Microbes in the inflamed gut

Koopman, N.

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Chapter One

General introduction

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The gut microbiome

Microbes were already on the planet more than three billion years before humans [1]. Animal life emerged and co-evolved in endosymbiotic relationship with these microbes and therefore we cannot live without all the trillions of microbes that inhabit our bodies. The majority of these bacteria, fungi, viruses, protozoa and archaea reside in the gut and are together called the gut microbiota. The number of bacteria in an individual's gut is estimated to be around 10^{13} to 10^{14} cells which is equal to the number of human cells in our bodies. There are more than 1,000 species known to reside in the gut. Altogether, the collective genome of these species contains more than two million genes which outnumbers the number of human genes by a hundred-fold. Each person is populated by approximately 15% of these species [2–4]. The microbiota and their 'theatre of activity', which includes microbial structural elements, metabolites, as well as environmental conditions, are collectively called the microbiome [5].

The microbiota interacts with the human body via various metabolites and proteins, resulting in a complex interaction network of signaling compounds that act locally and systemically. This makes the gut microbiota key to many aspects of human health. Diet, medication and lifestyle can all influence the composition of the gut microbiota [6–8]. Examples of metabolites that can be modulated by both the host and microbes are short chain fatty acids, indole derivatives, bile acid metabolites and vitamins [9,10]. Disturbances and lower diversity in the gut microbiota composition have been associated with various conditions and diseases such as obesity [11,12], cancer [13], neurological disorders [14], and inflammatory bowel disease (IBD) [15–17].

The gut microbiota and the immune system develop in interaction

The development of the gut microbiota already starts during birth and is shaped by gestational age, mode of birth and type of feeding after birth. Maternal transmission, also called vertical transmission, provides the first bacteria in the gut which are mainly dominated by species from the Bacteroidetes and Actinobacteria phyla, the genus *Bifidobacterium* more specifically. Over time, more bacterial species colonize the gut. The gut microbiota matures gradually during the first months of life and becomes more diverse in composition and functionality until it becomes relatively stable after weaning approximately one year after birth [8,18–20]. Bacterial phenotypic characteristics such as aerotolerance, hardened cell walls and the ability to from endospores (called sporulation) are beneficial in the transmission from person to person. Although, spore forming bacteria are abundant in the microbiota of adults, they do not seem to be maternally transmitted and are not prevalent and abundant in neonates indicating that this property is of importance for familial transmission or acquirement from the environment later in life [20–22].

In parallel to the gut microbiota, the adaptive immune system is developed in the first months of life. The adaptive immune system is trained by microbes it encounters and *vice versa* the adaptive immune system selects for colonization of certain species while others are eradicated and contributes thereby to the development of the gut microbiota composition. The adaptive immune system enables the body to recognize and remember both beneficial and pathogenic species and either promote or suppress the growth of a species.

This complex interplay between microbes in the gastro-intestinal tract and the adaptive immune system starts immediately after birth when both systems are still maturing. The maturation of the infant mucosal immune system takes months; thus, the transmission of immunity plays a significant role in protecting against pathogens that might cause disturbances in the natural development of the microbiota of the infant [18]. Breastfeeding allows for passive transfer of maternal antibodies, especially secretory Immunoglobulin A (IgA), providing immunity for the infant and thereby affecting the developing immune system of the infant and the microbiota that are able to colonize the gut. The infants innate immune system responds to microbial antigens bound to maternal secretory IgA in a

tolerogenic way, which trains the infant immune system to recognize beneficial commensals that were also tolerated by the immune system of the mother [18].

