are at present only available in some microbiota expert teams. The multi-omics approach is of particular importance for translational research (microbiome analysis into clinical applications), because chronic human illnesses or diseases associated with a perturbed microbiota are unlikely to be caused by a single bacterium, protein or pathway.

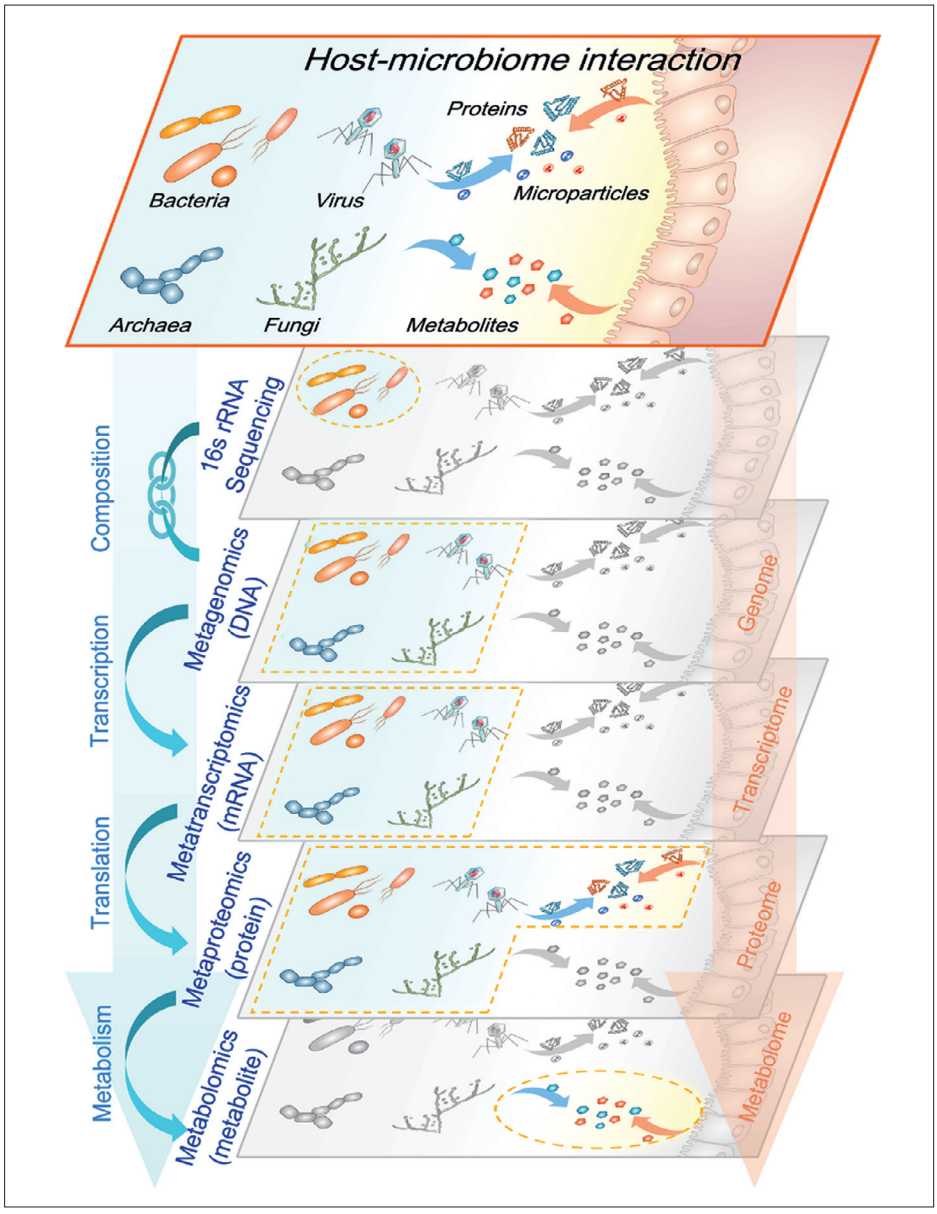

Figure 4. Multi-omics approach of microbiota analysis.

Adopted from Xu Zhang and co-workers [36].

The human gut consists of host and microbial cells, as well as secreted proteins, metabolites, and microparticles, all of which may interact with each other to impact human health. Different meta-omic approaches each examine different aspects of this intestinal ecosystem at different levels with their own advantages and disadvantages.

Molecular techniques to study bacterial strains in depth

To interpret the metagenomic (annotated) data, culturing is still essential. To determine the functions and phenotypes of unknown bacterial genes, culturing of the microbial communities (culturomics) has revived and regained interest [37, 38]. In addition, complete genetical and phenotypical characterisation of cultured isolates is essential to define microbiota interactions (between bacterial species or between bacteria and host) that are difficult or yet impossible to predict unless tested biologically [39].

Once isolated, the complete DNA of a bacteria can be determined with whole genome analysis (WGS). The introduction of next-generation sequencing (NGS) machines had made WGS attainable in terms of costs and time. The generated WGS data can then be subjected to a variety of molecular analyses to characterise the bacterium in terms of antibiotic resistance, molecular epidemiology (e.g. typing) and virulence. Due to its high resolution and inter and intra-reproducibility, WGS is highly suitable as typing method. One means of exploiting WGS data is the identification of single nucleotide polymorphisms (SNPs) that vary among isolates. An alternative approach is multi locus sequence typing (MLST). With common MLST, a limited number of housekeeping genes is sequenced and every sequence variant of a housekeeping gene (locus) is assigned as a distinct allele. For each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST). To increase the resolution and to make optimal use of WGS, many more loci can be employed with core genome MLST (cgMLST) (for *E. coli* around 2500 of total 7700 genes) and whole genome MLST (wgMLST) [40]. An advantage of MLST, cgMLST and wgMLST is that loci used in the schemes are readily conserved and shared among laboratories using online databases. In addition to online (commercial or free available) databases for typing, also many online databases exist to further characterize bacterial isolates with respect to genes or mutations in chromosomes or plasmids associated with resistance, serotypes, plasmids or specific gene functions. A new trend of microbiological characterization is the application of long read sequencing by rapid methods, such as the "MinION" nanopore. Analysis with longer read lengths will alleviate numerous computational challenges surrounding genome assembly as short-read methods can miss some randomly-distributed segments of genomes present in phages, plasmids and virulence factors. Therefore long-read sequencing provides the tool to study the presence and composition of antibiotic resistant genes containing plasmids [41].

Table 1. Techniques to study the microbiota, description, advantages and limitations.

