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INTESTINAL MICROBIOTA DEVELOPMENT IN CHILDHOOD: IMPLICATIONS FOR HEALTH AND DISEASE

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ACADEMIC DISSERTATION

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Abbreviations

BMI Body mass index
CD Crohn's disease
DNA Deoxyribonucleic acid
FXR Farnesoid X receptor
HMO Human milk oligosaccharide
IBD Inflammatory bowel disease
IBDU Unclassified inflammatory bowel disease
IgA Immunoglobulin A
LGG *Lactobacillus rhamnosus* GG
LPS Lipopolysaccharide
OTU Operational taxonomic unit
SCFA Short-chain fatty acid
PCoA Principal coordinates analysis
PCR Polymerase chain reaction
RBB Repeated bread beating
rRNA Ribosomal ribonucleic acid
TGR Transmembrane G protein-coupled receptor
TLR Toll-like receptor
TNF-α Tumour necrosis factor alpha
UC Ulcerative colitis

Abstract

The intestine is a major interface between the human body and the environment, and harbours a dense population of immune cells, as well as a diverse microbial ecosystem. The immune system of the infant develops in tight interaction with the intestinal microbiota, and the early-life microbiota succession is considered important for immune maturation. Many common practices, such as birth by Caesarean section, antibiotics, and lack of breastfeeding, influence the development of the infant's microbiota. The altered early microbiome may have long-term effects on the later health of the child.

This thesis characterises the development of the intestinal microbiota in healthy children. The influence of four common factors potentially modulating the microbiota – prenatal stress, breastfeeding duration, antibiotic use, and probiotic use – were investigated, as well as the association between early-life microbiota composition and the development of BMI. In addition, the microbiota in healthy children was contrasted with that that of children with IBD, characterising the association between treatment response and microbiota.

DNA-based methods were used for microbiota profiling from frozen faecal samples. The bacterial composition was studied using two methods, a phylogenetic microarray, HITChip, as well as 454-pyrosequencing of the 16S rRNA gene amplicons. In addition, real-time qPCR was conducted to measure bile-salt hydrolase genes and antibiotic resistance genes. Bacteria were cultured anaerobically from the faecal samples for antibiotic susceptibility testing.

The results showed that the microbiota in childhood are sensitive to modulating factors, and are predictive of later-life health. Maternal stress during pregnancy was associated with altered microbiota development over the first months of life. Short duration of breastfeeding was associated with fast microbiota maturation, high BMI, and frequent antibiotic use in pre-school age. The results indicate that some of the benefits of breastfeeding are microbiota-dependent. Antibiotic use emerged as a central regulator of the microbiome, with potential effects on the metabolic development of the child. LGG supplementation prevented some of the penicillin-associated changes, but failed to prevent the macrolide-associated loss of bifidobacteria. In IBD patients, the microbiota composition varied along a gradient of intestinal inflammation. High microbiota similarity to healthy controls predicted positive response to anti-TNF-α treatment in IBD patients.

This work suggests that maternal wellbeing is the first step towards a healthy microbiota in the child. Promoting a natural microbiota development in childhood by breastfeeding, avoiding unnecessary antibiotics, careful selection of the antibiotic when it is needed, and possibly the use of specific probiotic strains, may have long-term health benefits, particularly in terms of

weight development and immune health. Furthermore, healthy children treated with antibiotics showed considerable microbiota similarities to IBD patients, suggesting that antibiotic courses may drive the microbiota towards an IBD-like state. Stratification of paediatric IBD patients based on the microbiota may enable tailored treatment and improved treatment responses.

Introduction

The intestine is a major interface between the human body and the environment, and harbours a dense population of immune cells and a diverse microbial ecosystem, consisting largely of bacteria but also of archaea and viruses (Qin *et al.* 2010, Lozupone *et al.* 2012). The intestinal microbiota have co-evolved with the host species (Xu *et al.* 2007, Ley *et al.* 2008). In this process the gut microbes have taken on many metabolic functions, which the host cells cannot perform (Qin *et al.* 2010, Sekirov *et al.* 2010). The human intestinal microbiota are composed mostly of bacteria belonging to four phyla: the Gram-positive Firmicutes and Actinobacteria, and the Gram-negative Bacteroidetes and Proteobacteria (Costello *et al.* 2009, Human Microbiome Project Consortium 2012, Rajilić-Stojanović and de Vos 2014). In addition, several other phyla are present at low levels. At finer taxonomic levels, there is substantial variation between individuals, and each adult has a unique and stable microbiota composition (Rajilić-Stojanović *et al.* 2013). Most intestinal microbes have not yet been cultured, although over 1000 species have now been described (Rajilić-Stojanović and de Vos 2014). Molecular, culture-independent approaches are therefore essential to comprehensively characterise the microbiota.

Microbial colonization of the infant may begin *in utero*, as bacterial DNA can be detected in the placenta (Aagaard *et al.* 2014) and in the meconium even in healthy pregnancies (Gosalbes *et al.* 2013, Ardissone *et al.* 2014). However, a massive colonization begins at birth, during and after which the infant is exposed to a rich diversity of parental and environmental bacteria. During the first weeks and months of life, there is large, inter- and intra-individual variation in the microbiota composition (Palmer *et al.* 2007, Eggesbo *et al.* 2011, Valles *et al.* 2012, Sharon *et al.* 2013). The causes of this variation are not fully understood. However, despite the vast diversity of environmental bacteria, to which the neonate is exposed, the intestinal colonizers are normally exclusively human-associated bacteria, and common patterns can be recognized in the process, even across cultures (Scholtens *et al.* 2012, Yatsunenko *et al.* 2012). It is becoming increasingly recognized that maternal vertical transmission of microbes is an important and finely orchestrated process, with evolutionary implications comparable to the inheritance of genetic material (Funkhouser and Bordenstein 2013). The immune system of the infant develops in tight interaction with the intestinal

microbiota, and the early-life microbiota succession is considered highly important for proper immune maturation (Martin *et al.* 2010).

The intestinal microbiota interact closely with the host's immune system and regulate metabolism (Hooper *et al.* 2012). The intestinal microbes play an essential role e.g. in the metabolism of bile acids and cholesterol, production of energy-rich short chain fatty acids from undigested fibre, and in the functioning of the intestinal epithelial and immune cell populations (Nicholson *et al.* 2012, Tremaroli and Bäckhed 2012). The symbiotic microbial communities living within the human host have been suggested to protect against allergic diseases and aberrant immune responses (Beyan *et al.* 2012, Hoermannsperger *et al.* 2012). Furthermore, the intestinal microbes protect the host from bacterial and viral infections by stimulating the immune system (Kinnebrew *et al.* 2012), and the intestinal barrier (Wlodarska *et al.* 2011) and out-competing pathogens (Croswell *et al.* 2009, Endt *et al.* 2010). The individual composition of the microbiota is linked to the health of the host: altered composition or function has been associated with intestinal and systemic inflammatory, autoimmune and metabolic diseases (Festi *et al.* 2011, Iebba *et al.* 2011, Burcelin *et al.* 2012, Greenblum *et al.* 2012). Rather than being attributed to a single pathogenic bacterium, many disease states are associated with some degree of alteration in the overall composition and functioning of the microbiota as an ecosystem.

Literature review

Human microbiomes

The abundance of bacteria and the community composition vary widely along the intestinal tract due to changes in conditions such as pH, oxygen level, nutrient content, and peristalsis (Stearns *et al.* 2011). The mouth has a diverse microbiota consisting of both aerobic and anaerobic organisms, with different communities found in different habitats (Dewhirst *et al.* 2010, Stearns *et al.* 2011). All of the phyla inhabiting the intestine are also found in the mouth, and the mouth is considered a microbial gateway to other body sites, including in the intestine (Dewhirst *et al.* 2010). In the stomach a surprisingly rich ensemble of bacteria are found (Bik *et al.* 2006, Stearns *et al.* 2011); whether they are alive and resident in the stomach is questionable.

In the intestine, the abundance and diversity of the microbiota increase toward the colon (Stearns *et al.* 2011). The small intestine is an unstable environment and the microbiota composition fluctuates within daily time scales (Booijink *et al.* 2010, Zoetendal *et al.* 2012). There is a rich, but pulsatile supply of various types of nutrients that have not yet been absorbed by the host, including simple sugars, fatty acids and amino acids. The arriving food is mixed with acids and oxygen, and the host secretes bile and digestive enzymes. Furthermore, the transit in the small intestine is much

faster than in the colon. Coping with these conditions is necessary for survival especially in the proximal small intestine. Microbes with an opportunistic life-style and fast intrinsic growth rate, able to rapidly utilize available substrates (Zoetendal *et al.* 2012), the so-called r-selected species, thrive in this environment. The most abundant genera are *Streptococcus, Veillonella, Prevotella* and various Proteobacteria (Ou *et al.* 2009, Cheng *et al.* 2013, Wacklin *et al.* 2013, Dlugosz *et al.* 2015). The microbes compete for nutrients with the host, and furthermore, the small intestine is the site where nutrient absorption occurs; the host must balance between compartmentalising the bacteria strictly into the intestine while simultaneously efficiently taking up nutrients into circulation. The host therefore has to limit the growth of microbes in the small intestine. The ileocaecal valve limits the translocation of microbes from the colon where the microbial density is much higher than in the upper intestinal tract, and the host secretes IgA and antibacterial compounds to control the microbes and to keep their density low (Salzman *et al.* 2007).

Toward the distal ileum, the transit slows down, the level of anoxia increases, the pH increases (Evans *et al.* 1988), and the conditions begin gradually to resemble those in the colon. In a healthy state, the human colon is a very stable environment, with fairly continuous input of complex polysaccharides to be utilized for energy, as well as close-to-neutral pH, and very low levels of oxygen. Thus the dominant species are adapted to such conditions, i.e., can be expected to be so called K-selected species that are highly specialized and competitively dominant in their preferred, stable environment. The host has adapted to coexisting with these microbes, whose metabolic products feed the colonocytes and maintain intestinal health. The dominant components of the colonic microbiota are to be considered mutualistic symbionts, depending on the host for survival and providing the host with various benefits. The dominant members in adult humans are those belonging to Firmicutes, mainly Clostridium clusters IV and XIVa, and Bacteroidetes (Costello *et al.* 2009, Human Microbiome Project Consortium 2012, Rajilić-Stojanović and de Vos 2014).

In addition to the digestive tract, all external surfaces of the human body, including the skin and the urogenital tract are inhabited by their own microbiomes (Human Microbiome Project Consortium 2012). Skin surfaces are often dominated by *Propionibacterium, Corynebacterium,* staphylococci or Proteobacteria, with wide variations in community composition between different types of skin habitats (Grice *et al.* 2009). The vaginal microbiota are particularly important for early colonisation and transmission of microbes from mother to child, and therefore likely to have played a major role in the evolution of the human-microbe symbiosis. The vaginal microbiota are usually dominated by one of three *Lactobacillus* species: *L. iners, L. crispatus, L. gasseri*, (in a few cases *L. jensenii*) or alternatively characterised by low abundance of lactobacilli and high diversity. The latter composition is associated with high pH and Nugent scores, indicating bacterial vaginosis.

The dominant organisms and their metabolism have important effects on the vaginal immune functions and the mucosal barrier, and therefore affect e.g. resistance to pathogens (Doerflinger *et al.* 2014).

Factors influencing the intestinal microbiota

At the level of genera and species, the composition of the microbiota shows considerable inter-individual variation. Each individual has a unique microbial fingerprint, which tends to be stable over time and resilient, but not always resistant, to perturbations (Martinez *et al.* 2013, David *et al.* 2014a). The microbiota composition is influenced by host genetics and the environment (Benson *et al.* 2010, Kashyap *et al.* 2013, Carmody *et al.* 2015). In humans, genetic loci influencing fucosylation of secreted glycans (*FUT2*), and innate immunity (*MEFV*) have been shown to influence the composition and functioning of the microbiota, with health implications (Khachatryan *et al.* 2008, Tong *et al.* 2014, Wacklin *et al.* 2014). However, the similarity in microbial profiles between monozygotic twins has not consistently been found to be greater than that of dizygotic twins (Zoetendal *et al.* 2001, Stewart *et al.* 2005, Turnbaugh *et al.* 2009, Tims *et al.* 2013). Spouses tend to resemble each other in their microbiota profiles more than other people and more than their children (Song *et al.* 2013), suggesting that shared environment has an important role. The microbiota are capable of adaptation to different situations, and respond rapidly to changes in diet (David *et al.* 2014b). Diet influences the microbiota by providing substrates for microbial fermentation, inducing host secretions, and possibly also directly by providing incoming bacteria (Salonen and de Vos 2014, Zoetendal and de Vos 2014). Some food-borne bacteria remain viable and metabolically active in the gut (David *et al.* 2014b). In addition to diet, the microbiota composition varies according to e.g., country, health status, and age of the individual (Yatsunenko *et al.* 2012). Hunter-gatherer communities harbour a much greater intestinal microbial diversity and a different composition than humans living in modern environments (Schnorr *et al.* 2014, Clemente *et al.* 2015), suggesting that modernization has simplified the human-associated microbiota.

Functions of the intestinal microbiota

The intestinal microbiota function essentially as an organ, performing tasks that the host cells do not have the capacity for. Microbes modify the intestinal environment to suit their own requirements, e.g. by altering the pH, breaking down and metabolizing bile acids, producing bacteriocins and stimulating the immune system to inhibit the growth of competing bacteria. The human host has evolved to depend on many of these essentially selfish bacterial functions.

