GENERAL INTRODUCTION

The human microbiome and gut microbiota

In the late 17th century, Antoni van Leeuwenhoek was the first who observed differences between fecal and oral microbiota, and between samples from individuals in states of health and disease. However, it was not until 1977 that Joshua Lederberg first suggested the term ‘microbiome’ to define the totality of microorganisms and their collective genetic material present in or on the human body. The term ‘microbiome’ is frequently confused with ‘microbiota’, which refers exclusively to the organisms that are present in different parts of the human body. The composition and diversity of the human microbiota differ between body sites (e.g. the gut microbiota refers to the community of microorganisms that live in the gastrointestinal tract, the oral microbiota to that in the mouth; the skin microbiota to that on the skin and so on).

The gut microbiota contains the largest number of microorganisms compared to other body sites: approximately 1000 species of bacteria, and trillions of microbes inhabit the human gut. The composition of the gut microbiota is host specific, but may change over life-time as an effect of different factors, including aging, diet, use of medications, and environmental changes. In a healthy state, the diverse, and balanced gut microbiota modulates several physiological functions including gut development, nutrient processing and digestion, immune responses, and control of host energy and lipid metabolism. Additionally, a healthy gut microbiota is necessary for protection against pathogen colonization, since potentially pathogenic microorganisms continuously have to compete with the other species for space, nutrients, and an optimal environment (colonization resistance).

The bacterial community of the gut microbiota consists almost entirely of four phyla (a level of classification): Firmicutes, Actinobacteria, Proteobacteria, and
The Firmicutes and Bacteroidetes represent more than 90% of the relative abundance of the intestinal microbiota. Changes in the relative abundance of these two phyla have been associated with pathological conditions. For example, a decrease in Firmicutes is well documented in inflammatory bowel disease (IBD), obesity is characterized by an increase of Firmicutes diversity with concomitant reduction of Bacteroidetes, and a high Firmicutes/Bacteroidetes ratio is found in some patients with irritable bowel syndrome (IBS). However, also conflicting results have been reported, which highlights the current status of our understanding of the gut microbiota. Importantly, although these diseases or syndromes may be associated with a disturbance of the gut microbiota, it is not clear whether this is a cause or consequence of disease.

For the onset of Clostridium difficile infection (CDI), however, it is generally accepted that a disturbed gut microbiota is a prerequisite in disease development. In a stable condition, the gut microbiota can effectively inhibit colonization and overgrowth of C. difficile; in a disturbed gut microbiota, colonization resistance is decreased, which gives C. difficile the opportunity to grow, differentiate, and cause disease.

**Clostridium difficile (infection)**

*C. difficile* is an anaerobic, Gram-positive, spore-forming, toxin-producing bacillus, first isolated from the stool of a healthy infant by Hall and O’Toole in 1935. Yet, it was not until 1978 that *C. difficile* was identified as the primary cause of pseudomembranous colitis (severe inflammation of the colonic mucosa, Figure 1), and shown to be a primary isolate from the feces of patients who received antibiotic treatment. A series of reports followed, where a strong correlation between pseudomembranous colitis, antibiotic therapy, *C. difficile* colonization, and toxin production was shown. Together, these studies and observations revealed *C. difficile* as an emerging pathogen, to date the most common cause of healthcare associated diarrhea and infectious colitis. Despite prevention and control measures,
hospital-acquired CDI is a growing problem, leading to ever-increasing morbidity, mortality, and costs.\textsuperscript{21}