Adapted from **[42]**

Technique	Advantage	Limitation
Marker gene sequencing (16S, ITS)	• Low cost • Relatively quick and easy to manage and interpret • Also suitable for low-biomass and highly host-contaminated samples • Large, available public databases	• No discrimination between dead or alive bacteria • Only determination to Operational Taxonomic Unit (equivalent to genus or sometimes species level) • Amplification bias because of targeted primer (sensitivity and copy number among species differs) • Choice of primers and variable region (V1-V9) magnifies biases • No functional information
Metagenomic analysis	• Determination and relative abundance of microbial functional genes, microbial taxonomic and phylogenetic identity to species and strain level (for known organisms) • Captures the complete microbiota (bacteria, phages, viruses, plasmids, microbial eukaryotes) • Can be mined for novel gene families • Possibility to assemble population-averaged microbial genomes	• Relatively expensive, laborious and complex sample preparation and analysis. • No discrimination between dead or alive bacteria • Contamination from host-derived DNA and organelles may obscure microbial signatures. • Significant proportion of data cannot be functionally assigned due to a lack of close matches in reference databases. In particular with viral data (where over 80% of sequence reads have no known match) • Often difficult to assign function unambiguously based on sequence similarity alone • Can be difficult to assemble genomes, particularly from less abundant members of the microbiota or when a community contains many closely related species. This means that, even if a function can be ascertained, it may be difficult to assign it to specific species within the whole community. • Population-averages microbial genomes tend to be inaccurate owing to assembly artefacts
Metatranscriptomics	• Can estimate which microorganisms in a community are actively transcribing (if paired to marker gene or metagenomic analysis) • Can discriminate between active and alive versus dormant or dead microorganisms or extracellular DNA • Captures dynamic intra-individual variation • Directly evaluates microbial activity, including responses to intervention and event exposure	• Expensive, laborious and complex sample preparation and analysis • Rely on obtaining sufficient high-quality RNA from the sample (challenging due to ubiquitous RNAses in host-derived samples) = fast processing is mandatory • Saturated with less informative, highly abundant transcripts (i.e. ribosomal proteins, major outer membrane proteins) obscuring the detection of functionally important, but less abundant transcripts • Requires paired DNA sequencing to decouple transcription rates from bacterial abundance changes
Metaproteomics	• Can measure which proteins are produced by active members of the microbiota • Measures not only microbiota but also proteins of the host (essential for microbiota – host interactions)	• Expensive and complex data analysis • Lower depth of measurement compared to metagenomics and metatranscriptomics, and can only capture 10-20% of expressed protein. MS spectra can also be saturated with the highly abundant proteins from dominant species, issue likely to be resolved by increasing the speed of time of MS scanning. • Lack of universal guidelines and protocols for proper performance and analysis of metaproteomic experiments
Metabolomics	• Can measure which proteins are produced by active members of the microbiota • Measures not only microbiota but also proteins of the host (essential for microbiota – host interactions)	• Expensive and complex data analysis • Difficulty to distinguish host- and microbiome-origin metabolites and directly link metabolites to specific taxa. Co-variations between metabolites and microbial species not yet known.

Microbiota and colonization resistance

In a healthy state, there is a symbiotic relationship between the host and the microbiota. The microbiota fulfils many functions which are of benefit for the host. Similarly, host factors are required to maintain a balanced microbiota. The gut microbiota contributes to host health by, amongst others degradation of carbohydrates (food), synthesis of bioactive substances (for example vitamins) and colonization resistance against pathogens [43]. Colonization resistance is the mechanism whereby the microbiota protects against colonization of exogenous and often pathogenic microorganisms. The importance of a healthy indigenous intestinal microbiota for the presence of colonization resistance was first recognized in the 1950s, and it was initially referred to as "antibiotic associated susceptibility" [44]. When pre-treated with streptomycin, a minimal infectious dose of only 10 instead of 10^6 *Salmonella enterica* bacteria was sufficient for mice to become infected [45]. Together with the observation that the susceptibly decreased when mice were exposed to coprophagy with normal mouse feces led to the idea that antibiotics cause a perturbation of the gut microbiota [45]. Colonisation resistance is the result of direct or indirect factors. Direct colonisation resistance refers to the direct suppression of intestinal pathogens by competitive exclusion (competition for nutritional niches or space) and by antimicrobial activities like bacteriocins [46]. For instance, *Bacteroides thetaiotaomicron* consumes carbohydrates used by *Citrobacter rodentium*, a gastro-enteritis pathogen in mice [47]. *Bacteroides thuringiensis* secretes thuricin, a bacteriocin (small-spectrum antibiotic) that directly targets spore-forming Bacilli and Clostridia, including *Clostridium difficile* [48]. In addition, commensal bacteria can also indirectly control invading pathogens by enhancing host immunity and mucus production in the intestines. The microbiota plays an important role in the development, training and maintaining of the immune system [49]. An example of this has been observed with *B. thetaiotaomicron* which can induce the host to produce antimicrobial C-type lectins that target Gram positive bacteria [50].

Gut microbiota and disease

The significance and role of many bacterial species in health and disease are poorly understood, but it has become evident that the gut microbiota is disturbed in a wide

range of diseases. This perturbation in function and composition of the microbiota is called dysbiosis, which is still difficult to distinguish from homeostasis or healthy microbiota, because a healthy reference or core microbiota is not (yet) defined. Importantly, dysbiosis is not only associated with intestinal disorders, such as inflammatory bowel disease (IBD; morbus Crohn and ulcerative colitis) **[51-53]** and irritable bowel syndrome (IBS) **[54, 55]**, but also with a wide range of extra intestinal conditions, such as metabolic syndrome **[56-58]**, (non)-alcoholic fatty liver disease (NAFLD) **[59-62]**. Neurological diseases like Parkinson's disease and multiple sclerosis, and psychiatric disorders are also considered to be associated with intestinal dysbiosis via the so-called hypothetical gut-brain axis **[63-66]**. Whether the microbiota is truly involved in the pathogenesis of those disorders awaits to be seen, but for many diseases, a role in the development or course of the disease has been shown in animal models **[9]**. Interestingly, the microbiota is not only involved in the pathogenesis, but also alters the pharmacokinetics or may mediate (side effects of) certain drugs direct or via CYP-like proteins **[67, 68]**. The microbiota is therefore, of importance in drug discovery, risk assessment and dosing regiments for various infectious and non-infectious diseases.

It is foreseen that all above mentioned diseases have a disease-specific profile of dysbiosis, although a conclusive description of dysbiosis in specific disorders is still lacking. In general, dysbiosis is characterized by a reduced diversity of the microbiota, with a reduction of certain species of the normally abundant Firmicutes or Bacteroidetes phylum (such as Clostridium cluster IV an XIVa, *Faecalibacterium prausnitzii*, *Akkermansia*, *Eubacterium halli*) and a higher abundance of the normally less abundant (opportunistic) Proteobacteria (like *Escherichia coli* or *Klebsiella* species). Whether such a perturbed microbiota is involved (driving or maintaining) in the pathogenesis or an epiphenomena (a consequence of the disease) is not yet elucidated for many diseases. In this regard, *Clostridioides difficile* infection appears unique, as dysbiosis of the microbiota is mandatory. Infection with *C. difficile* represents the classic example of a disease that is caused by a dysbiotic microbiota, providing a model to study the dysbiotic microbiota and interventions targeting dysbiosis.