Many symbiotic microorganisms protect the host from the invasion of intestinal pathogens, a phenomenon called colonisation resistance (Buffie and Pamer 2013). Lactic acid bacteria are particularly well-studied examples of improved colonisation resistance and several mechanisms have been elucidated: they produce lactate which decreases the intestinal pH below the optimum for many Gram-negative pathogens (Fernandez *et al.* 2003), they produce antibacterial compounds (Cintas *et al.* 2001), compete for binding sites (Fernandez *et al.* 2003), and interact with the immune system, strengthening immune responses to pathogens (Wells 2011).

The intestinal microbial communities have an essential role in the digestion of food: it has been estimated that up to 10% of a person's daily calories on a Western diet come from microbial fermentation (McNeil 1984). The dominant members of the colonic microbiota are specialized degraders of complex polysaccharides, such as dietary fibres and resistant starch, releasing short-chain fatty acids (SCFA) as a result of polysaccharide degradation. Butyrate, acetate, and propionate are the most abundantly produced SCFAs (Macfarlane and Macfarlane 2003). Butyrate is produced by several species belonging to the Firmicutes, especially the Clostridium clusters IV and XIVa, propionate by Bacteroidetes and Clostridium cluster IX, and acetate by various bacteria (Louis *et al.* 2007). Methanogens are the only abundant archaea found in the intestinal tract and convert hydrogen and carbon dioxide into methane. The intestinal pH, which depends on diet, has a strong influence on the composition and activity of the microbiota: mildly acidic pH favours butyrate-producing Firmicutes, and neutral pH favours propionate-producing *Bacteroides* (Walker *et al.* 2005, Duncan *et al.* 2009). Bacteria-derived butyrate provides energy for colonocytes, helps them cope with hypoxia, improves the intestinal barrier, and has anti-inflammatory effects (Singh *et al.* 2014, Zheng *et al.* 2015). Propionate and acetate are transported to the liver and used for gluconeogenesis and cholesterol synthesis, respectively (Wolever *et al.* 1991). In addition to fermenting non-digestible polysaccharides, certain bacterial species are specialized in host-glycan utilization. In early life these species degrade oligosaccharides in breast milk (Ward *et al.* 2006, Marcobal *et al.* 2011), and in later life the abundant mucus-derived glycans. Species belonging to the genera *Akkermansia, Bifidobacterium, Bacteroides* and *Ruminococcus* are able to utilize mucin and are thought to represent key species in the intestinal mucosa, performing the first step in mucus degradation and thus unlocking an abundant energy source in the gut (Hoskins *et al.* 1985, Derrien *et al.* 2004).

Bile acid metabolism is one the key functions performed by the intestinal bacteria, with strong effects on host energy metabolism. The mammalian host produces primary bile acids, conjugated to taurine, or more commonly in adult humans, to glycine. Bacterial bile-salt hydrolases de-conjugate the bile acids, rendering them susceptible for further bacterial modification into secondary and tertiary forms (Ridlon *et al.* 2006). Primary bile acids are highly toxic to bacteria, and the modifications have been shown

to increase bacterial survival in the presence of bile (Jones *et al.* 2008). Bile salt hydrolases are enriched in the human intestinal microbiota (Jones *et al.* 2008), indicating that they confer a selective advantage in the intestinal environment. Modified bile acids have been implicated in colorectal cancer, but they also function as metabolic regulators by activating several key receptors with strong effects on the host's energy metabolism, such as FXR-α and TGR5 (Fiorucci *et al.* 2009). Bile-salt hydrolase activity of the microbiota has been shown to reduce host weigh gain, insulin resistance and blood cholesterol, via FXR-α and TGR5 signalling (Smet *et al.* 1998, Joyce *et al.* 2014). Microbial activity in the intestine thus has the potential to regulate host energy metabolism via extraction of energy from non-digestible compounds and via controlling energy expenditure and storage. SCFAs, particularly butyrate, regulate satiety and energy expenditure via gut hormones (Lin *et al.* 2012) and may be involved in the epigenetic programming of metabolism-regulating genes (Remely *et al.* 2014).

The intestinal microbes are not only beneficial but produce also compounds with harmful effects on the host. The microbiota include several pathobionts, species with the potential to negatively influence the host, most notably by inducing inflammation and even infections. The host actively inhibits the translocation of the microbes into systemic sites (Slack *et al.* 2009). However, many of their metabolic products enter the bloodstream and have systemic effects. The translocation of bacterial compounds depends on the integrity of the intestinal barrier, which can be weakened e.g., during infection by pathogens, stress or obesity (Soderholm and Perdue 2001, Brun *et al.* 2007, Groschwitz and Hogan 2009). Lipopolysaccharide (LPS), an endotoxin produced by Gram-negative bacteria, stimulates systemic inflammation and promotes adiposity, and may play an important role in metabolic diseases (Cani and Delzenne 2009). In a healthy microbiota, butyrate-producing Firmicutes (Peng *et al.* 2009), *Akkermansia muciniphila* (Everard *et al.* 2013), and bifidobacteria (Cani *et al.* 2007, Ewaschuk *et al.* 2008) may improve the intestinal barrier function, which limits the amount of inflammatory bacterial antigens that pass into circulation. When these protective functions of the microbiota are disrupted, the microbiota may promote inflammation systemically.

Apart from the above-mentioned processes, bacteria have a wide range of effects on the host, from vitamin production (LeBlanc *et al.* 2013) to the production of harmful substances from dietary compounds (Humblot *et al.* 2007, Koeth *et al.* 2013) and even to influencing behaviour (Heijtz *et al.* 2011). Studies on germ-free mice have revealed that the presence of bacteria has a profound impact on host phenotype, affecting all organ systems in the body (Evans *et al.* 2013).

Microbiota analysis methods

The intestinal microbiota are most conveniently and non-invasively surveyed from faecal samples. The faecal microbiota are representative of the colonic luminal community, while the bacterial composition in the mucosal layer and in the small intestine cannot be directly inferred from the faecal samples, but would require more invasive procedures (Zoetendal *et al.* 2002, Eckburg *et al.* 2005). However, the majority of the intestinal bacteria reside in the large intestine, where most of their metabolic activities take place. Therefore, faecal samples are a useful source of information on the intestinal bacterial community. Traditionally, the composition of the microbiota was analysed by culture-based methods, which is labour-intensive, causes bias towards easily cultivated species, and the results are highly sensitive to culture conditions. Because of these difficulties, DNA-based methods, utilizing marker genes for taxonomic identification, are commonly used to characterise the microbiota (Table 1). Most large studies on infant microbiota have used qPCR, which is a targeted, non-global method, but due to the simplicity of the microbiota in young children, often suitable. Smaller studies have usually conducted next-generation sequencing of variable regions on the 16S rRNA gene, which allows for comprehensive microbiota analysis. Infant microbiota studies abound, but studies with preschool or school age children are scarce (Table 1).

Development of the intestinal microbiota in childhood

The infant microbiome is simple, often dominated by a single species. Aerobic and facultative bacteria, such as staphylococci, streptococci, enterococci and enterobacteria are among the first colonizers (Palmer *et al.* 2007, Bäckhed *et al.* 2015, Dogra *et al.* 2015a). Bifidobacteria, which are anaerobic, normally begin to increase in abundance a few days or weeks after birth (Eggesbo *et al.* 2011, Bäckhed *et al.* 2015, Dogra *et al.* 2015a). The infant microbiome is enriched in genes encoding enzymes for the utilization of milk-derived glycans and the production of B vitamins (Bäckhed *et al.* 2015). However, even the fully breastfed infant has the microbial genetic potential for plant-derived polysaccharide degradation (Kurokawa *et al.* 2007, Vaishampayan *et al.* 2010, Koenig *et al.* 2011), which becomes necessary when solid foods are introduced. At weaning the microbiome responds to the change in diet by an increase in the abundance of Bacteroidetes and Clostridium clusters IV and XIVa, and a decline in Bacilli, Proteobacteria, and Actinobacteria (Koenig *et al.* 2011, Bergström *et al.* 2014, Bäckhed *et al.* 2015). By the age of 2-3 years, children reach a community composition, which is distinct from the infant community, but has not yet reached an adult-like composition (Ringel-Kulka *et al.* 2013, Korpela 2014). The development is gradual thereafter, and the adolescent microbiota composition still differs from that of adults (Agans *et al.* 2011).

Table 1. Recent DNA-based studies on the microbiota of children. # refers to the number of samples per child, Birth, Diet, AB, and Phe indicate whether the study included information on birth mode, diet, antibiotics, and child phenotype. Metagenomics = sequencing the whole metagenome, 16S seq = sequencing parts of the 16S rRNA marker gene. TRFLP = terminal restriction fragment polymorphism analysis, qPCR = quantitate PCR, FISH = fluorescence *in situ* hybridisation.

Ref	Location	Method	N	Age	#	Birth	Diet	AB	Phe
Yatsunenko et al. 2012	America, Malawi	Metagenomics		>0mo	1				
Tanaka et al. 2009	Japan	TRFLP, qPCR	26	0-2mo	5	x		x	
Dogra et al. 2015a	Singapore	16S seq	75	0-6mo	4	x	x		x
Bäckhed et al. 2015	Sweden	Metagenomics	98	0-12mo	3	x	x		
Jakobsson et al. 2014	Denmark	16S seq	24	0-24mo	6	x	x	x	x
Penders et al. 2006,	Netherlands	qPCR	1000	1mo	1	x	x	x	x
Lee et al. 2015	Korea	16S seq	20	1mo	1		x		
Fouhy et al. 2012	Ireland	16S seq, qPCR	18	1-2mo	2			x	
Fallani et al. 2009	Europe	FISH	606	1.5mo	1	x	x	x	
Azad et al. 2013	Canada	16S seq	24	4mo	1	x	x		
Nylund et al. 2013	Finland	Microarray	34	6-18mo	2		x		x
Bergström et al. 2015	Denmark	qPCR	300	9-36mo	3	x	x		x
Persaud et al. 2014	Canada	16S seq	184	12mo	1	x	x	x	
Ringel-Kulka et al. 2013	US	Microarray	28	1-4yr	1				
De Filippo et al. 2010	Burkina Faso, Italy	16S seq	29	1-6yr	1		x		

Factors influencing the early microbiota colonisation

Many common practices, such as birth by Caesarean section, antibiotic use, lack of breastfeeding, and exposure to the hospital environment, influence the development of the infant's microbiota (Biasucci *et al.* 2010, Dominguez-Bello *et al.* 2010, van Nimwegen *et al.* 2011, Persaud *et al.* 2014, Bäckhed *et al.* 2015, Dogra *et al.* 2015a). In addition to the above-mentioned effects, there is large inter-individual, geographic and methodological variation in microbiota composition, which are often difficult to separate (Fig. 1).

An infant receives its genome from both parents, but the mother is most likely the main donor of the infant's second genome, the microbiome. There is evidence that the early development of an infant's microbiome is influenced by prenatal factors, suggesting that the colonization process depends strongly on maternal effects. The maternal intestinal and vaginal microbiomes have been shown to change during pregnancy, becoming less diverse and more dominated by typical infant-colonizing bacteria such as lactic acid bacteria (Aagaard *et al.* 2012, Koren *et al.* 2012). Several factors, such as excessive weight gain, antibiotic use, and stress, may interfere with the development of the microbiota during pregnancy and therefore with the transmission of bacteria to the infant. Overweight mothers harbour intestinal microbiota with low abundance of bifidobacteria and high abundance of enterobacteria and staphylococci (Collado *et al.* 2008, Santacruz *et al.* 2010), which is reflected in the infant's early microbiota (Collado *et al.* 2010). The breast milk microbiome of overweight mothers also differs from that of normal weight mothers, indicating that the maternal guidance of the infant's developing microbiome may depend on maternal weight (Collado *et al.* 2012), and may be involved in the inheritance of obesity (Woo and Martin 2015). The effects of maternal antibiotic use on the infant's microbiome have not been thoroughly investigated, but maternal antibiotic use is known to increase the infant's risk of childhood overweight (Mueller *et al.* 2015).

Prenatal stress affects the immunological and psychological development of the infant, predisposing to various diseases including asthma (Cookson *et al.* 2009) and increased childhood adiposity (Dancause *et al.* 2015). In monkeys, prenatal stress has been found to influence the early development of the microbiome: the offspring of experimentally stressed females have a microbiome deficient in lactobacilli and bifidobacteria (Bailey *et al.* 2004). The mechanism is uncertain, but may be related to stress-induced inflammation and increased levels of cortisol. Enterobacteria, unlike many other intestinal bacteria, are able to thrive in the intestine during inflammatory states (Lupp *et al.* 2007) and may benefit from potential stress-associated inflammation, as well as the stress-associated hormones (Lyte *et al.* 1997). Furthermore, cortisol controls bile acid homeostasis (Rose and Herzig 2013), which directly regulates the microbiota (Islam *et al.* 2011).