Acquisition of \textit{C. difficile} occurs by ingestion of its spores via contact with infected patients, or contaminated objects (fecal-oral transmission). A spore is an inactive stage of \textit{C. difficile} that allows the bacterium to survive outside a host for years. Since spores are resistant to alcohol, heat, and acid, they are hard to eradicate, and consequently play a key role in disease transmission.\textsuperscript{22,23} Once ingested, \textit{C. difficile} spores germinate, and grow into vegetative cells. In the colon, \textit{C. difficile} adheres to the epithelial lining, and waits for the opportunity to expand and colonize the colon. Exposure to spores does not always lead to colonization: in its healthy state the gut microbiota offers protection against colonization with \textit{C. difficile}.\textsuperscript{24} However, after any event that causes a disruption of the gut microbiota, \textit{C. difficile} replicates, colonizes the colon, and releases two toxins that mediate colitis and diarrhea: toxin A, and toxin B.\textsuperscript{20} These toxins are largely responsible for the mucosal damage and characteristic symptoms of CDI: severe diarrhea, and pseudomembranous colitis.\textsuperscript{20,25,26} Nontoxigenic strains of \textit{C. difficile} do not cause disease.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{images/healthy_colon_pseudomembranous_colitis.png}
\caption{Healthy colon mucosa (left) versus pseudomembranous colitis (right)\textsuperscript{27}}
\end{figure}
**Clostridium difficile ribotype 027: a more virulent strain**

*C. difficile* ribotype 027 (also known as the B1/NAP/027 strain) was the causative agent of the largest outbreak of *C. difficile* reported to date, which occurred in Canada in 2003.\(^{28}\) Infected patients had a significantly higher morbidity and mortality, giving rise to the term ‘hypervirulent’.\(^{28-30}\) Since this first outbreak, ribotype 027 has spread rapidly, being responsible for multiple outbreaks in various countries in Europe; first identified in England in 2005\(^{31}\), shortly thereafter in The Netherlands\(^{32}\), and subsequently in many other European countries.\(^{33}\) The emergence of *C. difficile* ribotype 027 has changed the epidemiology of CDI significantly.

The increased virulence has been linked to hyper production of toxins A and B, the production of an additional binary toxin, and increased sporulation levels.\(^{34,35}\) Additionally, resistance to fluoroquinolones and erythromycin has been described.\(^{36-38}\)

**Risk factors for Clostridium difficile infection**

A disturbed gut microbiota is at the core of CDI pathogenesis.\(^{24}\) Especially antibiotics have profound effects on the structure and function of the gut microbiota by decreasing bacterial diversity, and causing an imbalance of the normal ratio’s between the different species.\(^{39}\) Within days after the start of antibiotics a disturbance of the gut microbiota is detectable. Although compositional changes may be dependent on the antibiotic used, as well as the underlying microbiota community of the individual, antibiotics from almost all classes have been associated with the development of CDI.\(^{40-43}\)

Increased age is another well described risk factor for CDI. While it is possible that age is an independent risk factor for CDI, increased age is also associated with use of antibiotics, more frequent hospital visits, and more comorbidity in general, all of which impact *C. difficile* susceptibility. In addition, increased age has been associated with a less stable and less diverse gut microbiota.\(^{44}\)
The use of proton pump inhibitors has also been correlated with higher CDI incidence in some studies, but other studies could not confirm this.\textsuperscript{45} Of note, it has been suggested that proton pump inhibitors, which increase the pH of the stomach, also may affect the gut microbiota.\textsuperscript{46}

A longer duration of hospital stay is another well-defined risk factor for the development of CDI. First and most likely because it is a marker of disease severity, which is also associated with CDI. Additionally, it increases the chance of exposure to \textit{C. difficile} spores either by indirect contact with a contaminated surface or by direct contact with an infected person.\textsuperscript{47,48}

**Clinical manifestations and diagnosis of \textit{Clostridium difficile} infection**

Disease severity ranges from mild diarrhea, to fulminant and sometimes fatal pseudomembranous colitis or toxic megacolon. The latter two manifestations are rare, but may lead to severe complications such as septic shock, perforation or death.\textsuperscript{49}