Clostridioides difficile infection as result of intestinal dysbiosis

Introduction & pathogenesis

Clostridioides difficile is a Gram-positive, spore forming, obligate anaerobic bacterium that was identified as part of the normal gut flora of healthy infants in 1935 [69]. The species name is derived from the initial difficulties to culture and identify *C. difficile*. The genus name was used for more than 80 years, but recently, based on phenotypic, chemotaxonomic and phylogenetic analyses, a novel genus *Clostridioides* gen. nov. has been proposed for *Clostridium difficile* as *Clostridioides difficile* [70]. Fortunately, the abbreviation remained intact. *C. difficile* is considered as part of the gut commensal microbiota of both humans as well as animals and is transmitted by spores via the fecal-oral route. Most vegetative *C. difficile* bacteria are killed in the stomach [71]. *C. difficile* spores are however acid resistant and will subsequently pass the stomach. After germination of the spores in the small intestine under the influence of bile salts, vegetative bacteria enter the colon where they can remain inactive (asymptomatic colonization) or cause an infection (CDI, *C. difficile* infection) varying from self-limiting and mild diarrhoea to life-threatening pseudomembranous colitis *(Figure 5)* [72]. Several virulence factors, including flagella and hydrolytic enzymes have been associated with disease [73].

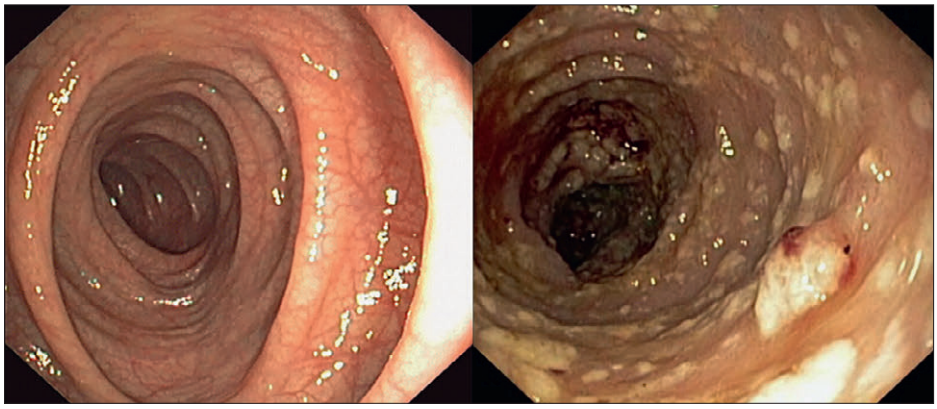

Figure 5. Healthy colon mucosa (left) versus Pseudomembranous colitis due to a *Clostridioides difficile* infection (right).

Adapted from Terveer and colleagues [74].

The two most important virulence factors of *C. difficile* are the exotoxins that are produced, toxin A and B. Both toxins are cytotoxic for a number of different cell types, increase vascular permeability by opening tight junctions between cells, and cause apoptotic cell death [72,73]. In addition, the toxins induce an inflammatory response mediated by tumour necrosis factor-alpha (TNF-α) and pro-inflammatory cytokines which contribute to the characteristic formation of pseudo-membranes [73].

Colonization versus infection

C. difficile infection (CDI), previously described as *C. difficile*-associated diarrhoea (CDAD), is the most common cause of healthcare associated diarrhoea in Western countries [75]. By shedding of spores from symptomatic patients, *C. difficile* can spread within healthcare facilities, which can subsequently result in new symptomatic patients and eventually clusters or outbreaks. Not all newly diagnosed CDI patients can be linked to other symptomatic CDI cases. Two studies using multi-locus sequence typing and whole genome sequencing with single-nucleotide typing respectively, could only link 25 %-40 % of CDI patients to a previously identified CDI patient [76,77]. Recent studies show that also asymptomatically *C. difficile* colonized patients contribute to the spread of *C. difficile* spores via healthcare workers or the environment [78-80]. Riggs showed that *C. difficile* spores were present on the skin of asymptomatic carriers and easily transferred to investigator's hands and the environment [80]. Although transmission events from asymptomatic carriers as index patient remains rare [79], asymptomatic carriers may still importantly contribute to transmission, as they likely outnumber symptomatic CDI patients. In addition, the *C. difficile* carriers have themselves a higher risk of progression to CDI [81-83]. Approximately 5 % (0-15 %) of healthy individuals are asymptomatically colonized with *C. difficile* [82]. The incidence increases during prolonged hospitalization to 4 - 21%, and in nursing home residents colonization rates of 4 – 10 %, but up to 51 %, have been reported [82]. These observations prompted to survey *C. difficile* colonization rates in the healthy population and amongst nursing home residents in the Netherlands. Asymptomatic carriership is usually undetected, as routine screening is not performed. Interestingly, in Canadian tertiary institution in Quebec City, isolation precautions for colonized patients identified upon admission, decreased healthcare associated CDI [84], suggesting that asymptomatic *C. difficile* carriers significantly contribute to spread of CDI.

Microbiological diagnosis of *Clostridioides difficile*

The diagnosis of CDI is not always easy and straightforward. Because of the possibility of asymptomatic colonization and the potential presence of other causes for diarrhoea, detecting the presence of *C. difficile* in the feces does not necessarily defines disease. The main problem is absence of a fast and easy to perform test that differentiates between colonization and infection with high positive and negative predictive value. Of note, the presence of two gold standards, each with their own benefits and drawbacks also complicates the interpretation of research in this area. The cell cytotoxicity assay (CCNA) as gold standard test, detects the presence of free *C. difficile* toxin by a cytopathic effect in cell culture that is neutralized by the presence of antitoxin [85]. Detection of free toxin in the stool correlates best with CDI symptoms [86], however is labour intensive and non-standardized [86]. Toxigenic culture (TC), the second gold standard, evaluates the potency of cultured isolates to produce toxins in vitro [86, 87]. TC is considered the most sensitive of both assays, however in return less specific as asymptomatically colonized individuals are also tested positive. Given these drawbacks, one can question whether a true gold standard exists at all. Due to its labour intensiveness and required expertise, the availability of both tests in routine clinical microbiology laboratories is limited. Furthermore, the test results are only available after several days, often too late for clinical decision making. In Table 2, estimates of pooled sensitivity and specificity for different diagnostic CDI tests compared to the gold standard are shown. These were used to calculate positive and negative predictive values (PPV and NPV) for the different tests at varying hypothetical CDI prevalence's, depicted in Table 3. The most rapid, and easy to perform diagnostic tests are toxin enzyme immunoassays (EIAs), but it has become evident that these assays lack sensitivity to accurately diagnose CDI (Table 2), especially in a low prevalence disease setting ranging between 5-10 % (Table 3) [88]. In contrast a glutamate dehydrogenase (GDH) EIA or toxin nucleic acid amplification test (NAATs) display high sensitivity, but also lack specificity. The European diagnostic guidance document advises therefore a two-stage algorithm, using a NAAT or GDH EIA as sensitive screening assay, in combination with tests to detect the presence of free toxins in stools as marker of disease activity [88]. Using this guidance document, the European Center for Disease Prevention and Control (ECDC), formulated a practical advice to apply the two-step algorithm in studies of CDI (Table 4) [89].

