CHAPTER 14

Summary and general discussion

This thesis focuses on the composition and dynamics of the intestinal microbiome in children, both in healthy state and in several gastro-intestinal diseases in which microbiota are supposed to play a critical role. Detailed characterisation of microbiota, from infancy towards adulthood, is essential to enlarge knowledge on microbiota-related pathology, to assess the potential of microbiota as a diagnostic tool for disease activity and to develop scientifically based therapeutic interventions linked to microbiota composition. To this end we used two complementary analytical techniques. The molecular microbiota analysis technique IS-pro was used for the identification of bacteria, based on the 16S-23S IS-region of their ribosomal DNA.[1] In addition an electronic nose device (eNose) was used to analyse faecal volatile organic compounds (VOC), which are mainly produced by intestinal bacteria and are considered to reflect not only microbiota composition, but also their function and host-microbiome interaction.[2,3]

PART I

Characterising paediatric microbiota in health and disease

In the first part of this thesis we explored the intestinal microbiome in the healthy state and in a variety of gastro-intestinal diseases. In **chapter two**, gut microbiota composition in a cohort of healthy Dutch children aged 2-18 years at different time points has been described. Here we defined reference values for microbial composition, diversity and stability in different age groups throughout childhood. This study was the cornerstone of the studies described in the subsequent chapters, in which the microbiota in gastrointestinal conditions has been explored. While microbiota composition fluctuated substantially over short intervals, it was characterised by a relatively high overall long-term compositional stability. We observed that stability was highly phylum-specific, with *Bacteroidetes* as the most stable component, followed by the phyla *Proteobacteria* and *Firmicutes*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* (FAFV). Comparable variation in stability patterns of bacterial phyla has recently been described in a cohort of younger children, up to four years of age, possibly reflecting physiological developmental steps.[4] We also observed a strongly positive correlation between diversity and stability, which is of great interest from an ecological point of view, as this has been a long-lasting dogma regarding the dynamics of ecosystems.[5] This finding underlined the importance of microbiota composition in health and disease; low microbial diversity has been linked to increased risk of various conditions, including inflammatory bowel disease (IBD), auto-immune disorders, obesity and infections

such as *Clostridium difficile*-associated diarrhoea.[6] We also identified the presence of an age-independent shared core microbiome, consisting of a limited set of species which were present in the great majority of children, primarily belonging to the phylum *Bacteroidetes*. A (functional) core microbiome has been described in adults.[7,8] while the emergence of a core set of species has recently been described in children up to four years of age, with *Bacteroidetes* as main representatives, which is in concordance with our observations.[4] The identification of a core microbiome may be considered an essential step to define the healthy state, as it is suggested that they play a critical role in maintaining health.[9] The significance of the concept of a health-related microbial core lies in the possibility of the development of preventative, diagnostic and, maybe, even therapeutic strategies focusing on maintenance or restoration of this core set. Interestingly, to our knowledge, none of the probiotic preparations currently in use contain these core species, which might explain their limited effect on most (paediatric) gastro-intestinal disorders. Whether the efficacy of probiotics could be improved by adding core species to probiotic mixtures warrants investigation in future studies.

The aim of **chapter three** was to describe microbiota composition and dynamics in a cohort of newly diagnosed, treatment-naïve paediatric IBD patients, including the main phenotypes ulcerative colitis (UC) and Crohn's disease (CD). We observed that IBD could be discriminated from controls with high accuracy, which confirms findings from previous studies and emphasises the potential of microbiota analysis as a complementary technique in the diagnostic work up of paediatric IBD. A novel finding in this study was that IBD patients could be discriminated from controls based on loss of the microbial core described in chapter two, in which *Bacteroidetes* dominate. This suggests that IBD is principally characterised by the loss of 'beneficial' microbes rather than the appearance/introduction of pathogens. We observed that these core species tended to regain expression after three months of follow-up, when disease course tended to return to the remission phase. This restoration of the core microbiome appeared to be incomplete, however, suggesting that disturbance of the bacterial core might be a structural or pathogenic problem in IBD patients. Interestingly, at six weeks follow-up microbiota profiles from patients with persistent active disease could not be differentiated from those in clinical remission. This seems to limit the potential for microbiota analysis as a tool to monitor disease activity in clinical practice, although there are some reports describing a relation between microbiota composition and disease activity over time.[10,11] Disturbance of the core microbiome as a critical aberration in IBD may provide opportunities to explore preventive and therapeutic interventions directed at maintenance or restoration of the microbial core.