Testing for \textit{C. difficile} or its toxins should only be performed in patients with diarrhea; testing of stool from asymptomatic patients is not clinically useful.\textsuperscript{50} Currently, two methods for diagnosing \textit{C. difficile} are considered as gold standard: the cytotoxicity assay, which detects the presence of free toxins in fecal samples, and the toxigenic culture, which evaluates the potency of cultured isolates to produce toxins \textit{in vitro}.\textsuperscript{51} Toxigenic culture is the most sensitive diagnostic tool, however, it cannot distinguish between individuals with asymptomatic colonization, and patients with an actual infection due to \textit{C. difficile}. Rapid toxin tests are more clinically relevant (since toxins mediate disease), but lack sensitivity.\textsuperscript{20} Molecular tests, such as polymerase chain reaction (PCR), are rapid, more sensitive and specific, and can target toxin genes. However, also PCR detects \textit{C. difficile} bacteria regardless of toxin production, making it unclear whether positive PCR results reflect clinical disease.\textsuperscript{52,53} Therefore, a two-
step algorithm with a screening test (PCR), followed by a toxin test to confirm active infection is the most reasonable diagnostic strategy.\textsuperscript{53}

**Treatment and recurrent *Clostridium difficile* infection**

Once a patient has been diagnosed with CDI, first, if possible, the ongoing antibiotic treatment should be discontinued.\textsuperscript{50} Second, common infection prevention measures, and cleaning of the environment should be performed to prevent further spread of *C. difficile*. When clinical symptoms do not resolve rapidly, antibiotic treatment for CDI should be administered. Standard antibiotic treatment for CDI involves antibiotics that suppress *C. difficile*: metronidazole for mild-moderate CDI, and vancomycin for severe infection.\textsuperscript{50} Unfortunately, about 20-30\% of patients experience recurrent disease; recurrence risk rises to 65\% in patients with multiple recurrences.\textsuperscript{54} The most common risk factors for the development of recurrent CDI include advanced age, CDI due to ribotype 027, multiple comorbidities, and use of antibiotics during or after initial treatment of CDI.\textsuperscript{55} It is suggested that these risk factors interfere with the ability of the gut microbiota to recover fully and re-establish colonization resistance.\textsuperscript{24,56} Alternatively, recurrent CDI may reflect failure of the host to mount a protective immune response against *C. difficile*.\textsuperscript{24} A subset of patients with recurrences falls into a downward spiral, including several episodes of recurrent diarrhea, and the risk of complications as well as re-hospitalization. The social impact is high, because patients with CDI are usually isolated in single hospital rooms. Recurrent episodes of CDI led to prolonged isolation; the economic impact on the healthcare system is significant, because CDI doubles the average length of hospitalization, and increases the cost of treatment.\textsuperscript{57}

A first recurrence of CDI is usually treated with vancomycin; subsequent recurrences with a tapered regimen of vancomycin, or fidaxomicin. Additionally, over the last decade, fecal transplant has received increasing interest as potential therapy for recurrent CDI.\textsuperscript{58,59}
Fecal Microbiota Transplantation

In the fourth century, in Chinese medicine, patients with food poisoning or severe diarrhea were treated with the so named ‘yellow soup’, a less unpleasant name for human fecal suspension.\(^6^0\) Later, in the 16\(^{th}\) century, Li Shizhen described using fermented fecal solutions, fresh fecal suspensions, or dried feces for treatment of severe diarrhea, fever, pain, vomiting, and constipation.\(^6^0\) Much later, Arab desert nomads recommended the consumption of fresh, camel feces as a remedy for bacterial dysentery; its efficacy was anecdotally confirmed by German soldiers in Africa during World War II.\(^6^1\) In scientific literature, infusion of donor feces was first described in 1958 by the surgeon B. Eiseman, who reported successful treatment of four patients with severe pseudomembranous colitis due to CDI, with fecal enemas.\(^6^2\) The transfer of feces from healthy donors to patients is now termed Fecal Microbiota Transplantation (FMT, Figure 2).