Table 2. Pooled sensitivities and specificities of categories of tests

Type	Compared to CCNA			Compared to TC			Compared to culture		
	N studies	Sensitivity (95% CI)	Specificity (95% CI)	N studies	Sensitivity (95% CI)	Specificity (95% CI)	N studies	Sensitivity (95% CI)	Specificity (95% CI)
EIA GDH									
total	12	0.94 (0.89-0.97)	0.90 (0.88-0.92)	8	0.96 (0.86-0.99)	0.96 (0.91-0.98)	11	0.94 (0.86-0.97)	0.96 (0.92-0.98)
well-type	5	0.94 (0.91-0.97)	0.92 (0.92-0.93)	1	0.94 (0.93-0.96)	0.94 (0.94-0.95)	4	0.89 (0.86-0.91)	0.91 (0.90-0.92)
membrane-type	7	0.98 (0.78-1.00)	0.90 (0.87-0.93)	7	0.97 (0.84-1.00)	0.96 (0.90-0.99)	7	0.93 (0.84-0.97)	0.98 (0.95-0.99)
EIA Tox A/B									
total	27	0.83 (0.76-0.88)	0.99 (0.98-0.99)	29	0.57 (0.51-0.63)	0.99 (0.98-0.99)			
well-type	18	0.85 (0.77-0.91)	0.98 (0.96-0.99)	16	0.60 (0.52-0.68)	0.98 (0.97-0.99)			
membrane-type	9	0.79 (0.66-0.88)	0.99 (0.98-0.99)	13	0.53 (0.45-0.61)	0.99 (0.97-1.00)			
NAAT	14	0.96 (0.93-0.98)	0.94 (0.93-0.95)	32	0.95 (0.92-0.97)	0.98 (0.97-0.99)			

CI: confidence interval
CCNA: cel cytotoxicity neutralization assay
EIA: enzyme immunoassay
GDH: glutamate dehydrogenase
NAAT: nucleic acid amplification test
TC: toxigenic culture

Table 3. PPV and NPV for different categories of index tests at hypothetical CDI prevalence's of 5, 10, 20 and 50%

Type test	CDI prevalence 5%		CDI prevalence 10%		CDI prevalence 20%		CDI prevalence 50%	
	PPV	NPV	PPV	NPV	PPV	NPV	PPV	NPV
Well-type EIA GDH	38	100	54	99	72	98	91	94
Membrane type EIA GDH	34	100	52	100	71	99	91	98
Well-type EIA tox A/B	69	99	83	98	91	96	98	87
Membrane type EIA tox A/B	81	99	90	98	95	95	99	83
NAAT	46	100	64	100	80	99	94	96

Pooled estimates of sensitivity and specificity compared to cell cytotoxicity neutralization assay were used to calculate the predictive values.
CDI: *Clostridium dificile* infection
EIA: enzyme immunoassay
GDH: glutamate dehydrogenase
NAAT: nucleic acid amplification test
NPV: negative predictive value
PPV: positive predictive value

Table 4. Practical advice to apply the two-step algorithm by the ECDC

Categorization of CDI diagnosis	CDI diagnostic algorithm		
	Screening test	**Confirmatory test**	**Optional third test**
ESCMID-recommended	NAAT	Toxin A/B EIA	N/A
	GDH EIA	Toxin A/B EIA	NAAT or toxigenic culture
	GDH and Tox A/B EIA	NAAT or toxigenic culture*	N/A
Not recommended	All other algorithms		

CDI: Clostridium difficile infection
ESCMID: European Society for Medical Microbiology and Infectious Diseases
NAAT: Nucleic acid amplification test
Toxin A/B EIA: enzyme immunoassay that test for both toxins A and B
GDH: glutamate dehydrogenase
N/A: not applicable
* In this testing strategy, NAAT or toxigenic culture is an optional second test (there is no third test option)

Gut microbiota & *Clostridioides difficile* infection

The propensity of *C. difficile* spores to colonize the intestinal tract and subsequently outgrow and produce toxins, is highly influenced by the host microbiota and metabolome. In healthy individuals, the immune system along with a complex interplay of the gut microbiota, by competition for food and place, excretion of metabolites and bacteriocins, suppresses the growth of *C. difficile*. This defence mechanism is also known as colonization resistance [90]. Upon disruption of the microbiota due to exogenous factors such as antimicrobials or other medication as proton pump inhibitors or chemotherapy, colonization resistance decreases and *C. difficile* can proliferate, produce toxins and cause disease [24, 91]. Of the antibiotics, clindamycin, fluoroquinolones and cephalosporins, monobactams and carbapenems are in particular notorious for serious microbiota disruption [92]. By disrupting the microbiota, antibiotics have a selective effect on several key factors of the microbiota to suppress *C. difficile*. For example, microbiota changes can inhibit conversion of the primary bile acids with *C. difficile* spore-germinating capacity, to the *C. difficile* inhibiting secondary bile acids, enabling the outgrowth of *C. difficile* spores [93, 94]. These disruptions of the microbiota and consequent vulnerability of disease progression are more common in the fragile elderly population. The precise microbes responsible for inhibition or progression from *C. difficile* colonisation to infection have not been identified. However,

few suggestions have been made [95]. For example, some bacteria, like for instance *C. scindens*, convert the *C. difficile* enhancing primary bile acids to the inhibiting secondary bile acids [93, 94]. Moreover, several studies have reported the recovery of Bacteroidetes and members of the Firmicutes phylum; the families Lachnospiraceae (formerly known as Clostridium cluster XIVa) and Ruminococcaceae (formerly known as Clostridium cluster IV), including *Faecalibacterium prausnitzii, Eubacterium rectale*, *Roseburia intestinalis* and other known butyrate-producing bacteria along with successful clinical recovery from CDI [96-99]. Additionally, the level of Bacilli and Proteobacteria, generally found at high levels in patients with CDI, decreased after successful recovery [98]. Those observations will guide the future development of bacterial mixtures to prevent and treat CDI. Finally, the involvement of host immunity in the gut microbiota-mediated colonization resistance to CDI is incompletely understood but has recently been studied in mice, suggesting that IL-22-mediated host glycosylation stimulates the growth of commensal bacteria that compete with *C. difficile* for the nutritional niche [100].