The development of the gut associated lymphoid tissue is also affected by gut microbiota and their metabolites. Microbe-associated molecular patterns (MAMPs) activate toll like receptors expressed on gut epithelial cells and immune cells, aiding in mucosal immunity maturation. Mature lymphoid follicles release immunoglobulin producing plasma cells into the lamina propria from where the immunoglobulins are transported into the intestinal lumen to maintain homeostasis [18,23–25]. For example, IgA can cross-link pathogenic bacteria which allows for clearance and thereby prevents their translocation across the epithelial layer [26]. MAMPs also promote epithelial cell proliferation, deepening of the crypts and increased density of Paneth cells in the small intestine, which release antimicrobial peptides. Furthermore, MAMPs stimulate goblet cells to produce mucus, adding a defense layer against microbes [27]. Dendritic cells phagocytose bacteria or sample microbial antigens and travel to lymph nodes, inducing T cell differentiation. T cells can be differentiated into different types of T helper cells that promote pro-inflammatory responses by producing cytokines or into regulatory T cells producing anti-inflammatory interleukin 10 (IL-10). Regulation of the regulatory T cell and helper T cell balance is crucial in maintaining homeostasis in the gut and discriminating between pathogens and commensals [9]. A balanced immune system maintains IL-10 producing cells and limits pro-inflammatory responses upon commensal microbe detection [28]. The development of the adaptive immune system in relation to microbes and immune responses towards gut bacteria are in more detail reviewed in Maynard *et al.*, 2012 [18] and Ruff *et al.*, 2020 [29].

Altogether, the immune system and the gut microbiota interact with each other in a complex network sensitive to disturbances. When the development of the immune system is disturbed, there might be a risk of accumulating disease-causing pathobionts or having inappropriate immune responses against commensal gut microbes leading to less diversity and reduced microbial functioning. Inflammatory bowel disease is such a disease involving aberrant immune responses against gut microbes.

(pediatric) Inflammatory bowel disease

Inflammatory bowel disease is a collective term for chronic inflammatory conditions in the gastro-intestinal tract affecting millions of people worldwide [30,31]. Classically, inflammatory bowel disease is divided into Crohn's disease and ulcerative colitis, although distinguishing is not always easy, the disease is therefore sometimes classified as intermediate or unclassified. Inflammatory bowel disease can occur at any age, although it is estimated that at least 25% of the diagnoses are in children [32]. The prevalence of inflammatory bowel disease is on the rise, with numbers of 77 per 100,000 children diagnosed in the United States [33].

Both ulcerative colitis and Crohn's disease are known for reoccurring 'flares' of inflammation that alternate with periods in which there is no inflammation observable. In ulcerative colitis inflammation is present over the whole length of the colon but remains limited to the epithelial layer, while in Crohn's disease transmural inflammation in multiple distant areas over the entire gastrointestinal tract can occur [34,35]. Periods with inflammation are called active disease and periods without inflammation remission. Active disease often comes with symptoms including, but not limited to, abdominal pain, diarrhea, blood in stools, fatigue, and weight loss. Some of these symptoms of pain present even in the absence of inflammation [36].

Inflammation in the gastrointestinal tract can be observed by endoscopy. In pediatric patients, endoscopy is not routinely used to confirm inflammation as this is an invasive procedure for the patient. Instead, the biomarker calprotectin is measured in feces which is a protein produced by neutrophil granulocytes [37]. Fecal calprotectin levels show a strong correlation with disease activity observed by endoscopy [38–40]. In addition to this biomarker, for Crohn's disease the Pediatric Crohn's Disease Activity Index (PCDAI) and for ulcerative colitis the Pediatric Ulcerative Colitis Activity Index (PUCAI), or abbreviated versions, are used as clinical disease activity measure taking into account patient experiences and physical examination [41–43]. However, the PCDAI and PUCAI correlate weakly with endoscopic severity in children with Crohn's disease [44,45], and in line, clinical symptoms do not always correlate with biomarkers for inflammation including fecal calprotectin levels [45].

Inflammatory bowel disease does not have a single cause and is considered to be an immune mediated multifactorial disease. Genome wide association studies have identified >200 alleles associated with increased susceptibility to inflammatory bowel

disease in genes playing role in innate immunity, recognition of microbes, mucosal barrier functioning and host defense mechanisms [46]. However, not all carriers of these mutations develop disease implicating that other factors are involved as well. Incidence of inflammatory bowel disease is increasing worldwide and has been associated with the adoption of a western lifestyle and urbanization [30,47]. Also, a diet low in dietary fibers and high in saturated fat has been associated with inflammatory bowel disease [48]. The cause of inflammatory bowel disease is therefore thought to be an interplay between genetic susceptibility, the gut microbiome and environmental triggers such as diet, antibiotic exposure and infections leading to a dysregulated immune response in the gastro-intestinal tract.