![Figure 2. Fecal microbiota transplantation](image)

After numerous studies proving that human feces has therapeutic potential\(^5^8,5^9\), recently, in a small open label randomized controlled trial, it has been shown that FMT is a highly effective therapy for recurrent CDI, with cure rates around 85-90\%.\(^6^3\) It is hypothesized that the healthy, balanced, diverse donor feces restores the disrupted intestinal microbiota of the patient, resulting in colonization resistance, which
prevents germination of residual spores of *C. difficile*. This hypothesis is supported by the observation that the gut microbiota of the recipient resembles that of the donor, indicating that the donors’ microorganisms are capable of restoring the structure and function of the gut microbiota of the patient.\textsuperscript{39,63} Although FMT is proven to be an effective therapy for recurrent CDI, large scale implementation in daily clinical practice is troubled by the lack of uniform guidelines, concerns about safety, and remaining uncertainty about long-term side effects. Additionally, important barriers for FMT include the costs of donor screening, and limited time window to recruit and screen a suitable donor and prepare the fecal suspensions.

Our growing understanding of the gut microbiota in health and disease suggests that it will play an important role in diagnosis, treatment, and possibly also prevention of human disease. The concept of modulating our gut microbiota, for example by FMT, is currently a topic that is receiving considerable interest.
OUTLINE OF THIS THESIS

This thesis is divided into five parts.

PART I consists of three chapters, in which we describe an outbreak of *C. difficile* ribotype 027 that occurred in the VU University medical center (VUmc) between 2013, and 2014.

Chapter 2 describes a case-control study where we compared CDI patients infected with *C. difficile* ribotype 027, with non-CDI controls, and controls with CDI due to other ribotypes for distribution of hospital-associated risk factors, and clinical outcome parameters.

Chapter 3 addresses risk factors associated with recurrent CDI during the outbreak.

In Chapter 4 we provide insight into the financial burden that the CDI outbreak brought upon the hospital.

PART II consists of two chapters, in which we focus on prediction tools for patients diagnosed with CDI.

In Chapter 5 we test published prediction models for complicated CDI for clinical use and provide external validation for three existing prediction tools.

Chapter 6 explores whether recurrent CDI could be predicted by on-site profiling of fecal volatile organic compounds.

PART III consists of three chapters, in which we address treatment of CDI with FMT.

Chapter 7 discusses complications, effectiveness, and long term follow-up of FMT as treatment for recurrent CDI.

In Chapter 8 we present, and discuss evidence supporting the curative use of early FMT in severe or complicated CDI to modify clinical course, and prevent colectomy.

Chapter 9 describes a child with Down syndrome treated with FMT for recurrent CDI. Furthermore, it addresses the impact of FMT on the gut microbiota composition, prior to and following FMT, linked to microbial communities in the donor feces.
PART IV consists of four chapters in which we address the development and first experiences of the Netherlands Donor Feces Bank (NDFB), and other potential areas where FMT might be of interest in the future.

Chapter 10 discusses the therapeutic potential of FMT in treating IBD, IBS, metabolic syndrome, graft versus host disease, depression, autism, and in eradication of carriage of multidrug resistant microorganisms.

In Chapter 11 we report the results of a pilot study in which we have treated 10 patients with post-infectious or antibiotic-induced IBS with FMT. Furthermore, we discuss the impact of FMT on the gut microbiota composition and diversity, linked to that of the donor.

In Chapter 12 we describe a patient with refractory celiac disease type II who received FMT as treatment for recurrent CDI, and remarkably showed a full recovery of duodenal villi and disappearance of celiac symptoms.

Chapter 13 describes the development and first experiences of the NDFB. Current donor recruitment and screening, preparation of the fecal suspension, legislation of FMT, transfer of fecal suspension to the patient, and follow up of patients treated with donor feces of the NDFB are addressed.

PART V is the final part of the thesis, and contains a general discussion and summary, and a Dutch summary.
REFERENCES


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