Treatment of *Clostridioides difficile* infection

In 2014, the CDI treatment guideline of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) advised metronidazole, vancomycin and to a lesser extent fidaxomicin as the cornerstones of the CDI treatment [101]. However, after publication of this guideline, a large multicentre RCT showed that metronidazole is inferior to vancomycin in the treatment of both severe and non-severe CDI [102], with cure rates of respectively 81% versus 73%. The lower cure rate could be partly explained by the poor intestinal concentration of metronidazole in the lower gastrointestinal tract [103]. As result, metronidazole is currently replaced by vancomycin in most guidelines as first line CDI therapy [104]. Similarly, the IDSA guidance document recommended either vancomycin or fidaxomicin over metronidazole for an initial episode of CDI [105]. After treatment of an initial episode of CDI, recurrence occurs within eight weeks in 15-25% [102, 106, 107]. For a patient with one or two recurrences, the risk of further recurrences is increased to 40-65% [106, 108]. Fidaxomicin seems evenly effective as vancomycin in curing the symptoms of a first CDI episode, though due to its small antibiotic spectrum, relapses occur significantly less, 25.3% versus 15.4% respectively [102, 109, 110]. In addition, in patients with a first relapse, less second

relapses occurred in the fidaxomicin treated versus a vancomycin treated group (19.7 % versus 35.5 %) [107]. A variation of the 10 days fidaxomicin treatment is a so-called extended-pulsed fidaxomicin (200 mg oral tablets, twice daily on days 1-5, then once daily on alternate days on days 7-25). Extended-pulsed fidaxomicin was superior to standard-dose vancomycin for sustained cure of *C. difficile* infection with a cure rate of 70 % versus 59 % at 30 days after end of treatment [111]. Although the initial treatment costs of (extended-pulsed) fidaxomicin are very high (€ 1680,68 2dd200mg 10 days), it may be cost-effective as first line therapy in older patients, in comparison to vancomycin (€ 410.73 4dd250mg, 10 days), due its increased efficacy [111, 112]. Nevertheless, due to the high costs, fidaxomicin is mainly prescribed for patients with recurrent CDI in the Netherlands. A new, interesting treatment strategy is provided by bezlotoxumab, an anti-*C. difficile* monoclonal antibody, which can be prescribed as additive to standard antibiotic therapy. In a large phase 3 study, bezlotoxumab significantly lowered the rate of recurrence within 12 weeks in comparison to standard therapy (17 % versus 27 %) [113]. As for fidaxomicin, the current price of bezlotoxumab in combination with the limited additional beneficial effects hampers its broad scale implementation in clinical practice. Despite high expectations, treatment approaches directed to bind or neutralize *C. difficile* toxin in the intestinal tract, were not successful. In most cases, the design of the studies was not optimal or insufficient number of patients were included. Tolevamer, a *C. difficile* toxin binding polymer, was inferior to antibiotic treatment in two RCTs comparing tolevamer with vancomycin or metronidazole [102]. Unfortunately, tolevamer was not tested in combination with anti-CDI antibiotics. Several attempts have been made to develop immune whey with anti-CDI antibodies obtained by vaccination of cows. Though in vitro studies were promising, only one clinical trial was started but stopped due to bankrupt of the company [114, 115].

Despite of the above described treatment modalities, a subgroup of patients suffers from persisting CDI, with continuing relapses after cessation of antibiotics. Recurrent CDI is characterized by a permanently disturbed microbiota, enabling the outgrowth *C. difficile* spores once the anti-CDI antibiotic is stopped. Modifying the gut microbiota to break this cycle and prevents relapses to occur. Of all gut modifying therapies, Faecal microbiota transplantation (FMT) shows at present the best cure rates of over 85 % [96, 116, 117]. In Table 5, FMT and other microbiota modifying therapies are discussed.

Table 5. Overview of gut microbiota modifying therapies for treatment of recurrent CDI

Gut modifying therapy	Comment	Evidence in CDI treatment
Prebiotics	Dietary components that foster the growth of beneficial micro-organisms and stimulate a healthy microbiota. For instance, fibres of which Short Chain Fatty Acids (SCFA) are produced, which are healthy for the host.	An RCT with oligofructose amongst 132 patients showed that a relapse of diarrhoea occurred in 8.3% of oligofructose treated patients versus 34.3% of placebo treated patients [118]
Probiotics	A live microbial feed supplement which could be of benefit for the host. Limited efficacy. One bacterial mix beneficial for all conditions seems too simple. Generalizability of the results is challenging due to varied probiotic preparations in the research. Examples of studied probiotics: *Saccharomyces boulardii*, *Lactobacillus plantarum*, non-toxigenic *C. difficile* strain, a multi-strain preparation of Lactobacilli and Bifidobacteria.	Systematic review of RCTs: Two studies have found benefit to probiotics in prevention of rCDI: *S. boulardii* [119, 120] and a non-toxigenic *C. difficile* strain M3 [121] when compared to placebo. Cochrane systematic review (n=8672, 31 RCTs). Moderate certainty evidence: probiotics effective to prevent CDAD (NNTB = 42 patients, 95% CI 32-58). Post hoc subgroup analysis: probiotics effective with CDAD baseline risk >5% (NNTB = 12), but when baseline risk ≤5%. When probiotics administered together with non-CDI antibiotics RR reduction of 0.4 in acquiring CDI (1.5 vs 4%), NNT 40 [122].
Synbiotics	Combination of pre- and probiotics, for instance yoghurt containing *Lactobacillus sp.*	No
Defined bacterial consortium	Bacterial mix of healthy bacteria	Poof of principal Phase I study with bacterial spores (approximately 50, SER-109), effective in subset of patients [123]. However, failure to treat rCDI in phase II study. Multiple studies with rational selected bacterial consortia (VE303, Vedanta) underway.
Phage therapy	Transfer of bacteriophages	Proof-of-principle: Faecal filtrate (including meta-bolites/bacteriocins) was proved to cure (n=5) CDI patients [124]. In addition, FMTs with increased bacteriophage α-diversity were more likely to successfully treat rCDI [125]
Faecal microbiota transplantation	Transfer of complete healthy microbiota ecosystem to diseased microbiota, containing living bacteria, bacteriophages, metabolites and bacteriocins.	Established to prevent relapses in multiple, recurrent CDI.
Metabolites (postbiotics)	Transfer of beneficial microbial products that prevent germination, colonization and/or toxin production of *C. difficile*. Without risk of permanent engraftment of potential microorganism with risk, although question whether response permanent and chronic therapy needed.	Ursodeoxycholic acid (UDCA) prevented rCDI in 16 high-risk patients [126].

CDAD: *Clostridioides difficile*-associated diarrhoea

NNTB: number needed to treat to benefit one person

RR: relative risk

Faecal microbiota transplantation, a highly effective microbiota modulating therapy

Recurrent CDI is associated with an impaired immune response to *C. difficile* toxins and more importantly, with a persistent and severely perturbed colonic microbiota [127]. 16S rRNA gene sequence analysis of patients with recurrent disease showed a highly variable bacterial composition in comparison with the normal predominance of Bacteroidetes and Firmicutes in healthy individuals [98, 127]. Furthermore, patients with rCDI showed lower species richness compared with patients with an initial CDI episode or control subjects [97, 127]. This perturbed and diminished microbiota is essential in maintaining the disease, which is supported by the observation that replenishing the microbiota by Faecal Microbiota Transplantation (FMT) results in prompt resolution of rCDI.