Furthermore, the observation that new IBD patients are characterised by loss of microbial 'healthy expression' could be an interesting subject of future studies aimed at early detection or even prevention of IBD, for example by analysis of the core composition in children with a positive family history for IBD at regular intervals.

In **chapter four** we describe microbiota composition in a cohort of children with functional constipation according to the Rome III criteria in comparison with that of healthy controls. In this cross-sectional study we found that acceptable discrimination of both groups could not be performed by conventional statistical techniques, such as unsupervised clustering methods and principal component analysis. By means of supervised machine learning, however, both subgroups could be discriminated with modest accuracy based on a set of species belonging to the phyla FAFV, *Bacteriodetes* and *Proteobacteria*. Notably, these discriminative species did not belong to the healthy core microbiome described in chapter one. The fact that outcome is highly dependent on the selected statistical analytical methods (supervised versus unsupervised approaches), has increasingly been appreciated in recent studies.[12-14] Application of supervised models, such as PLS-DA, Bayes Net and Random Forest have been shown particularly useful as classifier model in highly complex data sets, which is the case with intestinal microbiota studies.[13] Such models may represent a useful approach to identify the most discriminative bacterial species between different subgroups, which can subsequently be used to construct a prediction algorithm. However, the test performances of these various prediction models have been shown to vary considerably, and it has therefore been questioned whether a certain classifier model may have universal applicability in different populations.[13] Further studies are needed to assess which model provides optimal accuracy in different research and clinical settings. The observation that microbial profiles in children with functional constipation differ from those in controls does not automatically implicate a causal relation; it could also be a consequence of, for example, delayed motility or changes in life style secondary to abdominal complaints. Although in vitro studies have demonstrated the influence of specific species on motility patterns[15-17] the results of interventions studies in constipated children aiming at manipulation of the microbiota, for example by prebiotics or probiotics, showed contradictory and mostly disappointing results.[18-20] Future studies are needed to externally validate our findings, preferably using a longitudinal design, allowing to address the relationship between dysbiosis and the course of clinical symptoms, and to evaluate whether targeted correction of the microbiome could improve symptoms.

The duodenal mucosa-associated microbiota in untreated paediatric coeliac disease as compared to those of healthy controls is described in **chapter five**. Recently the pathogenesis

of coeliac disease has increasingly been linked to (alterations of) the gut microbiota, based on observations that neonatal infections and recurrent rotavirus infections are associated with an increased prevalence of coeliac disease,[21,22] while other studies found an inverse association between coeliac disease and *Helicobacter pylori* infection.[23,24] The majority of studies investigating microbiota in coeliac disease reported compositional differences, such as increased abundance of *Bacteroides* and *Proteobacteria* spp. in affected subjects,[25-27] while in other studies no relevant differences were detected.[28] Notably, the patterns of microbial dysbiosis in affected subjects vary widely between studies, possibly due to differences in age of subjects (children versus adults), sample harvesting, collection and storage and microbial detection techniques. Such a wide variation in outcome may raise the question about the potential role of microbiota in the development of coeliac disease. In our study, we did not observe differences in composition or diversity between affected children and controls. Nevertheless, research on microbiota-targeted strategies to manipulate disease onset and course has emerged over the past years, mainly involving prebiotics and probiotics. The basis of treatment for coeliac disease, however, remains lifelong adherence to a strict gluten-free diet. Therefore, potential benefits from microbiota-targeted interventions in coeliac disease might only be expected from manipulation of disease onset rather than modulating clinical symptoms. Anyhow, available evidence regarding the use of probiotics in coeliac disease is as yet insufficient to recommend their administration in clinical practice.[29]

In **chapter six** we investigated gut microbiota in newly diagnosed juvenile idiopathic arthritis (JIA) patients, prior to the prescription of disease-modifying antirheumatic drugs. Partial least squares discriminant analysis (PLS-DA) enabled discrimination of microbiota profiles between JIA patients and controls, based on a limited set of species exclusively belonging to the phylum *Bacteroidetes*. In this case as well, these discriminative species were representatives of the core microbiome in healthy children. Our findings add to the increasing notion that (changes in) gut microbiota may be involved in the pathophysiology of JIA,[31] although causality has not incontestably been demonstrated. Larger studies, preferably with a longitudinal instead of cross sectional design, are needed to increase understanding of the potential role of microbial communities in the aetiology of JIA.