Caesarean section, which is unnecessarily common in many parts of the world (Zizza *et al.* 2015), affects the early microbial colonization of the

infant. Caesarean-born infants are first exposed to skin and hospital surfaces, rather than the birth canal, and consequently are colonized by bacteria residing on these surfaces, while vaginally born infants are colonized by vaginal and faecal bacteria of their mothers (Dominguez-Bello *et al.* 2010, Bäckhed *et al.* 2015). The normally dominant members of the infant intestinal microbiome, bifidobacteria, often show delayed development, and colonization by *Clostridium difficile* is more common than in vaginally born infants (Grönlund *et al.* 1999, Penders *et al.* 2006, Biasucci *et al.* 2010). In addition to bifidobacteria, *Bacteroides* spp. form a significant part of the vaginally born infant's intestinal microbiota, but appear late in C-section born infants (Bäckhed *et al.* 2015). The microbiome differences between C-section born and vaginally born infants persist at least 6-12 months (Grönlund *et al.* 1999, Bäckhed *et al.* 2015, Dogra *et al.* 2015a). However, the fact that C-section-born infants generally develop normally and eventually reach a microbiota composition comparable to vaginally born infants indicates that major microbial colonisation and re-organisation occur after birth, guided by post-natal exposures.

Breastfeeding

Breastfeeding continues the maternal guidance of the developing microbiota by providing and nourishing specific bacteria (Grönlund *et al.* 2007, Marcobal *et al.* 2010, Garrido *et al.* 2012). The natural breastfeeding duration in humans is estimated to be 2-3 years, extending sometimes up to 6 years (Kennedy 2005). Although breastfeeding is known to promote the health of the infant and the mother (Labbok 2001, Hornell *et al.* 2013), modern children are often weaned before the age of 6 months (Callen and Pinelli 2004). During pregnancy, microbial translocation from the intestine to the breast tissue increases, and breast milk contains many taxa that are commonly found in the infant intestine (Donnet-Hughes *et al.* 2010). Breast milk may therefore be a source of colonizing microorganisms.

In addition to microbes, breast milk contains a rich cocktail of immunologically active compounds and cells. Formula-fed infants are essentially immune-deficient before the maturation of their own immune system, as they lack the maternally derived IgA, cytokines, hormones, leucocytes, human milk oligosaccharides (HMOs), and bactericidal enzymes present in breast milk (Hanson 1998, Hanson 2000, Newburg and Walker 2007, Blustein *et al.* 2013). Indeed, breastfeeding is known to protect against infections in early life (Duijts *et al.* 2009, Abrahams and Labbok 2011, Hornell *et al.* 2013), and in pre-industrial times infant survival was strongly dependent on breastfeeding (Macadam and Dettwyler 1995).

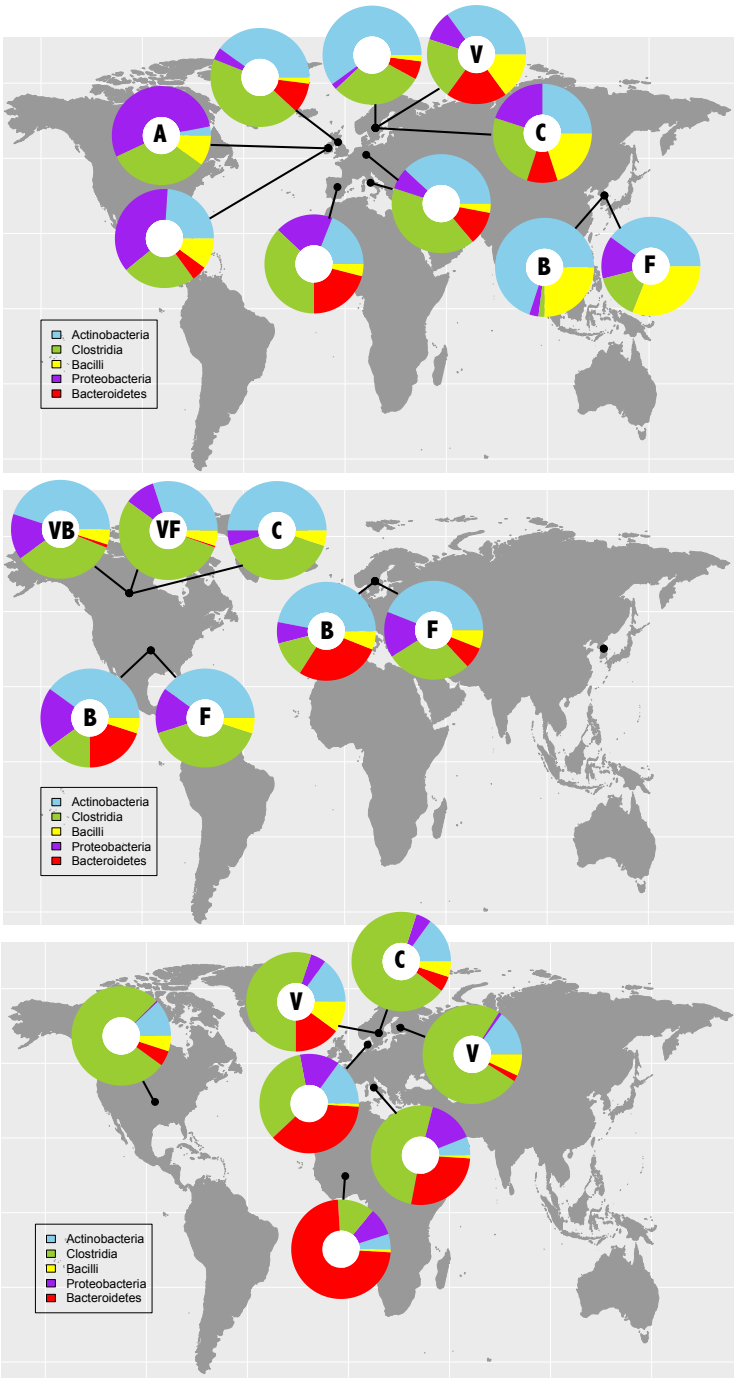

Figure 1. Summary of recent early-life microbiota studies (data from papers in Table 1). Top panel: 1 month, middle panel: 3-4 months, and bottom panel: >6 month old infants. A = antibiotics, V= vaginal delivery, C = caesarean section; B = breastfed, F = formula-fed. Firmicutes are divided into clostridia (including erysipelotrichi) and bacilli.

Importantly, breast milk contains considerable amounts of various complex fucosylated and sialilated glycans that are non-nutritious for the host, but utilized as energy sources by *Bifidobacterium, Bacteroides,* and *Akkermansia* spp. in the infant intestine (Ward *et al.* 2006, Marcobal *et al.* 2011, Ottman 2015). These glycans are not present in formula milk, and even oligosaccharide-supplemented or probiotic-supplemented formulas may not have the same effect on the infant microbiota as human milk (Euler *et al.* 2005, Brunser *et al.* 2006). Maternal *FUT2* genotype affects the composition of breast milk glycans, and infants of mothers with the inactive allele (non-secretors) show delays in their acquisition of bifidobacteria and differences in bifidobacterium species composition (Lewis *et al.* 2015). This indicates that the mother guides the early development of her infant's microbiota via breast milk. Breastfed infants often have higher abundances of bifidobacteria and lactobacilli and lower abundances of clostridia (including *C. difficile*), enterobacteria and enterococci than formula-fed infants (Ahrné *et al.* 2005, Euler *et al.* 2005, Brunser *et al.* 2006, Azad *et al.* 2013, Bergström *et al.* 2014, Bäckhed *et al.* 2015). The compositional differences are reflected in the functional differences: breastfed infants have more microbial genes coding vitamin B (e.g. folate) production (Bäckhed *et al.* 2015). The cessation of breastfeeding, rather than the introduction of solid foods, initiates a change in the microbiota towards an increased abundance of fibre-degrading Firmicutes and Bacteroidetes (Palmer *et al.* 2007, Bergström *et al.* 2014), which form the majority of the adult microbiota.

Antibiotics and probiotics

Antibiotics account for the majority of prescription medication used by children in western countries (Chai *et al.* 2012). Several human studies have shown dramatic changes in the intestinal microbiota of adults in response to oral antibiotic treatments (De La Cochetiere *et al.* 2005, Dethlefsen *et al.* 2008, Jakobsson *et al.* 2010, Jernberg *et al.* 2010, Dethlefsen and Relman 2011). In adults, the intestinal microbiota usually, but not always, recover after discontinuation of the antibiotic treatment. However, in infants, perinatal antibiotic use is associated with changes in the intestinal microbiota composition persisting for up to 1 year (Persaud *et al.* 2014), indicating that early-life antibiotic use may permanently disturb the colonization process. In the short term, infants with antibiotic exposure often have reduced abundance of bifidobacteria, normally the dominant member of the infant microbiota, and increased abundance of potentially inflammatory bacteria such as *E. coli* (Penders *et al.* 2006, Tanaka *et al.* 2009, Fallani *et al.* 2010).

Probiotics are defined as live microorganisms that when administered in adequate amounts confer a health benefit on the host (Hill *et al.* 2014). Lactic acid bacteria and bifidobacteria are among the most commonly used probiotics. *Lactobacillus rhamnosus* GG is one of the best-studied strains

marketed as a probiotic (Saxelin *et al.* 2005). Extensive meta-analyses have shown this bacterium to be effective in the treatment of many gastrointestinal illnesses in children: it reduces gastrointestinal pain (Horvath *et al.* 2011), *Clostridium difficile* -associated diarrhoea (Segarra-Newnham 2007), healthcare-associated diarrhoea (Szajewska *et al.* 2011), antibiotic-associated diarrhoea (Hawrelak *et al.* 2005), and the duration of infectious diarrhoea (Szajewska *et al.* 2007). In adults, probiotic use has not resulted in large changes in faecal microbiota composition (Kim *et al.* 2013, Lahti *et al.* 2013a). This suggests that the mode of action may be related to altered microbial metabolism or direct interaction with the host (Gerritsen *et al.* 2011). *L. rhamnosus* GG produces pili with the mucus-binding protein SpaC (Kankainen *et al.* 2009). These pili have been shown to be important for the adhesion of the bacteria to mucus and to enable close contact with intestinal cells and effective stimulation of the immune system (Lebeer *et al.* 2010, Gerritsen *et al.* 2011).

Health effects of the early microbiome development

The early development of the microbiome is emerging as a key factor involved in the immunological (Grönlund *et al.* 2000, Sjögren *et al.* 2009, Russell *et al.* 2012) and metabolic (Cox *et al.* 2014) programming of the host, with potential long-term health impacts (Reinhardt *et al.* 2009, Willing *et al.* 2011, Scholtens *et al.* 2012). The factors affecting the early microbiome development, such as delivery by Caesarean section (Bager *et al.* 2008, Cardwell *et al.* 2008, Thavagnanam *et al.* 2008, van Nimwegen *et al.* 2011, Mueller *et al.* 2015), lack of breastfeeding (Harder *et al.* 2005, Hornell *et al.* 2013), prenatal stress (Dancause *et al.* 2015, Hohwü *et al.* 2015), and early-life antibiotic use (Hviid *et al.* 2011, Virta *et al.* 2012, Mueller *et al.* 2015, Saari *et al.* 2015, Gerber *et al.* 2016) have been associated with increased incidence of various metabolic and immunological conditions, such as increased weight gain, overweight, asthma, type 1 diabetes, celiac disease, and inflammatory bowel disease (IBD). The type of antibiotic and the timing of the course appear to be important for later metabolic effects (Saari *et al.* 2015, Gerber *et al.* 2016). The increased risk for allergic diseases in C-section born infants has been shown to be associated with an increased abundance of *C. difficile* in early life in a large Dutch infant cohort (van Nimwegen *et al.* 2011). Animal experiments have demonstrated that early-life antibiotic use disrupts the microbiota and consequently immune function, predisposing to the development of asthma (Noverr *et al.* 2005, Russell *et al.* 2012).

In production animals, antibiotic use increases weight gain at least partly by suppressing subclinical infections (Dibner and Richards 2005). In laboratory mice, the antibiotic-induced weight gain was demonstrated to result from the altered gut microbiome (Cox *et al.* 2014). Epidemiological studies have confirmed the positive relationship between antibiotic use and

weight gain in humans (Thuny *et al.* 2010, Ajslev *et al.* 2011, Trasande *et al.* 2013, Gough *et al.* 2014, Saari *et al.* 2015, Gerber *et al.* 2016) and indicated that even prenatal antibiotic exposure predisposes to childhood overweight (Mueller *et al.* 2015). This suggests that maternal microbes may play a significant role in the metabolic programming of the infant. Pre- and perinatal maternal and environmental factors are being recognized as important contributors to the long-term metabolic programming and weight development of infants, and multiple lines of evidence indicate that childhood overweight may be strongly dependent on early-life exposures (Cottrell and Ozanne 2008). The intestinal microbiota, acquired initially during birth from the mother and nurtured by breast milk, are emerging as an important modulator of early metabolic programming, with long-lasting health consequences.

Paediatric inflammatory bowel disease

One of the most extreme examples of aberrant microbiota development is inflammatory bowel disease (IBD), a term encompassing Crohn´s disease (CD), ulcerative colitis (UC) and unclassified colitis (IBDU). The incidence of paediatric IBD is rapidly increasing in Europe and North America (Benchimol *et al.* 2011). In Finland the incidence of paediatric IBD has increased by 5-8% annually, reaching 15/100 000 in 2003 (Lehtinen *et al.* 2011). The incidence of UC has continued to increase since (Jussila *et al.* 2012). The causes of IBD are unknown. Several genes involved in immune defence are associated with disease risk, but their total effect is fairly small (Jostins *et al.* 2012). Childhood environment and exposures have been shown to predict IBD incidence (Gearry *et al.* 2010), and common to these predictive factors is that they influence the intestinal microbiota. The interactions between the immune system and intestinal microbiota are most likely of central importance in the aetiology of IBD (Jostins *et al.* 2012). Disturbed microbiota development, caused possibly by frequent antibiotic use, lack of breastfeeding, Caesarean birth or dietary patterns, may lead to the development of inflammatory microbiota, to which the immune system reacts aggressively in genetically susceptible individuals (Gearry *et al.* 2010, Kostic *et al.* 2014).