The use of human stool as therapy for (mainly) gastrointestinal disorders, such as food poisoning and diarrhoea, was first reported in ancient China [128]. In the fourth century, Ge Hong orally administered faecal suspensions to treat severe diarrhoea, later referred to such suspensions as 'yellow soup" [128]. In 1958, Western literature described the first patients with severe antibiotic-induced colitis who were successfully treated with donor stool containing enemas [42]. With the increased CDI incidence in the 21st century, this 'ancient' therapy regained interest. In 2013, the first randomized trial using FMT to treat recurrent CDI demonstrated a remarkable efficacy in comparison to vancomycin [96]. FMT was successful in 81% of rCDI patients after just one FMT infusion, 94% after multiple infusions, while vancomycin was successful in only 31% of patients [96]. This high efficacy was confirmed in many independent studies that followed [116, 117, 129], and FMT is now advised in guidelines for treatment or recurrent CDI [104, 105]. A meta-analysis by Quraishi, including seven RCTs and 30 case series, showed that FMT was more effective than vancomycin in resolving recurrent and refractory CDI with a relative risk of 0.23 and a clinical resolution of 92% [116]. The second meta-analysis by Moayyedi, included ten RCTs with a total of 657 patients with *C. difficile*-associated diarrhoea and demonstrated that FMT was significantly more effective compared with placebo or vancomycin treatment, with a relative risk of 0.41 [117]. After FMT, patients show an increase in microbiota diversity, reaching levels that are observed in healthy donors [96, 98]. In conclusion, FMT is a highly effective treatment for patients suffering from multiple recurrent CDI [105].

Table 6. Overview of the outcome of FMT studies performed in patient with various conditions

Disorder	Type of study (references)	Outcome	Comments and important unresolved questions
Recurrent CDI	RCT [96, 129, 133, 134] Meta-analysis [116]	Highly effective, cure rate single infusion >80%	Advised in guidelines for recurrent rCDI [101, 105]
Severe CDI	Case series [135-138]	Effective and safe. An FMT program significantly decreases CDI-related mortality (9.1% versus 21.3%) and colectomy (2.7% versus 6.8%) in patients with fulminant CDI.	May be lifesaving. Probably sequential FMT infusion needed [139]
UC	RCT [140-143] Meta-analysis [53, 144, 145]	Pooled response rate of 29% for achieving endoscopic remission	Optimization of protocol required: Is rational selection of donors required? Is it possible to select patients who are more likely to respond? Should FMT be offered as induction or maintenance treatment? Is pre-treatment (with IBD medication) needed?
CD	Cohort studies [146-148] RCT [149] Meta-analysis [53]	Pooled clinical response rate of 53%. No endoscopic remission achieved	In very small RCT no effect, larger RCTs and rational donor selection needed
IBS	RCT [150-152] Meta-analysis [153, 154]	Large variation in treatment, placebo (auto-FMT/water) and definition and follow-up of treatment effect. According to meta-analysis of 5 RCTs no significant improvement in IBS symptoms with FMT (50% assigned to FMT responded, 56 assigned to placebo responded. In recent RCT with donor selection high response rates: 23.6%, 76.9% ($p<0.0001$) and 89.1% ($p<0.0001$) of the patients who received placebo, 30 g FMT and 60 g FMT, respectively [151]	Larger RCTs needed Which patients may benefit? Is repeated FMT required? How should patients be pre-treated before FMT? Rational donor selection needed?
HE	RCT [155]	Safe, no SAEs related to FMT, no new episodes of HE 150 days post-FMT	Confirmative study needed Rational donor selection needed
MDRO	Cohort studies [156-159] RCT [160]	Suggestive of some effectivity eradicating VRE and ESBL bacteria	Rational donor selection needed. Larger RCT needed with sufficient number of patients
Metabolic syndrome	RCT [161-163]	No effect on clinical endpoints. Transient increased insulin sensitivity	Strictly experimental, first results do not seem promising
Autism	Open-label trial [164]	Effect noted on psychiatric and GI symptoms	Underpowered. Further studies are needed
GvHD	Case series [165-167]	Steroid-refractory GVHD: decreased symptoms. Higher progression-free survival.	Seems safe Underpowered. RCTs are needed

Abbreviations: CD, Crohn's disease; CDI, Clostridioides/Clostridium difficile infection; ESBL, extended-spectrum beta-lactamases; FMT, faecal microbiota transplant; GI, gastrointestinal; GVHD, graft-versus-host disease; HE, hepatic encephalopathy; IBS, irritable bowel syndrome; MDRO, multidrug resistant organism; RCT, randomized controlled trial; SAE, serious adverse event; UC, ulcerative colitis; VRE, vancomycin-resistant enterococci. NAFLD: Non-alcoholic Fatty Liver Disease. Based on van Ooijevaar and colleagues [132]

Large heterogeneity exists among the included studies with respect to donor faeces volume, FMT preparations, route of administration, pre-treatment and numbers of FMTs **[116, 117, 130]**. This underlines the need for standardization of FMT to facilitate FMT, and increase the safety of this new treatment modality, stool banks such as the Netherlands Donor Feces Bank (NDFB) are emerging to standardize and centralize the process of donor selection and screening and to provide ready-to-use donor faeces suspensions to treating physicians. In addition, initiatives are undertaken to further standardize the process of FMT in Europe.

For now, CDI remains the prime disease for which there is a consistent body of evidence supporting treatment by FMT. However our growing understanding of the gut microbiota in health and disease suggests FMT, or more precisely; the concept of modulating the gut microbiota, could have great potential in treating other diseases than CDI **[131, 132]** *(see Table 6)*.

Colonisation with multidrug resistant organisms; unknown association with intestinal dysbiosis

Antibiotic resistance - Introduction

Antibiotic resistance, the ability of a bacterium to resist the action of one or more antibiotics, threatens effective prevention and treatment of infections, and is considered a major threat to public health worldwide **[4, 168]**. Bacterial resistance of several antibiotic classes is nowadays also becoming increasingly more common in the Netherlands **[169, 170]**. Infections with multidrug resistant organisms (MDROs) are not only more difficult to treat, but are accompanied with a rise in health care costs, patient morbidity and mortality **[171]**. Data based on the European Antimicrobial Resistance Surveillance Network (EARS-Net) show that each year, more than 670,000 infections occur in the European Union with antibiotic resistant bacteria, of which 64 % health care associated. Over 33,000 patients die annually as a direct consequence thereof **[172]**. The related cost to the healthcare system is around 1.1 billion Euro's **[173]**.