Currently, there is no cure for inflammatory bowel disease and the goal of treatment strategies is to reduce symptoms and induce and maintain clinical remission. Remission almost never occurs spontaneously and treatment with medication is needed. The most prescribed treatments include corticosteroids to induce remission, and immunosuppressants such as thiopurines to maintain remission. Biologicals, including anti-tumor necrosis factor-**α** agents, can be prescribed in both phases of treatment [49–52]. However, adherence to these treatments can be difficult due to side effects and immunosuppression. Approximately one third of the patients do not respond to these drugs or become non-responders during the treatment [53]. Additionally, specific diets can be prescribed. For example, in pediatric Crohn's disease a 6-8 week diet of liquid food called exclusive enteral nutrition (EEN) is often prescribed because it has few side effects. EEN is shown to be beneficial by improving nutritional status and reducing inflammation and disease symptoms [54]. However, not all patients respond to this diet, with responsiveness seeming to be influenced by their gut microbiota and metabolome prior to treatment [50].

In conclusion, the need for new treatment strategies is high. The multi-factorial nature of the disease asks for a comprehensive, holistic view of the disease in which the microbiome, immune response and metabolism of the patient are taken into consideration. Integrative approaches of advanced techniques including metabolomics, proteomics and microbiomics would facilitate the identification of key features and pathways acting on local and systemic level that influence the shift from active disease to remission. These insights could be pivotal in identifying new therapeutic targets and enhancing current treatment strategies.

Microbes in inflammatory bowel disease

As described above, interactions between microbes and the host contribute to the etiology of inflammatory bowel disease. Host polymorphisms in MAMP sensor genes such as toll-like receptor 4 (*TLR4*) [55] and nucleotide-binding oligomerization domain-containing 2 (*NOD2*), also known as caspase recruitment domain-containing protein 15 (CARD15) [56,57] are associated with disease susceptibility. Also, exposure to antibiotics during early childhood has been associated with an increased risk for inflammatory bowel disease [58].

Gut microbiota compositions of patients with ulcerative colitis or Crohn's disease are different from healthy individuals and show diminished diversity and stability [16,59–61]. On phylum level, decreases in Firmicutes and increases in Proteobacteria are observed in both ulcerative colitis and Crohn's disease patients [62,63]. In Crohn's disease specifically, decreased proportions of the Clostridia class, including the *Roseburia* and *Faecalibacterium* genera of the Lachnospiraceae and Ruminococcaceae families are observed [64–66]. Although a few bacterial strains have been shown to be specific to inflammatory bowel disease [67], it is unlikely that a single bacterial species is responsible for the metabolic changes observed in inflammatory bowel disease patients. In a lesser extent, the fungal composition, also called mycobiota, has been studied in the context of inflammatory bowel disease. Patients show increased total levels of fungi compared to healthy controls, compositional differences are not always consistent between studies however increased ratios *Basidomycota*:*Asomycota*, decreased abundances of *Saccharomyces cerevisae*, and increased abundances of *Candida albicans* and *Malassezia* were observed repeatedly [68–71]. Longitudinal cohort studies are needed to elucidate whether the alterations in microbiota precede the onset of inflammation or are more a reflection of the disease.

Over the past decade, the first sequencing and omics studies have been performed that provide insight into the interaction between microbiota and host in inflammatory bowel disease in correlation with inflammation. Over 2,700 metabolites have been identified as differentially abundant in inflammatory bowel disease, including differences among sphingolipids, short chain fatty acids, amino acids, bile acids and carboximidic acids [72–74]. Metabolites and metabolite classes were frequently lower in inflammatory bowel disease patients relative to non-inflammatory bowel disease controls, suggesting a loss of metabolic diversity among inflammatory bowel disease patients that parallels the loss of taxonomic diversity observed in inflammatory bowel disease.

Furthermore, over the course of the disease microbiota-host interaction seems to play a pivotal role as well. A longitudinal study revealed an interaction network of >2900 molecular host and microbial molecular interactors during inflammatory bowel disease. Especially, the taxa *Faecalibacterium, Roseburia, Subdoligranulum, Alistipes* and *Escherichia,* the metabolite octanoly carnitine and several lipids and short chain fatty acids were identified as key regulators of interleukins [75]. Although these studies provide insight, they are all performed in adults. Less is known about the interaction between the microbiota and host over the course of disease in children.