The microbiota in paediatric IBD patients is often characterized by increased abundance of Gram-negative organisms and decreased abundance of butyrate-producers (Schwiertz *et al.* 2010, Papa *et al.* 2012). An increased abundance of bacteria, particularly Gram-negative bacteria, in the gut mucosa has been observed, suggesting a failure at the mucosal barrier (Conte *et al.* 2006). Some studies find an increase in *Bacteroides* (Schwiertz *et al.* 2010), while other report an increase in enterobacteria (Papa *et al.* 2012, Gevers *et al.* 2014), suggesting that the IBD-associated microbiota may take different shapes.

The treatment of IBD is currently centred on calming the immune response, using anti-inflammatory drugs such as 5-aminosalisylic acid, corticosteroids, immunomodulatory drugs, and reducing the abundance of bacteria in the intestine by antibiotics. Antibiotics have, however, been shown to aggravate rather than improve the dysbiosis associated with IBD (Gevers *et al.* 2014), and antibiotic use is a risk factor for IBD (Virta *et al.* 2012). The use of TNF-α-antagonists is becoming increasingly common in the treatment of severe paediatric IBD patients, and many patients respond positively. TNF-α is a pro-inflammatory cytokine, produced by activated macrophages and other immune cells. When bound to its receptor, TNF-α actives signalling cascades leading to a range of outcomes from immune responses to apoptosis or cell proliferation (Chen and Goeddel 2002). Its expression is increased in inflamed mucosa. However, nearly half of patients show no response, require dose escalation, or lose the response (de Bie *et al.* 2012). The reasons for this are currently unknown and response cannot be predicted (de Bie *et al.* 2012). The use of TNF-α-antagonists carries the risk of adverse effects, and is very expensive, and therefore selecting patients with a high likelihood of benefiting from the treatment would be important, but is currently not possible.

Aims of the thesis

This thesis aims to characterize the development of the human intestinal microbiota in healthy children and to identify factors, which are important for the natural development of the microbiota. Specifically, the influence of four common factors potentially modulating the microbiota – prenatal stress, breastfeeding duration, antibiotic use, and probiotic use – is investigated, as well as the association between early-life microbiota composition and the development of BMI. In addition, the microbiota in healthy children is contrasted with that that of children with IBD, characterising the association between treatment response and microbiota in IBD.

Material and Methods

Study cohorts

The data for paper I is derived from the Dutch BIBO study, which is a longitudinal study following 193 mothers and their children from the third trimester of pregnancy on (Beijers *et al.* 2011). Pregnant women were recruited through midwife practices in Nijmegen and surrounding areas (the Netherlands). A sub-cohort of 56 infants was selected for this study based on the availability of their faecal samples and their exposure to prenatal stress. All infants were healthy, born at full term (≥ 37 weeks) and had a 5-min

APGAR score ≥ 7. Caesarean-delivered children were excluded. Five faecal samples were collected from the infants from birth until ±115 days of life.

Table 2. Study cohorts. Microbiota refers to the number of children providing microbiota samples. Other response indicates how many children provided other response variables (specified below).

	Country	Age	Micro biota	Other resp.	Samples /subject	DNA extr.	Analysis platform
I	Netherlands	<5 months	56	56	3-5	RBB	HITChip
II	Finland	2-7 years	142	226	1-2	Pro	454
III	Finland	2-7 years	142	236	1-2	Pro	454
IV	Finland	2-7 years	88	231	2	Pro	HITChip
V	Netherlands & Finland	3 months	162	162	1	RBB	HITChip
VI	Finland	12-18 years	94	94	1-3	RBB	HITChip

RBB = repeated bead beating. Pro = enzymatic lysis procedure and subsequent purification using the Promega Wizard Kit (see Salonen et al. 2010 and Ahlroos & Tynkkynen 2009 for details).

Data for papers II-IV originate from a probiotic trial (Kumpu *et al.* 2012). The children were recruited at day care centres in northern Finland. The probiotic treatment group received milk supplemented with *Lactobacillus rhamnosus* GG (LGG; approximately 10^6 cfu/ml), and the control group received similar milk without the probiotic. The intervention continued for seven months. All participants attended a health check and were asked to provide a faecal sample at the beginning and end of the intervention period. Originally a total of 501 children participated in the study; for the microbiota studies, subsets were selected based on the availability of the relevant records and samples (Table 2).

For paper V, data from the BIBO cohort (N=87, based on availability of faecal samples and weight data) were combined with data from a Finnish infant study (N=75). The Finnish study was a large synbiotic trial involving ca. 1000 infants (Kuitunen *et al.* 2009), from which a sub-cohort was selected for the study from the vaginally born control group, based on availability of faecal samples and weight data.

For study VI, we invited 12-18 year old Finnish-speaking patients with IBD treated at the Children's hospital, Helsinki, to provide faecal samples for microbiota and calprotectin analyses. Age-matched healthy adolescents, and patients with juvenile idiopathic arthritis were invited as controls. IBD patients with acute severe colitis were excluded. One faecal sample was

analysed for most subjects, but a subset of 11 patients, beginning TNF-α-therapy, provided a faecal sample before, after 2 weeks and after 6 weeks from starting the therapy.

Written informed consent was received from the parents of all children participating in the studies. All studies were approved by the local ethical committees.

Analysis of the intestinal microbiota using faecal samples

DNA was extracted from the faecal samples using either the repeated bead beating (RBB) method, which relies on mechanical and chemical lysis of cells, as described previously (Salonen *et al.* 2010), or a modified version of the Promega Genomic Wizard DNA Purification Kit (Promega, Madison, WI, USA; Ahlroos and Tynkkynen 2009). In the Promega protocol, the purification of DNA is based on sequential precipitation of proteins and nucleic acids. Both methods have been shown to result in comparable microbiota compositions (Salonen *et al.* 2010). The bacterial composition was studied using two methods, a phylogenetic microarray, the Human Intestinal Tract Chip (HITChip) (papers I, IV, V, VI) as well as 16S rRNA gene amplicon sequencing using the Roche 454 pyrosequencing platform (papers II, III).
The HITChip is specifically designed for the analysis of the human intestinal microbiota (Rajilic-Stojanovic *et al.* 2009). The microarray consists of oligonucleotide probes targeting hyper-variable regions V1 and V6 of the 16S rRNA gene, allowing the identification, quantification and phylogenetic positioning of not only previously cultured and named, but also uncultured bacterial phylotypes. Microarray analysis of the bacterial DNA was conducted by collaborators in Wageningen, the Netherlands, as described (Rajilic-Stojanovic *et al.* 2009). Briefly, the DNA was amplified with PCR using the universal bacterial primers T7prom-Bact-27-for and Uni-1492-rev. The DNA was then transcribed to RNA, which was labelled and hybridized on the microarray. The signal intensities of the oligonucleotide probes were translated into abundances of 1038 species-level phylotypes, 130 genus level-taxa, and 23 phylum-level taxa and clostridium clusters using the fRPA pre-processing algorithm (Lahti *et al.* 2013b). The genus-level taxonomy was formed by grouping together related (>90% genetic similarity) organisms. The groups were named according to the nearest cultured relative. The microbiota data were transformed into relative abundances by dividing the signal intensities of each taxon by the total signal intensity of the sample.

Sequencing of the V4-V6 hypervariable region of the 16S rRNA gene was conducted using the 454 Titanium pyrosequencing on a GS FLX (Roche Diagnostics) instrument, using the primers S-D-Bact-0564-a-S-15/S and Univ-1100-a-A-15, which have high coverage among bacteria (Klindworth *et al.* 2013). The sequences were filtered for chimaeras with the Uchime program (Edgar *et al.* 2011). Reads shorter than 501 nucleotides and samples

with <1000 reads were filtered out. After pre-processing, we had a total of 2,262,107 reads from 257 samples (on average 8801 reads/sample, range 1469-14653 reads/sample). De novo OTU picking was done using Qiime (Caporaso *et al.* 2010). To avoid batch effects, we normalized the data following a method we have developed earlier (Korpela *et al.* 2014).

In addition, for paper III, functional profiling of the microbiome was conducted. Whole genome metagenomic sequencing was conducted by collaborators at the European Molecular Biology Laborary, Germany, on a subset of 20 samples using the Illumina HiSeq platform. The metagenomic analysis was used as a discovery tool to guide further qPCR-based analyses on a larger number of samples. Based on the metagenomic results, real-time qPCR was conducted to measure bile-salt hydrolase genes and antibiotic resistance genes. In addition, bacteria from the faecal samples were cultured anaerobically to test for antibiotic susceptibility of the bacterial communities.

Other sources of data

For paper I, stress experienced by the mothers during the third trimester of pregnancy was recorded in two ways: validated questionnaires measuring different types of stress, and salivary cortisol levels measured at different times of the day.

Papers II-IV included antibiotic purchase records, obtained from the national drug purchase registry maintained by Kela (the Finnish Social Insurance Institute). All reimbursed drug purchases, which is estimated to cover >95% of antibiotic purchases, are recorded in the registry, as well as the diagnoses for chronic illnesses. Paper II utilized questionnaire-based data on the duration of breastfeeding, as well as weight and height of the children measured during a physician's visit at the beginning of the study. Paper III included the registry-based information on chronic illnesses and weight and height data.

In paper V, the children were measured for weight and height at the age of 5-6 years. In addition, information on their antibiotic use was obtained from clinical records and questionnaires.

For study VI, faecal calprotectin was measured in a routine clinical laboratory using a quantitative enzyme immunoassay, as an indication of disease activity. In addition, patient records including diagnosis, location of disease, history or surgery, diet, antibiotic and probiotic use were available.

Statistical methods

Univariate analyses were conducted using general linear or generalized linear models, depending on the response variable. Normally distributed response variables were analysed with linear regression or analysis of variance models;

count variables were analysed with generalized linear models, using the negative binomial distribution. Fixed effects models were used when only one time point for each individual was analysed and mixed effects models with subject as random factor, when multiple samples were analysed from the same individuals. Potentially confounding variables such as probiotic use, age, and BMI were included as covariates in the models. Multivariate analyses were conducted with principal coordinates analysis (PCoA), usually using the Bray-Curtis dissimilarities, hierarchical clustering, and multivariate permutational analysis of variance. All statistical analyses were conducted in R (R Core Team 2012), using the packages vegan (Oksanen *et al.* 2013), MASS (Venables and Ripley 2002), glmmADMB (Skaug *et al.* 2012), and nlme (Pinheiro *et al.* 2013).

Results and Discussion

Microbiota development during the first years of life

Combining different data sets of altogether 222 healthy children, it is possible to roughly characterize the general pattern of microbiota development during the first 6 years of life (Fig. 2). While there are marked individual differences in the development, on average the development tends to progress through three distinct phases: successional phase 1 is represented by a dominance of Bacilli, mainly staphylococci, streptococci, and enterococci. This phase begins a few days after birth and is estimated to last in general for 2-3 weeks, when bifidobacteria become the most abundant group. The dominance of bifidobacteria represents the second successional phase, and continues until about 1 year of age. During the first two phases, Proteobacteria represent a relatively abundant taxon, on average 10% of the microbiota. At the age of approximately 6 months, the abundance of bifidobacteria and Proteobacteria begin to decline, and the abundance of Clostridium clusters (hereafter 'clostridia', referring collectively to the Firmicutes classes *Clostridiaceae*, *Erysipelotrichaceae* and *Ruminococcaceae*) and Bacteroidetes begin to increase gradually. By the age of approximately 1 year, bifidobacteria, although still very abundant, have been replaced by clostridia (mainly Clostridium cluster XIVa) as the most abundant bacterial group, which marks the initiation of the third successional phase. During this phase, clostridia continue to increase in abundance, reaching a relative abundance of 50% at approximately 2 years, and the infant-type taxa, Bacilli, Proteobacteria, and bifidobacteria, decline to <10% by the age of 6 years. The timing of the transition from phase 2 to phase 3 likely depends on the timing of weaning (Koenig *et al.* 2011, Bergström *et al.* 2014, Bäckhed *et al.* 2015), but what induces the transition from phase 1 to phase 2 is not well known. These results are averages based on 455 samples from a cohort of 222 children originating from two European countries, but they appear to be fairly general

among healthy, vaginally born, breastfed children (Dogra *et al.* 2015b). Similar patterns appear to occur in non-European children, especially with regard to bifidobacteria (De Filippo *et al.* 2010, Yatsunenko *et al.* 2012). However, individual variation in the timing of the phases is considerable and likely to be of importance for the metabolic and immunological programming of the child.