In conclusion, we have shown in chapters two to six that there are differences between the microbiomes in healthy children and in children with various gastro-intestinal and auto-immune diseases. Healthy children are characterised by the preservation of a microbial core which is dominated by a limited number of species belonging to the phylum *Bacteroidetes*. This core microbiome is disturbed in *de novo* IBD and JIA. Based on alterations of (a limited set

of) core species, healthy and disease state could be discriminated with high accuracy using statistical supervised classification models. This enforces the potential of microbiota profiling as a diagnostic tool. How this core might play a role in preserving health, whether core disturbances are causative or rather consequences of disease, and whether manipulation of this core can influence the course of disease, are issues that need to be subject of future studies.

PART II

Application of molecular microbiota detection techniques in clinical practice

In this second part, we illustrate the potential of molecular microbiota detection methods in clinical practice. Currently, conventional culture techniques are still the cornerstone of gut microbiota identification in diagnostic processes. The majority of gut microbes, over 80%, however, cannot be identified with such methods. Over the past decades, the emergence of DNA-based microbiota detection techniques has tremendously increased our knowledge of the role of microbiota in health and disease. These methods have, however, not yet found their way towards implementation as routine diagnostic tools in clinical practice. One major issue is the lack of standardisation, hampering its current use as diagnostic tool in clinical practice.[32] In **chapter seven**, we describe two children with severe ulcerative gastritis and oesophagitis. Histological examination of mucosal biopsies showed the presence of microorganisms with a morphology suggestive of *Sarcina ventriculi*.[33] Culture of biopsies was negative, but the presence of *S. ventriculi* was confirmed by means of IS-pro analysis. Both patients were treated with targeted antibiotics and symptoms resolved completely within a few days. *S. ventriculi* infection is very rare, but life-threatening complications have been reported.[34] Molecular microbiota detection methods provide the opportunity to confirm infections by microorganisms which cannot be cultured, such as *S. ventriculi*. Recognition of disease-specific microbial signatures may allow for the implementation of DNA-based microbiota detection techniques as diagnostic tools in clinical practice. IS-pro is of particular interest, as this technique has the capacity to generate results within five hours following a rigorous sampling protocol, allowing for rapid initiation of targeted treatment.[1]

In **chapter eight**, we show the impact of faecal microbiota transfer (FMT) on microbiota composition in a child with recurrent *Clostridium difficile* infection (CDI). The patient was successfully treated with FMT, which had already been proven a safe and effective treatment for recurrent CDI in adults,[35,36] and seems to be similarly effective in children, although

paediatric studies are limited.[37] Before and after FMT the faecal microbiome was analysed using IS-pro, as was the faecal microbiome of the donor. The patient acquired a unique, very stable microbiota profile, despite CDI recurrence and subsequent antibiotic therapy. *Bacteroidetes* were acquired from the donor and remained stably present, while post-FMT FAFV and *Proteobacteria* were unique to the patient. Recurrent CDI is characterised by decreased overall microbial diversity; suggested mechanisms of FMT include the restoration of diversity, preventing (re)colonisation with pathogenic *C. difficile*. Microbiota profiling in the follow-up of patients treated with FMT could provide insight into the degree of transmission and implementation of donor microbes to the host. Longitudinal microbiome analysis linked to clinical response may increase our understanding of optimal microbial conditions and may allow for improvement of FMT efficacy. Furthermore, monitoring of microbial diversity through time may allow to predict individual recurrence risk and provide a window of opportunity for timely intervention.

PART III

Faecal VOC are mainly produced by microbes and therefore are considered as an interesting candidate to serve as a biomarker for the detection of pathogens.[38,39] However, studies evaluating the potential of VOC are limited so far. In the third part of this thesis we show the potential of faecal volatile metabolomics as a diagnostic tool and for monitoring disease activity in various paediatric gastro-intestinal conditions. In **chapter nine** we provide a review of the available literature on the evidence on faecal VOC as biomarker for paediatric gastrointestinal diseases, including irritable bowel syndrome, infectious diarrhoea, liver diseases, IBD and necrotising enterocolitis (NEC). Although most studies report profound differences between healthy and diseased states, environmental factors such as diet and medication have not been taken into account, increasing the risk of type 1 errors. In **chapter ten**, we show that faecal VOC profiling may discriminate paediatric patients with *de novo* UC, CD and controls, both during active disease as well as in clinical remission, with promising sensitivity and specificity. Interestingly, UC and CD may be discriminated from one another with high accuracy, which has not been acquired with other non-invasive biomarkers. Instead of, for example, breath or urine samples, we choose to use faecal samples for VOC analysis as IBD is a gastrointestinal condition linked to gut microbiota alterations. We hypothesised that faecal VOC could provide a more direct and integral view into these processes than VOC from other body substrates, but comparative studies are lacking so far. When comparing VOC results with microbiota

outcome in faecal samples (chapter two), both techniques show differences between IBD and controls, independently of disease activity. The study groups were too small to draw firm conclusions regarding the potential of VOC profiling to monitor disease-activity. We provisionally conclude that the eNose technology holds promise as a novel, non-invasive technique in the diagnostics of IBD. This will be topic of future studies.