Sporobiota

As mentioned above, some bacterial species have the ability to form endospores. These species are collectively called the sporobiota and may constitute up to 50% of bacteria within the gut [76,77]. The sporobiota include pathogens as well commensal and beneficial species contributing to gut homeostasis. Spore forming species interact, like other species, with the host in their vegetative form but also in their spore form. The resistant nature of spores towards harsh conditions like low pH in the stomach and oxygen makes them interesting to exploit as vehicle for drugs delivery or as probiotic in diseases including inflammatory bowel disease. However, sporobiota in the gut remains an understudied topic. Insight in the sporulation process, spore properties and germination, transmission dynamics, and consequences of gut colonization is important in providing a complete understanding of the gut microbiome and its overall function in health and disease.

To conclude, it is likely that the microbiome contributes to the interindividual variability of inflammatory bowel disease, the disease course and the response to treatment. Therefore, it should be considered for the development of future treatments strategies, especially when aiming for a personalized medicine approach. To achieve the development of effective treatment strategies a comprehensive approach of studying the gut microbiota in the context of the patient is needed. In addition to analyzing the composition of the microbiota, the functional roles should be elucidated by analyzing the interactions with the host and its environment, including diet and drugs.

Outline of this thesis

The overall aim of this thesis is to study the gut microbiome in a disease driven perspective. Multi-omics, advanced sequencing and bioinformatics techniques are used to explore the interaction network between microbes and host in inflammatory bowel disease. This thesis can be divided into two parts. In **part I** of this thesis, entitled **"Interactions between microbiota and host in inflammatory bowel disease"**, the role of the gut microbiome in pediatric inflammatory bowel disease is explored in a holistic context, where the patient's proteome and metabolism were also taken into consideration.

In **chapter 2**, data from 6 different -omics datasets in 3 different anatomical compartments of pediatric patients with Crohn's disease patients are integrated to obtain insight in the shift from active disease to remission. In **chapter 3,** a similar -omics datasets from ulcerative colitis patients is assessed to gain insight in this type of inflammatory bowel disease and compare the findings with what is found in Crohn's disease patients as described in **chapter 2**.

Chapter 4 consists of a literature review on the multi-faceted role of serotonin in gut homeostasis. The synthesis, release, and reuptake of serotonin in the gut is and how this is modulated by the gut microbiota and how serotonin affects the gut microbiota *vice versa* are discussed. This chapter also gives detail on the mechanisms by which serotonin could affect the disease course in inflammatory bowel disease and describes the effects of interventions targeting serotonin signaling.

Part II of this thesis, entitled **"Sporobiota in the gut in health and disease"** focusses on a specific feature that many bacteria residing in the gut possess, the capacity to produce spores. As elucidated above spore forming bacteria resemble an important fraction of the gut microbes found in the gut. This part starts with **chapter 5** where literature on spore properties and the processes of sporulation and germination is in the light of the human gut environment is reviewed. Furthermore, the role of sporobiota in transmission, health and disease is described and both pathogenic and beneficial spore formers are discussed. Lastly, in this chapter the potential of sporobiota as treatment in the form of probiotics and vaccine and drug delivery systems and the corresponding challenges are discussed.

In **chapter 6**, the presence of spore formers within the gut microbiota composition is studied. We present the sporulation potential as measure to determine the capacity of an

individual's microbiota to sporulate and investigate whether there are differences in healthy individuals and patients with inflammatory bowel disease. In addition we address whether fecal microbiota transplantation can restore the sporulation potential in Crohn's disease.

In chapter 7, a widely cited anecdote on the use of camel feces, supposedly containing *Bacillus subtilis* spores, to treat dysentery during World War II is investigated. The potential of *B. subtilis* spores as probiotic is briefly reviewed and we investigated the presence and abundance of *B. subtilis* spores in feces from the Egyptian dromedary camel, *Camelus dromedarius*.

Finally, this thesis is closed with chapter 8 providing a general discussion and perspectives on the topics discussed in part I and part II.

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