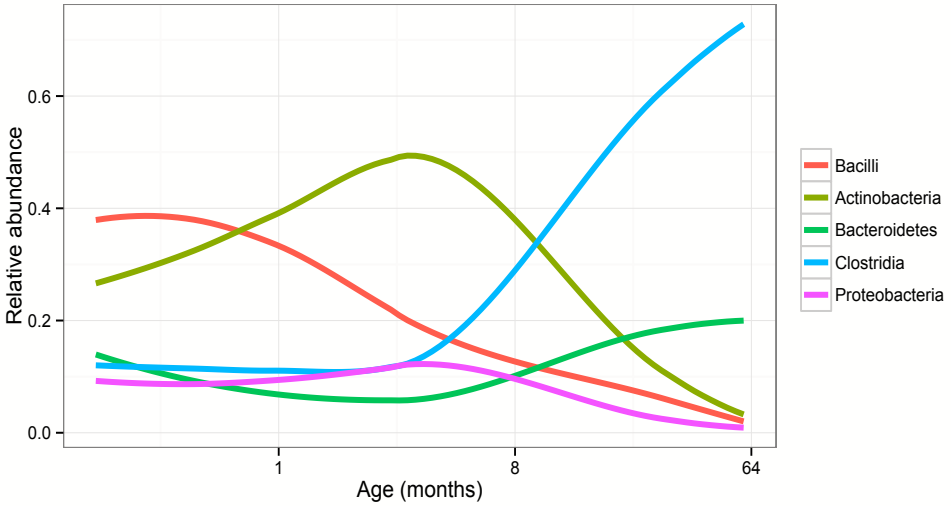

Figure 2. Microbiota development during the first 6 years of life (x-axis is on log-scale). The figure shows averages based on 455 faecal samples of 222 children of different ages.

Maternal stress during pregnancy is associated with the infant's early microbiota development (I)

Maternal pregnancy-related stress was strongly associated with the early development of the infant's intestinal microbiota (I), slowing the transition to a *Bifidobacterium*-dominated composition (Fig. 3). Together, two stress-indicators, experienced pregnancy-related stress and salivary cortisol showed associations with 78% of the genus-level bacterial groups in the infants, and prenatal stress was comparable to feeding type (breastfed or formula-fed) in terms of the strength of the microbiota association. The results suggest that maternal stress, or something co-occurring with maternal stress, is a major contributor to the early development of the microbiota. Cortisol levels and reported stress were only modestly correlated, indicating that they measure different types of stress. However, they appeared to have similar and additive effects on the microbiome: infants born to mothers with high reported stress and high cortisol had the most divergent microbiota development, compared to infants born to mother with low levels of both stress indicators. The infants born to mothers with one high and one low indicator had an intermediate microbiota composition. The bacterial taxa most strongly associated with

prenatal stress were bifidobacteria and lactobacilli, both of which were significantly reduced in the high stress group, and enterobacteria and enterococci, which were significantly increased (Fig. 3).

As this study is descriptive, the mechanism behind the observed association cannot be delineated. The altered infant microbiota may be caused by changes in maternal microbiota, or changes in the infant's physiology. In favour of the former hypothesis speak the observation that the abundance of Proteobacteria increases during pregnancy in some, but not all, mothers (Koren *et al.* 2012), and the composition of infant meconium is characterized by dominance of either lactobacilli or Proteobacteria (Gosalbes *et al.* 2013). Whether the maternal microbiota composition correlates with maternal stress, and translates to a higher abundance of Proteobacteria and lower abundance of lactobacilli and bifidobacteria in the infant's intestine, is yet to be confirmed. However, in mice, exposure to as little as 2 hours of stress alters the colonic mucosal microbiota, reducing the abundance of lactobacilli (Galley *et al.* 2014).

Stress is strongly related to inflammation: both psychological and physiological stress may induce inflammation also in the intestinal mucosa, and vice versa, inflammation may induce stress (Black 2002). Stress may thus directly affect the microbiota of the mother, and maternal transmission of bacteria. This pathway may lead to a selection of bacteria tolerant of inflammation and stress hormones, such as the enterobacteria (Lyte *et al.* 1997, Lupp *et al.* 2007), which were elevated in the stress-exposed infants. Stress may also influence bile acid levels, as cortisol is known to control bile acid production in the liver, and regulate cholesterol and bile acid homeostasis (Rose and Herzig 2013), thus influencing the maternal microbiota (Islam *et al.* 2011). Furthermore, maternal stress may influence her lifestyle (e.g., diet, sleep, exercise), which is likely to affect her microbiota. It is also possible that the maternal stress correlates with or is dependent on some other factor that also alters the infant microbiota. Furthermore, reverse causation is possible: inflammation is known to regulate glucocorticoid secretion, and LPS produced by the intestinal microbiota may activate the HPA axis (Black 2002). Microbiota-derived inflammation in the mother may thus affect her stress measurements, in which case maternal stress and the infant microbiota would both be dependent on the maternal microbiota.

Although causation cannot be established in observational human studies, experiments with non-human primates show a very similar effect of prenatal stress on the microbiota (Bailey *et al.* 2004), which suggest that the effect is causal and fairly universal. Both the stress response and the inflammation response are highly conserved across the animal kingdom (Maier and Watkins 1998), and it is possible that the response of the host-associated microbiota to stress and possibly inflammation may be equally conserved. If so, the apparent negative effects observed in prenatally stressed infants could be adaptive and shaped by evolution. It is possible that prenatal

stress prepares the infant to cope in a stressful environment, and changes in the microbiota may be involved in the early programming.

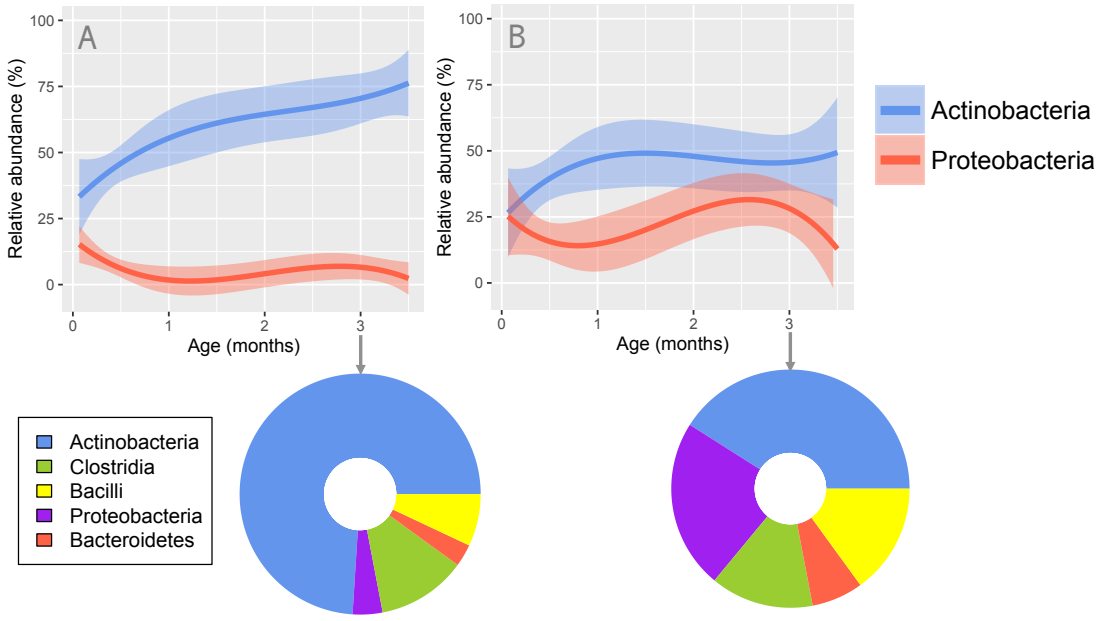

Figure 3. The development of the relative abundance of selected bacterial groups during the first 4 months of life in low prenatal stress (A) and high prenatal stress (B) infants in paper I. The panels show the best-fit lines (based on linear 3rd polynomial models) and standard errors. The average relative abundance of the main bacteria phyla, Firmicutes divided into bacilli and clostridia, at 3 months of age is shown below the panels.

Breastfeeding duration is associated with long-term microbiota development (II)

Among the 2-7 year old Finnish cohort, the duration of breastfeeding was associated with the microbiota composition, when the effect of other factors such as health and antibiotic use were eliminated (II). A clear distinction in the microbiota composition was evident between children breastfed for 0-6 months and those breastfed for 8-16 months: the abundance of bifidobacteria and *Akkermansia* were significantly higher, and the abundance of clostridia significantly lower among the children with longer breastfeeding duration, indicating a slow transition from phase 2 to phase 3 (Fig. 4). *Bifidobacterium* spp. and *Akkermansia muciniphila* are known to specialize in host-glycan utilization and both genera contain species with the ability to degrade mucin (Derrien *et al.* 2004, Ruas-Madiedo *et al.* 2008). Several *Bifodobacterium* spp. are capable of growing in breast milk (Zivkovic *et al.* 2011), as has also been found for *Akkermansia muciniphila* (Ottman 2015).

Although we matched the children based on age, antibiotic use history, and BMI, we cannot exclude the possibility that some of the observed effects

of breastfeeding may have been due to potential confounding effects of birth mode, infant birth weight, lifestyle, diet, maternal education, or maternal BMI (Riva *et al.* 1999, Scott *et al.* 2001, Bertini *et al.* 2003, Cernadas *et al.* 2003, Wojcicki 2011).

Antibiotic use is associated with microbiota development (II, III)

Early-life antibiotic use appeared to interfere with the development of the infant's microbiota and to disrupt the maternal guidance via breast milk (II). Children who had been weaned late but had antibiotic courses before weaning had at 2-7 years of age a microbiota composition similar to those with short duration of breastfeeding (Fig. 4), particularly with respect to the abundance of bifidobacteria and clostridia. The transition from successional phase 2 to 3 was thus faster in the children who had received antibiotics during their early life, regardless of breastfeeding duration (II). Children who had received antibiotics before weaning or shortly after had an increased abundance of Bacteroidetes (mainly *Bacteroides* spp.) at the age of 2-7 years compared to those who did not receive antibiotics in early life (II).

Antibiotic use continued to affect the microbiota development in later childhood (III). The microbiota composition in children exposed to penicillin or macrolide –type antibiotics during the 2 years before faecal sample donation was compared the composition in children with no recent antibiotics and low lifetime antibiotic use. The most dramatic apparent effect of antibiotic treatment was the decline in the relative abundance of *Bifidobacterium* after a macrolide course (Fig. 5), which normalized within 2 years. As bifidobacteria declined, the relative abundances Bacteroidetes and Firmicutes were elevated after a macrolide course; the increase in Bacteroidetes was highly significant and consistent between individuals. The abundance of Firmicutes was strongly dependent on age, and the antibiotic effect was not statistically significant in the total cohort.

Bacteroides spp. were thus elevated if the child had received antibiotics in early life, or if the child had recently received macrolides, suggesting that *Bacteroides* spp. may benefit from the antibiotic-induced disruption of the normal microbial balance. The abundances of *Collinsella, Lactobacillus*, and *Anaerostipes*, as well as the total richness and maturity of the microbiota, also remained reduced for up to 2 years after a macrolide course. Furthermore, macrolide resistance was increased and the abundance of bile-salt hydrolase genes decreased after macrolide treatment. The former observation is indicative of causality and demonstrates that antibiotic use promotes antibiotic resistance within the individual; the latter indicates that antibiotic use may alter bacterial metabolism of host-affecting biomolecules, potentially influencing the microbiota-host interaction.

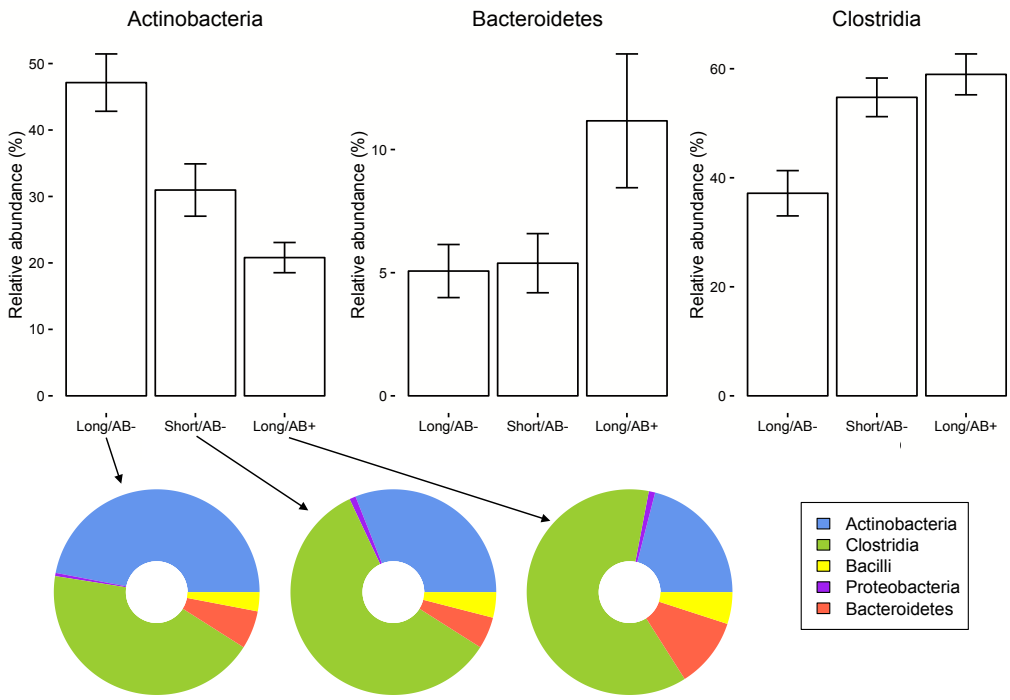

Figure 4. Relative abundance of selected bacterial groups in children breastfed for 8-16 months without antibiotics (Long/AB-), 0-6 months without antibiotics (Short/AB-), and 8-16 months with antibiotics (Long/AB+). Data from paper II. The bars show the group means and the whiskers show the standard errors. The average relative microbiota composition in the three groups is shown on the lower panel.