The Centers for Disease Control and Prevention (CDC) has outlined and prioritized the threats posed by specific MDROs of which drug resistant *Neisseria gonorrhoeae*, carbapenem resistant Enterobacterales (CRE) and *C. difficile* were the most urgent [5]. Although resistance is considerably more common in the Netherlands than 20 years ago, resistance rates are much lower compared to many other European countries [169]. This is partly because of the limited antibiotic use, both in the community as well as in the hospital [1]. Nevertheless, much effort is put in maintaining this low prevalence rate of MDRO. Low prevalence rates give the opportunity to combat resistance, for example with an active search-and-destroy (decolonisation) policy regarding "Methicillin Resistant *Staphylococcus Aureus* (MRSA)". With support of medical microbiologists, infection prevention workers and infectious disease specialists, only 1.2 % of *Staphylococcus aureus* isolates cultured from infections was resistant against methicillin in the Netherlands in 2018 [169]. In contrast, infections with Extended Spectrum β-lactamase-producing (ESBL) Enterobacterales (previously known as Enterobacteriaceae [174]) are much more frequently encountered, both in healthcare facilities and in the community [175] *(Figure 6)*. In the Netherlands, Enterobacterales resistant to both fluoroquinolones and aminoglycosides, are also considered a MDRO,

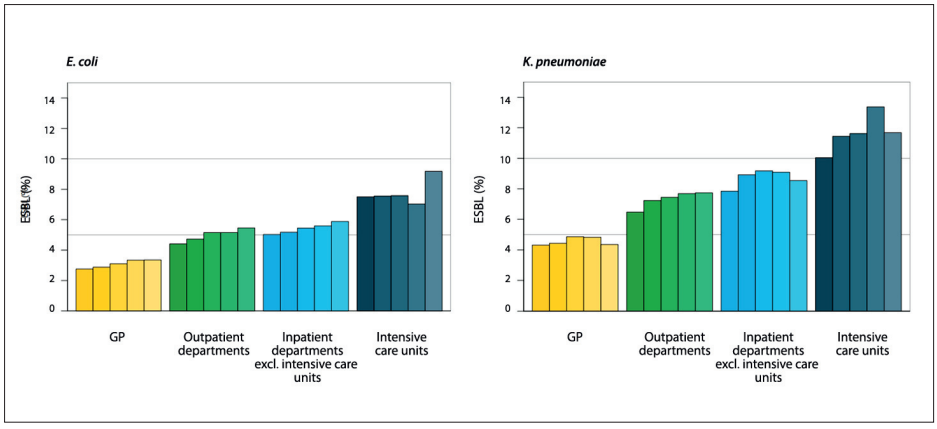

Figure 6. Trend in ESBL producing *E. coli* (left) and K. pneumoniae (right) in the Netherlands in different patient populations (from left to right 2015 to 2019). Isolates of patients attending the general practitioner are depicted in yellow, the outer patient clinic in green, the hospitalised patients (minus intensive care) in light blue and intensive care in dark blue. Adopted from Nethmap [170].

as these antibiotics are important antibiotics used in hospital settings [170, 176]. Of infections with Enterobacterales, urinary tract infections constitute the main clinical syndrome, followed by bloodstream infections with often a urinary or biliary tract origin. On the other hand, similar as *C. difficile*, also MDR Enterobacterales can asymptomatically reside in the gut. The intestinal tract is considered as an important reservoir of human Enterobacterales colonization and infections [177, 178].

Antibiotic resistance - General mechanism of action

Antimicrobial resistance is ancient and is the result of a million years of microbial co-evolution [179]. Most antimicrobial compounds are (derived) from microorganisms. The co-resident target microorganisms have therefore evolved mechanisms to overcome the antimicrobial action, referred to as 'intrinsic resistance'. Intrinsically resistant bacteria are however not the focus of the resistance problem. In contrast to 'acquired resistance', in which a bacterium that was originally susceptible to the antimicrobial compound, gained resistance. There are several major resistance mechanisms, namely 1. Destruction or modification of antibiotic molecule (for instance β-lactamases) 2. Modifications of antimicrobial target and/or binding place (for instance, alteration of Penicillin Binding Protein) 3. Prevention to reach the antimicrobial target by actively extruding by efflux-pumps or decreasing penetration (for instance by porins) or upregulation of the target 4. By-pass of target molecule by microorganism by change in metabolic pathway (for instance some sulphonamide resistant bacteria switch to using preformed folic acid) [180]. Development of acquired resistance can occur 'de novo' or by acquisition of exogeneous resistance genes. Bacteria acquire external DNA through three strategies 1. Transformation (incorporation of naked DNA), 2. Transduction (phage mediated) and 3. Conjugation (bacterial sex). Emergence of resistance in the hospital environment often involves conjugation, a very efficient method of gene transfer that involves cell-to-cell contact and is likely to occur at high rates in the gut microbiota under antibiotic treatment [181]. This is referred to as horizontal spread. Conjugation makes use of mobile genetic elements as vehicles to share valuable genetic information. The most important mobile elements are plasmids and transposons [181].

Antibiotic resistance to cephalosporins by "Extended Spectrum β-lactamases (ESBLs)"

β-lactam agents such as penicillin's, cephalosporins, monobactams and carbapenems, are the most frequently prescribed antibiotics. β-lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolysis and are the predominant mechanism of resistance in Gram-negative bacteria [180]. Extended-spectrum β-lactamase-(ESBL) producing bacteria have the ability to hydrolyse oxyimino-cephalosporins, and monobactams, but not cephamycin's or carbapenems. ESBLs were first described in 1983 and emerged especially in *Klebsiella pneumoniae* and *Escherichia coli* [182]. The genes encoding ESBL resistance, are frequently located on plasmids. Large plasmids carrying both ESBLs and several other resistance genes (e.g. aminoglycosides and fluoroquinolones) are frequently present in the Enterobacterales family [183]. Plasmid-mediated spread of ESBL and other antibiotic resistance genes (ARGs) contributes to the global spread of resistance against many frequently used antibiotics. The global pooled prevalence of ESBL colonization in the intestinal tract of asymptomatic individuals is 14 %, with an increasing trend of 5.4 % annually [2]. Driving factors for this rise are the globalization and the pandemic spread of CTX-M as most dominant ESBL enzyme [184], both in the hospital as well as in the community [2, 175]. CTX-M originated from chromosomal β-lactamases genes from different *Kluyvera* species [185]. *Kluyvera* spp. are ubiquitous found in the environment [186], and the probable environmental reservoir of the resistance genes. These β-lactamases genes were captured and mobilized on a variety of mobile genetic elements mediating rapid dissemination [187]. In the Netherlands, approximately 4.5-8.6 % of the healthy individuals is asymptomatically colonized with ESBL-producing Enterobacterales [188-191]. The prevalence of ESBL colonization varies largely per population; geographically as well as in diseased versus healthy individuals. Travel, kitchen hygiene (for instance not changing the kitchen towel each day) and antibiotic use are important risk factors for carriership of ESBL producing *E. coli* or *K. pneumoniae* in healthy individuals [192-194]. Carriership showed even a seasonal variation, that could not be explained by travel and antibiotic use [193]. Spontaneous clearance of the ESBL varies per bacterial species and per ESBL enzyme. One study reports a long duration of carriage in patients with a clinical infection, since 43 % remained ESBL positive after 1 year [195]. Another study revealed that intestinal colonization in the general population persisted

for >8 months in 32.9 % [193]. However, a study amongst 633 Dutch travellers who acquired ESBL during travel, concluded that the median duration of colonisation after travel was 30 days, and only 14.3 % and 11.3 % remained colonized at 6 and 12 months after return, respectively [190]. A second large community survey showed that the average duration of carriage was 0.35 years (4.2 months) amongst 4177 Dutch community-dwelling subjects [192]. Spontaneous clearance of certain subclones appears to be more difficult, as colonization of *E. coli* subtype (ST) 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 [196]. Of particular interest is the recognition of individuals with a higher risk of asymptomatic carriage and potential spread to the healthcare facility or community of MDROs. Nursing home residents as well as patients attending the hospital have multiple risk factors for colonization and infection with MDRO and *C. difficile* [178, 197-203]. They are thought to be a potential reservoir for spread and transmission in the hospital [80, 204, 205]. Frequent contact between residents due to communal living, high frequency of healthcare contact and presence of factors that facilitate MDRO spread such as incontinence present additional opportunities for transmission [206].