In **chapter eleven** we assessed the potential of VOC profiling in the early detection of NEC in premature infants. We showed that faecal gas analysis has potential as an early non-invasive biomarker of NEC. VOC profiles of infants who developed NEC can be discriminated from control infants up to three days prior to the onset of clinical symptoms. So far only one pilot study has addressed this topic, showing that VOC profiles assessed with GC-MS of six NEC patients differed from those of seven control infants in the four days preceding the clinical onset of NEC.[40] Our findings are in line with previous microbiota profiling studies in NEC patients, all showing that profound microbial alterations precede NEC.[41-44] Interestingly, we observed that the VOC profiles in infants who develop late-onset sepsis can be differentiated not only from those in controls, but also from the VOC profiles in future NEC patients, a distinction which is currently a clinical challenge. Obviously, our findings need to be externally validated in a larger cohort to prove the potential of VOC analysis as diagnostic tool for NEC. We have initiated a validation study in which nine neonatal intensive care units (NICUs) in the Netherlands and Belgium participate. The identification of NEC-specific (key) volatiles by GC-MS could lead to future development of tailor-made NEC-specific eNose sensors. Early, pre-clinical detection of NEC would provide a window of opportunity to manipulate NEC onset and course, hopefully leading to decreased mortality and morbidity.

The aim of **chapter twelve**, based on our findings in chapter eleven, was the evaluation of the potential of faecal VOC analysis as an early diagnostic tool for late-onset sepsis in preterm infants. We showed that faecal VOC profiles in preterm infants with sepsis and controls can be discriminated up to three days before clinical onset of the disease. In a follow-up study performed in six NICUs in the Netherlands, we confirmed that faecal VOC profiling has potential for the detection of late-onset sepsis in a pre-clinical stage, with, interestingly, different VOC patterns for different pathogens (in preparation). Our observations suggest, that at least in some cases of late-onset sepsis in preterm infants, intestinal microbiota may play an aetiological role.

In **chapter thirteen**, an overview of diagnostic biomarkers for NEC is described, with the focus on early microbial alterations. Although in most studies profound changes in microbial

signatures between NEC subjects and controls were described, starting up to weeks prior to NEC onset, differences in study design (used microbiota detection techniques, sampling intervals) hampers reliable comparison between study results.

In conclusion, in the third part of this thesis we have shown that subjects with IBD, NEC and neonatal sepsis can be discriminated from controls using faecal VOC analysis, at least in NEC and neonatal sepsis even in pre-clinical stage. Recognition of the potential of VOCs as a marker of bacterial metabolism may open avenues towards the development of primed eNose sensors which can be used in the early detection of diseases associated with a disturbed gut microbiome.

FUTURE PERSPECTIVES

Differences in methodology between studies on intestinal microbiota- and VOC analysis hamper reliable comparison of study results. Therefore, standardisation of methodology should be given priority in future studies. This urgency is illustrated in recent work by our group, in which it has been shown that differences in collection, storage, and preparation of faecal samples profoundly impact VOC outcome.[45] It has now become evident that the intestinal microbiome harbours a tremendous potential as biomarker for disease activity for a variety of (gastro-intestinal) diseases. Classical bacterial identification methods are, however, time-consuming and require expensive microbiological equipment and expertise. IS-pro technology may overcome these limitations, as detailed results can be obtained within hours following sampling, while usage of eNose devices may even allow for bedside analysis. In future studies we will focus on simultaneous analysis of gut microbiota and faecal VOC in order to obtain complementary information about microbial composition and microbe-host interaction. This could be used to develop diagnostic algorithms with high accuracy. This approach may in particular be beneficial for diseases in which gut microbial disturbance may precede clinical symptomatology, such as NEC and neonatal sepsis. Increased information on microbiota composition may lead to the development of novel, microbiota-targeted therapeutic interventions, while the identification of disease-specific key VOC may allow for the development of tailor-made eNose sensors.

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