Recent penicillin or amoxicillin use had partly different and clearly weaker overall association with the microbiota than macrolide use (Fig. 5). Indications for the different antibiotic types did not differ: both macrolide and penicillin-type antibiotics were mostly prescribed for respiratory infections. This suggests that the antibiotics, and not the illness that prompted their use, caused the patterns observed in the microbiota. Previous mouse experiments support our results, and indicate that the observed effects of antibiotics on the microbiota are likely to be causal. A reduction in bile-salt metabolism has been observed in mice experimentally treated with streptomycin (Antunes *et al.* 2011). Early-life treatment with penicillin and macrolide –type antibiotics has been shown to alter the microbiota of mice in a similar way (Nobel *et al.* 2015), as seen here in children.

The differences between the antibiotics in their apparent effects on the microbiota are likely due to the differences in mechanism of action, spectrum of susceptible organisms, and pharmacokinetics. Penicillin-type antibiotics are betalactams, which kill bacteria by inhibiting cell wall synthesis. The beta-lactam ring binds to peptidoglygan in the bacterial cell wall, inhibiting cross-linking between peptidoglycan chains. Beta-lactams tend to be more effective against Gram-positive bacteria, while Gram-negative bacteria with the outer membrane protecting the peptidoglycan structure, are often less susceptible.

To be effective, betalactams need to be maintained at a sufficient concentration over time. Penicillins are well absorbed from the small intestine and excreted via urine. Therefore their concentration in the colon is likely to be mostly too low for strong bactericidal effects, particularly against Gram-negative organisms.

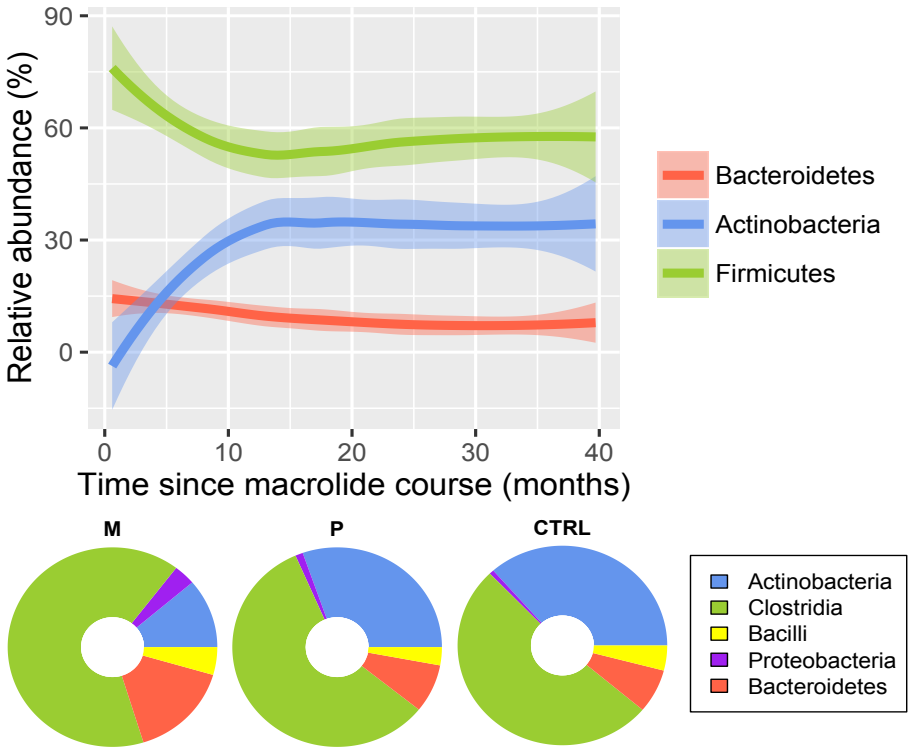

Figure 5. Antibiotic use and phylum-level microbiota. Data from paper III. Top panel shows the association between the abundance of the dominant phyla and time since the most recent macrolide course (locally averaged smooth fit with standard errors). Bottom panel shows the average relative microbiota composition in children with a macrolide course (M) or penicillin course (P) <6 months ago, and children with no antibiotics for >2 years (CTRL).

Macrolides inhibit bacterial protein synthesis in bacterial cells by binding to the 50S-ribosome subunit. To be effective, they need to permeate the cell wall, which renders many Gram-negative bacteria resistant due to their cell wall structure. Macrolides concentrate in macrophages, and in many tissues and excretions, such as saliva and stomach (Scholar & Pratt 2000). Furthermore, macrolides are metabolised in the liver and excreted in bile, often undergoing enterohepatic cycling. These characteristics mean that that macrolides may reach high concentrations in the intestine (Scholar & Pratt 2000). They have a long half-life, so their effect may last longer than that of penicillins. Macrolides are thus more likely to reach sufficiently high concentrations in the intestine for sufficint periods of time to have negative effects on the

microbiota. Macrolides also have a range of effects on host cells (Kanoh & Rubin 2010).

A full recovery of the microbiota from an antibiotic course appeared to take longer than the average interval between courses (the children received on average 1.8±1.5 courses/year). This suggests that many children may not fully recover from antibiotic disturbance, but are in a continuously disrupted state throughout their early life. Whether this continuously distorted microbiota composition in childhood has any permanent life-long effects on the microbiota composition or function is currently not known, but mouse studies indicate that even temporary disruption of the microbiota in early life has long-term metabolic consequences (Cox *et al.* 2014).

L. rhamnosus GG alleviates penicillin-associated changes in the microbiota (IV)

The results have shown that antibiotic use is a major determinant of microbiota development and composition in children. A potential solution to mitigate the effects of antibiotics on the microbiota is probiotic use. However, its efficacy in preventing antibiotic-associated microbiota disturbance in children has not been clearly demonstrated. Furthermore, the consequences of long-term probiotic use on the microbiota of children have not been investigated. We found that long-term daily probiotic consumption altered the microbiota composition of children, but did not appear to have any detrimental effects. The observed changes occurred largely among the bacteria residing in the small intestine: several species of lactic acid bacteria increased in abundance, while the relatives of *E. coli* decreased (Fig. 6). This may be a beneficial change, as many species related to *E. coli* are potentially pathogenic and produce the inflammatory lipopolysaccharide (LPS) (Raetz and Whitfield 2002). However, such changes have not been observed in previous studies with adults (Kim *et al.* 2013, Lahti *et al.* 2013a), indicating that the adult microbiota may be more resistant to modulation than the developing microbiota of preschool children, or that long-term supplementation is necessary for changes in the microbiota to occur. Importantly, the probiotic treatment prevented many of the penicillin-associated changes in the microbiota such as the increase in *E. coli* and *Haemophilus* (Fig. 6). However, the macrolide-associated reduction in bifidobacteria was not prevented by LGG consumption. Previously, *Lactobacillus acidophilus-Bifidobacterium spp.* supplementation in adults during *H. pylori* eradication therapy (amoxicillin-clarithromycin-lansopratzole) and during amoxicillin treatment has been shown to prevent some of the antibiotic-associated changes in the microbiota (Plummer *et al.* 2005). Our results show that one lactobacillus strain alone is not sufficient to prevent the microbiota changes associated with all antibiotic types; different species may be efficacious in association with different types of antibiotics.

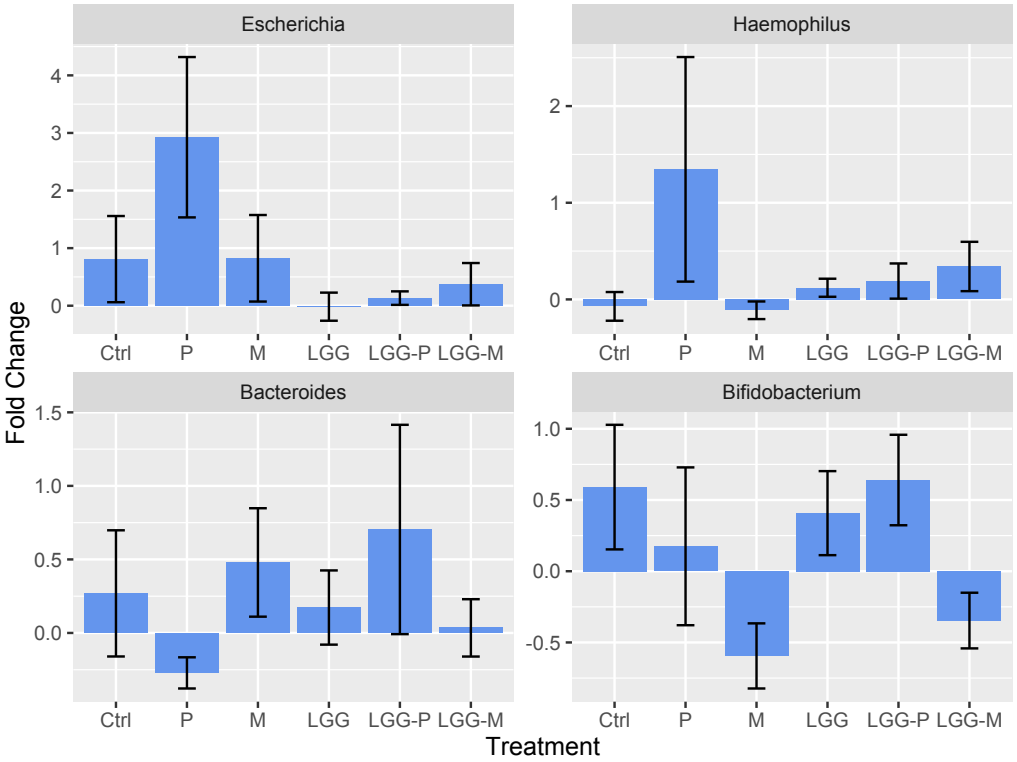

Figure 6. Change in relative abundance of selected bacterial taxa from baseline to the end of the intervention in children treated with different combinations of antibiotics (P = penicillin, M = macrolide) and probiotic (LGG = *Lactobacillus rhamnosus* GG) during the intervention (paper IV). The bars show the group means and the whiskers show the standard errors.

Breastfeeding and probiotic use may prevent infections (II, IV)

Long duration of breastfeeding was associated with reduced rate of infections (mainly respiratory infections) throughout the preschool age, as indicated by reduced antibiotic use (II). However, if the child received antibiotics during breastfeeding or shortly after weaning, the protective effect of breastfeeding was weaker. These results suggest that part of the long-term protection against infections conveyed by long breastfeeding is attributable to the beneficial effects of breast milk on the microbiota. In support of these results, we discovered that daily consumption of LGG significantly reduced the infection rate of preschool children for up to three years after the cessation of the probiotic trial (IV).

The observed protective effects of breastfeeding and LGG supplementation are in concordance with current knowledge on the immunomodulatory capacity of lactic acid bacteria. Several strains of lactobacilli and bifidobacteria have been shown to regulate systemic

immunity in animals and humans (reviewed in Cross 2002, Vouloumanou et al. 2009). The host's immune cells sample the microbiota and respond to microbial signals. Bacterial metabolites and components, such as the cell-wall peptidoglycan, can elicit immune cell activation, immunoglobulin production, and cytokine production. Administration of probiotic strains has been shown to strengthen immune responses in the gut against specific enteric pathogens, but also in extra-intestinal mucosal sites non-specifically against viral and bacterial pathogens (reviewed in Cross 2002, Vouloumanou et al. 2009). In a previous study on daycare-attending children, analogous to our LGG-supplementation study, LGG supplementation was shown to reduce the frequency of respiratory infections during the intervention (Hatakka et al. 2007).

Together these results suggest that certain strains of bacteria, such as LGG taken as a supplement and those promoted naturally by breastfeeding, may regulate the development of the immune system and hence contribute to the life-long immunological programming occurring in early life (Martin *et al.* 2010). This suggests that LGG supplementation may help particularly those children who did not receive sufficient breast milk for optimal immune development, or who were given antibiotics during the breastfeeding period.

Microbiota and metabolic programming (II, III, V)

Long duration of breastfeeding appeared to protect against high BMI in later childhood, but only among the children who did not receive antibiotics before weaning or immediately after (II). This suggests that the protection against overweight endowed by breastfeeding, which has been shown in several cohorts (Metzger and McDade 2010), is microbiota-dependent, and that antibiotic use may disrupt the beneficial metabolic effects of breastfeeding. This also offers an explanation to the controversy regarding the metabolic benefits of breastfeeding (Owen *et al.* 2005); antibiotic use has not been analysed in previous studies. Furthermore, lifetime antibiotic use was associated with increased BMI only among the children who received macrolide courses during their early life (III), indicating that early-life microbiota composition affects the child's later susceptibility to antibiotic-associated weight gain. Macrolide use itself was associated with changes in the microbiome (III) that have been linked with increased BMI, adiposity, obesity or metabolic diseases in children or adults: low richness (Le Chatelier *et al.* 2013), reduction of bacterial bile-salt metabolism (Joyce *et al.* 2014), and reduction in bifidobacteria (Kalliomäki *et al.* 2008, Bergström *et al.* 2014, Dogra *et al.* 2015a), increase in *Bacteroides* (Scheepers *et al.* 2015, Haro *et al.* 2016), increase in *Erysipelotrichaceae* (Zhang *et al.* 2009), and decrease in *Christensenellaceae* (Goodrich *et al.* 2014). Furthermore, mice treated with macrolides, but not those treated with amoxicillin (a penicillin-type antibiotic), have an increased susceptibility in later life to excess fat

accumulation (Nobel *et al.* 2015). The microbiota changes associated with macrolide treatment may explain the obesogenic effect of early-life broad-spectrum antibiotic courses (Bailey *et al.* 2014). These results, supported by earlier experiments with mice (Cho *et al.* 2012, Cox *et al.* 2014, Nobel *et al.* 2015), suggest that the early-life microbiota composition is integrally involved in the long-term metabolic programming of infants.