Resistance to polymixins and carbapenems

Infections with MDROs resulted in an increasing demand to use carbapenems. Subsequently, carbapenem resistance developed [207]. Carbapenemases can be produced by Enterobacterales and non-fermenters, and are classified into three classes according to Ambler classification; class A, B and D. Of the genes conferring carbapenem resistance, the carbapenemases pose the most threat, because of the possibility of horizontal gene transfer by plasmids or other mobile genetic elements. Carbapenemases are still sporadically observed in the Netherlands, both in the hospital [169] as well as in the community [194]. However, national surveillance detected a small cluster of eight closely related New Delhi metallo-beta-lactamase producing *K. pneumoniae* in patients without epidemiological link, indicating unnoticed spread [208]. Due to the rise of carbapenemase producing MDRO's, some of the older antibiotics such as polymyxins (colistin and polymyxin B) regained interest for patients' treatment. Colistin, also known as polymyxin E, is bactericidal and demonstrates activity against most Gram negative bacteria [209]. However, its nephrotoxicity

and neurotoxicity has prevented the use of colistin in regular patient treatment [210]. Therefore, colistin usage was mainly limited to veterinary medicine for treatment of gastrointestinal infections in food producing animals [211]. In the Netherlands, polymyxin B is frequently used for selective gut decontamination in Intensive Care Units and stem cell transplantation patients [212, 213]. The polymyxins are cationic peptides with fatty acid tails. Electrostatic interaction occurs between the positively charged groups on the polymyxin and the negatively charged groups of the lipid A component of the lipopolysaccharide (LPS). The fatty acids interact with the lipid components of the outer membrane. These interactions result in a change in the permeability of the outer membrane, and the polymyxins gain access to the cytoplasmic membrane. The breach in the permeability barriers, result in leakage of intracellular contents and subsequently cell death [209]. Some important Gram-negative bacteria, such as *Serratia marcescens*, *Burkholderia* and *Proteus* species, are intrinsically resistant to the action of polymyxins. Colistin resistance in Gram-negative bacteria is primarily due to post-translational modification of the lipopolysaccharide (LPS) molecules [214]. Substituents (such as 4-amino-$_{L}$-arabinose, phosphoethanolamine and/or galactosamine) which are positively charged reduce the negative charge of the outer membrane, resulting in less binding between the bacterial LPS and the colistin [214]. While the genes necessary for most of these additions are chromosomally encoded, the identification of a plasmid harbouring a novel colistin resistance gene, *mcr-1* in November 2015, is of concern as it threatens to increase the rate of colistin resistance [215]. Since the discovery of the *mcr-1* gene, ten *mcr* genes types (*mcr-1* to *mcr-10*) have been detected in Enterobacterales isolates of human, animal and environmental origin with worldwide distriution [216, 217]. The emergence of colistin resistance is currently analysed in a large European survey coordinated by ECDC and preliminary data indicate that its prevalence in the Netherlands amongst clinical isolates of *E. coli* and *K. pneumoniae* is higher than expected (March 2020, source RIVM).

Multidrug resistant organisms; rationale for microbiota modifying therapies

Most infections with ESBL producing Enterobacterales have high morbidity and mortality and are preceded by intestinal colonization [171, 177, 178]. Prevention and eradication of ESBL producing Enterobacterales from the intestinal tract is therefore of

interest. Since spontaneous decolonization occurs infrequently, innovative strategies for decolonization of MDR bacteria are needed. A published guidance document by the ESCMID could not recommend an antimicrobial intervention strategy for decolonization **[218]**. However, Millan et al., observed that FMT for treatment of patients with multiple recurrent CDI decreased the number and diversity of antimicrobial resistance genes **[219]**. This observation was followed by various case-reports of patients colonized with ESBL producing Enterobacterales who were successfully decolonized by FMT **[156-159, 220-225]**. However, only one RCT was performed which assessed decolonization of MDRO Enterobacterales by treatment of oral non-absorbable antibiotics or by FMT. Unfortunately, no statistically significant advantage of FMT was found, though the trial suffered from inclusion of insufficient number of patients **[160, 226]**.

Outline of this thesis

This thesis, entitled "Exploring the role of the microbiota in defence against *Clostridium difficile* and multidrug resistant Gram negatives", reports on the microbiological, epidemiological and clinical aspects of *Clostridioides difficile* and multidrug resistant organisms (MDROs). Part I of this thesis focusses on the epidemiology and diagnostic practices of asymptomatically colonized individuals, whereas part II focusses on eradication and/or treatment of these micro-organisms by restoring a healthy microbiota with "Faecal Microbiota Transplantation".

Chapter 1 is a general introduction on the gut microbiota in relation to colonization and infection with *C. difficile* and MDRO, and Fecal Microbiota Transplantation.

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

Chapter 2 evaluates the performance of several diagnostic *C. difficile* tests compared to the gold standard toxigenic culture of asymptomatically colonized patients at admission to three large hospitals in the Netherlands.

Chapter 3 reports on the prevalence of plasmid mediated colistin resistance genes; *mcr-1,2* [215, 227], in faecal samples of patients attending a tertiary care hospital in the Netherlands. Furthermore, it describes the genetic mechanism of phenotypically colistin susceptible *mcr-1* containing *E. coli*.

Chapter 4 determines the prevalence, risk factors and transmission within the nursing home of *C. difficile* and MDRO in asymptomatic nursing home residents in a high (Ireland) and low (the Netherlands) endemic country. Transmission of MDROs was studied with whole genome sequence analysis.

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

Chapter 5 describes the establishment of the Netherlands Donor Feces Bank (NDFB), a national operating non-profit stool bank facilitating FMT in the Netherlands. It addresses the current practice of donor recruitment and screening, preparation of the faecal suspension, logistics and transport of the faecal microbiota suspension to treating physicians in the local hospitals, and the follow-up of the outcome and safety of FMT in patients treated with FMT suspensions provided by the NDFB.

In **Chapter 6** the four years results of extensive donor screening and the outcome of FMT with suspensions provided by the NDFB are reported. In addition, the additional benefit of expert consultation, as provided by the working group of the NDFB is described. An attempt was made to understand the failures (post-FMT recurrence), and to identify donor and faeces suspension specific factors for optimal rCDI treatment.

Chapter 7 is an analysis of the effect of transmission of *Blastocystis* species from donors to patients by FMT, using a combination of PCR and subtyping techniques.

Chapter 8 describes an attempt of MDRO decolonisation from the intestinal tract with FMT in a patient suffering from recurrent urinary tract infections with a VIM-positive *Pseudomonas aeruginosa*. Microbiota analysis using 16S analysis was performed on both donor and patient stool before and after FMT.

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