We therefore investigated the association between early-life microbiota composition and BMI in later childhood. The associations between the intestinal microbiota composition at the age of 3 months and BMI outcome at the age of 5-6 years were investigated in two cohorts (V), and were found to differ between the groups with low and high lifetime antibiotic use (Fig 7). This suggests that weight development may be affected by different factors in children with low and high lifetime antibiotic use. Several genus-level groups in the phylum Bacteroidetes (*Bacteroides, Prevotella, Alistipes, Parabacteroides*) were positively associated with later BMI in the children with low lifetime antibiotic use, i.e. were presumably predictive of susceptibility to diet-induced overweight. Strikingly, the same phylum was increased in a mouse model of early-life antibiotic exposure, causing increased sensitivity to diet-induced adiposity (Cox *et al.* 2014). In previous human studies, *Bacteroides* species, and particularly *B. fragilis* have been associated with increased BMI outcomes in later childhood, especially in children with low fibre intake (Vael *et al.* 2011, Scheepers *et al.* 2015). This indicates that a high abundance of *Bacteroides* in early life, caused by antibiotic use or other factors, may predispose to later diet-induced weight gain.

Among the children with frequent lifetime antibiotic use, i.e. whose BMI both diet and the antibiotics likely affected, a low Actinobacteria-to-Firmicute ratio at the age of 3 months predicted increased BMI at 6 years. The early microbiota composition thus appears predictive of later antibiotic-associated weight gain. This supports the finding that macrolide use in early life, which we showed to practically eliminate Actinobacteria in children, is required for the later antibiotic-associated increase in BMI (III). Together these results and the finding that long duration of breastfeeding was associated with reduced BMI and increased Actinobacteria-to-Firmicute ratio at preschool age (II), indicate that early maturation of the microbiota, possibly due to early weaning or antibiotic use, may affect the child's metabolic development.

We cannot exclude the possibility that the associations between the early microbiota composition and later BMI development were caused by maternal effects, such as maternal diet influencing both the early microbiota and the BMI development. However, several lines of evidence support the idea that microbiota regulate metabolism and the development of the metabolic phenotype. In mice, antibiotic-induced weight gain, mediated by the microbiome, involves similar hormonal changes as diet-induced weight gain: increased levels of leptin and decreased levels of ghrelin and peptide YY (Cox

et al. 2014, Nobel *et al.* 2015). The common factor between an obesogenic diet and antibiotic use is that they both strongly alter the intestinal microbiota, which have the capacity to regulate the host's energy homeostasis via e.g., FXR- and TGR5-signalling by bile acids (Watanabe *et al.* 2006, Joyce *et al.* 2014), TLR4-signalling by lipopolysaccharide (LPS) (Song *et al.* 2006, Cani *et al.* 2007), Angptl4/Fiaf-signalling (Bäckhed *et al.* 2004, Camp *et al.* 2012), and gut hormone and adipose tissue regulation by SCFA production (Ichimura *et al.* 2009, Lin *et al.* 2012). There is thus ample evidence from animal studies supporting the notion that associations between breastfeeding, antibiotic use, early-life microbiota composition, and later BMI outcomes may be causal.

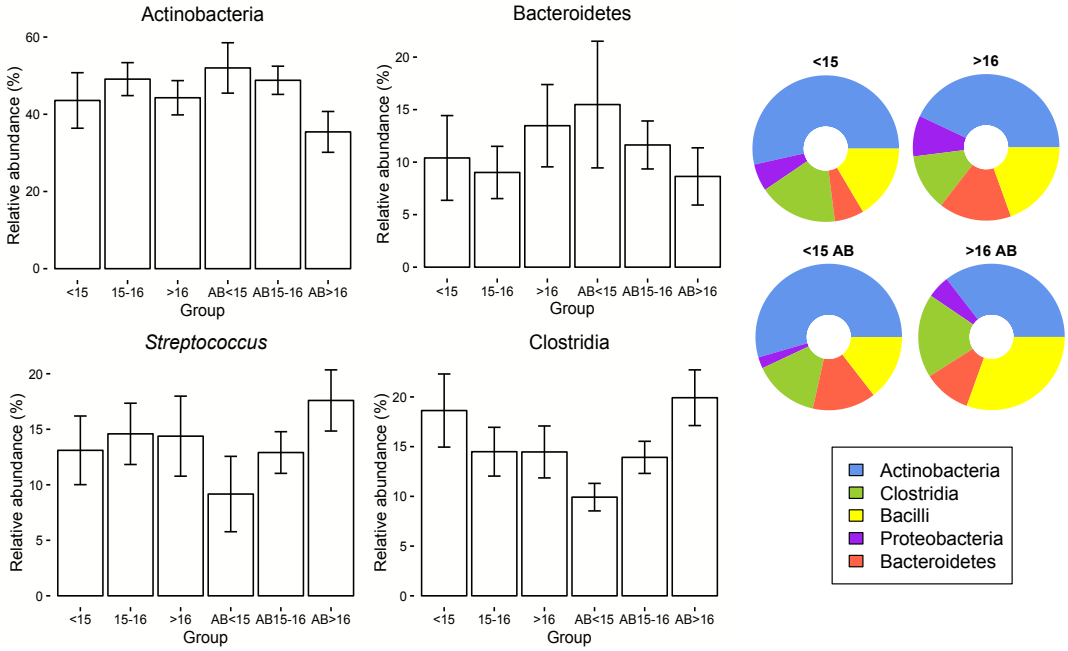

Figure 7. Relative abundance of selected bacterial groups at the age of 3 months in children with low BMI (<15), intermediate BMI (15-16), and high BMI (>16) at the age of 5-6 years, with low or high (indicated by AB) lifetime antibiotic use (V). The bars show the group means and the whiskers show the standard errors. The average relative microbiota composition in the different groups is shown on the right.

Increasing evidence is implying that bifidobacteria may have a key role in the metabolic programming of infants. Two Finnish studies based on one cohort have noted a negative, albeit not highly significant, association between abundance of *Bifidobacterium* spp. in infancy and later BMI development (Kalliomäki *et al.* 2008, Luoto *et al.* 2011). Furthermore, high abundance of Firmicutes and low abundance of bifidobacteria, suggestive of early microbiota maturation, has been associated with rapid growth and increased adiposity in infancy (Bergström *et al.* 2014, Dogra *et al.* 2015a). We found the

same associations among the children with frequent antibiotic courses. In mice, excessive abundance of clostridia in early life and rapid maturation of the microbiota has been achieved by antibiotic administration (Cho *et al.* 2012, Cox *et al.* 2014). Human infants born via C-section and those weaned early have a more rapid maturation of the microbiota than vaginally born infants and those with long duration of breastfeeding (Bäckhed *et al.* 2015), which could contribute to the adipose phenotype associated with early weaning and C-section birth. Rapid development of the microbiome toward an adult-like composition in early infancy may enable fast growth in infants, which is known to predispose to overweight in later childhood (Stettler *et al.* 2002, Baird *et al.* 2005). In addition, maternal overweight and stress during pregnancy, Caesarean delivery, antibiotic treatment during infancy and early weaning are all associated with increased risk of childhood overweight (Agras *et al.* 2004, Sloan *et al.* 2008, Li *et al.* 2010, Huh *et al.* 2012), and reduced abundance of bifidobacteria in the infant's intestine (Penders *et al.* 2006, Collado *et al.* 2008, Collado *et al.* 2010, Santacruz *et al.* 2010, Bergström *et al.* 2014). Our results support the protective role of bifidobacteria against childhood overweight, especially against antibiotic-associated weight gain.

The Gram-negative organisms, which we found increased in prenatally stressed infants (I), in children treated with antibiotics before weaning (II), in antibiotic-treated children (III, IV), in infants who later had high BMI outcomes (V), and in IBD-patients (VI), produce lipopolysaccharide (LPS), an inflammatory endotoxin. Circulating LPS induces inflammation, which affects glucose homeostasis and adipocyte metabolism (Geurts *et al.* 2014). Bacterially induced inflammation is considered an important component in the development of obesity and the related conditions, although the exact mechanisms are still unclear (Geurts *et al.* 2014). High-fat diets increase gut permeability and increase the amount of LPS leaking into circulation, thereby inducing low-grade inflammation (Geurts *et al.* 2014). Bifidobacteria reduce the leakage of LPS from the gut presumably by up-regulating tight-junction proteins and can thereby improve the metabolic health of the host and counter-act diet-induced weight gain (Cani *et al.* 2007, An *et al.* 2011, Neyrinck *et al.* 2012). In adult humans, obesity and related metabolic markers have been shown to correlate negatively with the abundance of bifidobacteria (Santacruz *et al.* 2010, Karlsson *et al.* 2012, Million *et al.* 2012). It is thus possible that bifidobacteria have a role in the early development of the intestinal barrier in infants and protect against LPS-induced systemic inflammation during a time when Gram-negative bacteria represent a considerably component of the microbiota. Frequent antibiotic use, increasing the abundance of Gram-negative bacteria and reducing the abundance of gut-protective bacteria, may involve recurrent LPS surges, similar to effects of a high-fat diet (Pendyala *et al.* 2012), which could contribute to the association between antibiotic use and weight gain.

Overall, the results indicate that the healthy infant microbiome undergoes succession in an orderly manner. Both delayed and too rapid

succession may be detrimental to the infant's metabolic and immunological development. Bifidobacteria may thus be seen as an indicator group, a biomarker of current and future health in infants. The results suggest that the early-life microbiota are involved in long-lasting metabolic programming, and can be used a biomarker of later obesity risk. The identification of infants with an obesogenic microbiota and tailored bacteriotherapy may thus represent a novel avenue in the battle against childhood overweight.

From an evolutionary perspective, the effect of early microbiota on the metabolic phenotype may be caused by adaptive phenotypic plasticity (Bateson *et al.* 2004). The microbiota may act as a signal to the infant of prevailing food availability and therefore adaptively guide the development of the metabolic phenotype. A diet low in breast milk in infancy would indicate prevailing food scarcity, as poor maternal nutritional condition limits her ability to produce breast milk (Rasmussen 1992). This would be reflected in the microbiota as a low abundance of HMO-utilising bacteria and a high abundance of Firmicutes. The adaptive response would be the development of an energy-conserving metabolic phenotype. However, in the modern world, breastfeeding and prevailing food availability are not coupled, potentially creating a mismatch between infant phenotype and the environment. Antibiotic use also disrupts the link between early-life nutrition and microbiota composition, by reducing the abundance of bifidobacteria even if the infant is breastfed (Tanaka *et al.* 2009). If the infant is metabolically prepared for scarcity but receives ample nutrition, the result is likely to be fast growth in early life and increased susceptibility to metabolic diseases.

Responses to Anti-TNF-α therapy in IBD depend on the microbiota (VI)

In paediatric IBD patients, we found that the microbiota varied along a gradient of intestinal inflammation. Higher levels of inflammation, measured by calprotectin, were associated with reduced microbial richness, reduced relative abundance of butyrate producers, and increased relative abundance of Gram-negative bacteria. Some IBD patients had a microbiota similar to healthy controls, while others had a dramatically increased abundance of Gram-negative organisms, either enterobacteria or *Bacteroides* spp, demonstrating that the microbiota of IBD patients can take different forms. The response to anti-TNF-α treatment was associated with the microbiota composition at baseline; high microbiota similarity to healthy controls predicted positive response to anti-TNF-α treatment (Fig 8). Among the most predictive organisms were *Bifidobacterium* and relatives of the butyrate-producing species *Eubacterium rectale*, whose high abundance predicted positive treatment outcome. The treatment response was also correlated with the microbiota response: an increase in diversity and similarity to healthy controls was accompanied by a decrease in inflammation. These results suggest that patients with a normal-like microbiota are likely to benefit from

the blockage of TNF-α. It is possible that in this subgroup of patients, IBD is primarily a result of an excessive immune reaction towards the normal microbiota, and dampening the immune reaction is sufficient to achieve a positive treatment outcome. Patients with a severely distorted microbiota may require microbiota modulation in addition to immune modulation to achieve positive treatment outcomes. An interesting option is faecal transplantation, which can be used to reset the microbiota to a new ecological state (Smits *et al.* 2013). However, the donor should be selected carefully to ensure that the source microbiota contain a large amount of taxa that are depleted in the patient and a minimal amount of IBD-associated taxa.

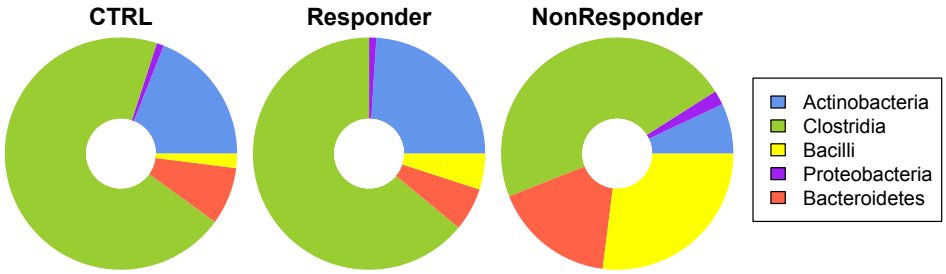

Figure 8. Average relative microbiota composition in healthy controls (CTRL) and baseline composition in IBD-patients who responded to anti-TNF-α therapy and IBD-patients who did not respond.

While the aetiology of IBD is unknown, antibiotic use during childhood is an identified risk factor (Virta *et al.* 2012). Two healthy controls in study VI, who had used antibiotics prior to sample donation, had microbiota compositions similar to the IBD cases. Correspondingly, one of the subjects in studies III and IV, with a history of repeated macrolide courses, was diagnosed with UC during the follow-up. In studies III and IV, we observed that antibiotics, and macrolides particularly, drive the microbiota towards an IBD-like composition characterized by low diversity and a high abundance of Gram-negative bacteria. It is an intriguing hypothesis that repeated antibiotic disturbances causing alterations in the microbiota might cause the immune system in genetically susceptible individuals to become increasingly hostile towards the altered microbiota, igniting an inflammatory response, which eventually prevents recovery and exacerbates the dysbiosis. Species related to *B. fragilis* and *B. ovatus*, the two groups that were strongly increased in the macrolide-users (III) and patients with Crohn's disease (VI), have been associated with aberrant immune function, including increased IgE levels and autoimmunity (Saitoh *et al.* 2002, Sepp *et al.* 2005, de Goffau *et al.* 2013, Murri *et al.* 2013, de Goffau *et al.* 2014), and with intestinal symptoms (San Joaquin *et al.* 1995, Manichanh *et al.* 2014). These results link the observed associations between antibiotic use, microbiota composition, and IBD.

How to encourage the natural microbiota development

The results indicate that to encourage a healthy infant microbiota development, maternal wellbeing during pregnancy is a key factor. Maternal stress, either directly or due to factors such as diet, metabolic health, or inflammation, may affect her microbiota composition and the transmission of bacteria to the infant. Early-life exposures, perhaps most prominently the duration of breastfeeding and antibiotic use, appear to have long-term consequences on the development of the child and the microbiota, confirming that breastfeeding for at least 12 months should be encouraged, and antibiotic use during infancy restricted to necessary cases. In general, antibiotic treatment of infants and children should be conducted with caution and the choice of antibiotic considered carefully. Penicillin should be the first choice whenever possible. The pharmacokinetics of the antibiotic may be a useful indicator of the potential collateral damage caused by the drug in the intestine. Specific probiotics may be helpful in reducing the antibiotic-induced damage in the intestinal microbiota of children, but attention should be paid to match the antibiotic with the right probiotic. LGG may help prevent or reduce the effects of penicillin-type antibiotics, but not those of macrolides. Hypothetically, *Bifidobacterium* supplementation may be helpful in ameliorating the effects of macrolides, although this should be investigated.

Limitations of the studies

Microbiota was analysed by DNA-based methods from faecal samples, which is currently the standard way to assess the intestinal microbiota. Many factors are known to potentially influence the reliability of the observed microbial composition (Thomas *et al.* 2015). The faecal microbiota is known to differ from the colonic luminal microbiota and even more from the colonic mucosal microbiota (Zoetendal *et al.* 2002, Eckburg *et al.* 2005). The mucosal microbiota are in close interaction with the host and may therefore be more important for the health of the host. However, the collection of mucosal biopsies is invasive and not possible in most cases. The use of faecal samples should not seriously confound the results, as the microbiota was consistently analysed from faecal samples in all cases; inter-individual comparisons are likely to be reliable although the absolute quantities of bacterial taxa in the intestine, and particularly in the mucosa, cannot be ascertained. Indeed, the results are based on relative abundances of bacterial taxa; measuring absolute abundances from faecal samples is complicated but could provide additional insights.

Timing of the faecal sample collection (Thaiss *et al.* 2014) and stool consistency (Vandeputte *et al.* 2016) are known to affect the microbiota composition. Neither was controlled in these studies. These are not likely to consistently vary between the different groups and therefore should not

seriously confound the results. Sample storage, DNA extraction method, and analysis platform including the primers used are also known to influence the observed composition (Thomas *et al.* 2015). In all studies the samples were collected at home and taken immediately to the study centre for storage in -70°C, or stored temporarily in the home freezer for a few hours. This protocol should cause minimal taxonomic bias, and ensure comparability between samples.

Different DNA extraction methods are known to be biased in their ability to retrieve DNA from different types of bacteria (Salonen *et al.* 2010, Thomas *et al.* 2015). Mechanical lysis using repeated bead beating is currently considered the best method to extracting DNA (Salonen *et al.* 2010, Santiago *et al.* 2014). This method was used for the infant samples. The samples from the 2-7 year old children were subjected to a modified protocol using the Promega Wizard kit, which has been shown to produce very similar results to the RBB method (Salonen *et al.* 2010). The platform used for quantifying the different taxonomic groups based on the DNA may exert additional biases. Two platforms targeting 16S rRNA amplicons, a microarray and pyrosequencing, were used, and their results compared. Both platforms provided corresponding results (III, IV) indicating that the results are not platform-specific: for example, the relative abundance of *Bifidobacterium* measured using the two platforms showed a between-platform correlation of 0.88.

The predictor variables, such as prenatal stress (I), duration of breastfeeding (II), antibiotic use (III), probiotic use (IV), and BMI (II, III, V) are likely to contain some amount of error. Prenatal stress was measured by validated questionnaires and measurements of cortisol, so the accuracy is likely to be fairly high: mothers with high scores on both experienced stress and measured cortisol most likely did experience more stress than those with low scores on both variables. Information on the duration of breastfeeding was obtained from the mothers up to 6 years after the child was born. It is possible that some mothers remembered the exact duration better than others, and recall bias cannot be ruled out as an alternative explanation to some of the results. If the mothers of antibiotic-using infants consistently recalled the duration of breastfeeding less accurately than the mothers of healthy infants, the apparent lack of association between breastfeeding duration and BMI could be explained by recall bias.

The drug purchase records are highly accurate and known to contain >95% of all drug purchases. However, a few antibiotic purchases were inevitably missed: drugs given during hospital stays would not be recorded nor would drugs purchased without showing the social insurance card. We compared the parent-reported number of antibiotic courses to the purchase records and found considerable differences. Even the antibiotic prescription data from the study physicians, which the families were advised to visit, did not fully match the purchase data, indicating that not all prescriptions were filled and that additional prescriptions were obtained from other physicians.

The antibiotic purchase data can be considered the most reliable and objective indicator of antibiotic use.

The probiotic intervention was well controlled. Most of the study milk was given to the children at the day care facility by the staff, which also monitored milk consumption. Compliance was further confirmed by analysing the amount of the probiotic strain in the faecal samples. Discordant samples were excluded. The BMI data for papers II, III and V were based on weight and height data measured by researchers or physicians and are therefore consistently measured and reliable.

Generality of the results

The results are based on four cohorts of children from two different countries, and they can be expected to generalize well to other, similar child cohorts. Similar or supportive findings have been discovered in animal studies, and in different cohorts of children and adults. However, all studies included in this work were restricted to healthy infants and children (with the exception of the IBD cases) in two European countries, and extrapolation to dissimilar cohorts may not be possible.

The prenatal stress study (I) excluded families with pregnancy complications, C-section birth, and serious infant health problems. Therefore, the results may only apply to vaginally born healthy infants. Antibiotic use was extremely rare during the study, and the results may not apply to infants taking antibiotics. The inter-individual microbiota variation among the Dutch infants was smaller than among the Finnish infants (V), suggesting that the results based only on the Dutch cohort may generalise only to a subset of infants. However, animal studies have shown very similar results (Bailey *et al.* 2004), indicating that the results on the effect of maternal stress on the gut microbiota generalise even across species borders.

The effect of breastfeeding duration on BMI, lifetime antibiotic use, and the later-life microbiota composition (II), as well as the associations between antibiotic and probiotic use and the microbiota composition (III, IV) were based on a fairly homogenous and healthy Finnish cohort, all attending day care. Children of other ethnicities or living in different environments may not show the same results. However, the association between early-life antibiotic use and later-life BMI has been found in a number of studies in different countries (Ajslev *et al.* 2011, Trasande *et al.* 2013, Azad *et al.* 2014, Bailey *et al.* 2014, Saari *et al.* 2015, Gerber *et al.* 2016), suggesting good generality. The association between duration of breastfeeding and BMI has also been observed in different cohorts (Arenz *et al.* 2004, Harder *et al.* 2005), although results to the contrary exist as well (Owen *et al.* 2005). To what extent the controversy is explained by antibiotic use, should be investigated in larger cohorts. The association between breastfeeding duration and microbiota composition (II) was based on a subset of only 42

non-overweight, non-asthmatic children with modest lifetime antibiotic use. In the total cohort the same patterns emerged, but were dampened by variations in antibiotic use, BMI, and health. The results thus may not fully apply to overweight or asthmatic children, or children with heavy antibiotic use history.

The associations between early-life microbiota composition and later-life BMI were based on children from two different countries with different childcare practices. The results are furthermore supported by previous studies on Finnish (Kalliomäki *et al.* 2008, Luoto *et al.* 2011) and Dutch children (Vael *et al.* 2011, Scheepers *et al.* 2015), suggesting that the results should generalise well at least among European children.

Causality not concluded

Data for papers I and V were collected as part of an observational study on child development and data for papers II-V were collected during probiotic trials. Apart from paper IV, in which the outcome of the probiotic trial was analysed, all papers present observational data. The observed correlations should not be taken as evidence of causality, nor can they reveal the causal mechanisms underlying the relationships. However, the results are supported by previous animal experiments and epidemiological studies, which provide ample support to the results presented here and evidence of causality. Furthermore, analysing bacterial bile-salt hydrolase and antibiotic resistance genes in paper III identified links to potential mechanisms. Antibiotic resistance showed a clear causal signal in the antibiotic treated children, which supports causality of the overall antibiotic-associated microbiota changes.

Future considerations

Further research in tightly controlled cohorts with detailed metadata and host parameters, including markers of immunological function, inflammation, and metabolism will be helpful to establish causality and to delineate the mechanisms underlying the observed associations. Humans and their microbiomes are known to exhibit a considerable amount of individuality due to differences in environment, genetics, and experiences. Therefore, assuming uniform responses in microbiome studies is inevitably too simplistic and likely to result in the missing of important signals in the data. Large cohorts with detailed metadata enable the accommodation of individuality in the microbiota and host responses by meaningful stratification of individuals into biologically comparable groups.

As bacterial bile acid metabolism and LPS-induced inflammation are implicated in the metabolic regulation of the host, measurements of bile acids in faeces and in blood, and markers of immune activation and inflammation

in blood should be undertaken in future studies, in conjuction with compositional and functional analyses of the microbiome. Whole-genome metagenomics and metaproteomics can reveal functions of the bacteria, but are still too expensive for most large-scale studies. The work presented here mainly consisted of compositional analyses, but some functional aspects were included, as well. The results show that fairly simple culture-based and PCR-based analyses can provide important complementary information to the high-throughput microarray or sequencing analyses. Whole-genome metagenomics was successfully used as a discovery tool to guide further analyses.

Although it is becoming evident that the early-life microbiome exerts long-term impacts on host health, it is still not clear which age is the most decisive for host development. A recent study found that antibiotic use during the first 6 months of life was less predictive of later weight gain than antibiotic use during the first 2 years of life (Gerber *et al.* 2016). We found that macrolide use during the first 2 years was most predictive of later antibiotic-associated weight gain and of asthma incidence. These results suggest that the sensitive time window is at least 2 years, if not longer, and that it may be necessary to look beyond the first few months.

Most importantly, in order to progress from pattern description to prediction-level understading of the dynamics and functions of the microbiome, long time series are essential. In macro-ecology, population monitoring through time series is considered the cornerstone of understanding ecosystem dynamics and functioning. Long time series collected at short intervals from well characterised individuals with simultaneous recording of host exposures, such as diet, living environment, medical treatments, and host health parameters will be a step towards estabilishing causality between host exposures and microbiota changes, and between microbiota changes and host parameters. Rather than analysing the relative abundance of bacterial taxa at given time points, the time series approach will enable the analysis of change in relative abundance, and consequently the predition of change, within the context of the individual composition.

Summary and Conclusions

The results show that the intestinal microbiota composition in early life predicts weight development in later childhood, and suggest that disruption of the natural microbiota development may have long-term health consequences. The microbiota in childhood appear sensitive to various modulating factors. Environmental factors begin to influence the microbiota even before birth, and therefore promoting maternal heath and wellbeing is the first step towards healthy microbiota in the child. Long duration of breastfeeding was associated with the microbiota development, as well as

reduced antibiotic use and BMI in preschool age, but antibiotic use before weaning weakened these associations. The results support the health-promoting effects of breastfeeding and indicate that some of the benefits of breastfeeding are microbiota-dependent. Antibiotic use during childhood emerged as a central regulator of the microbiome and its long-term development, with potential effects on the metabolic development of the child. Particularly macrolide use was associated with potentially obesogenic changes in the microbiome. Antibiotic use in early life, and particularly the use of macrolides, should be avoided if possible. Probiotic use during and after antibiotic courses may offer a way to mitigate some of the antibiotic-associated changes, but the probiotic should be matched with the antibiotic. LGG supplementation prevented some of the penicillin-associated changes, but failed to prevent the macrolide-associated loss of bifidobacteria. Supplementation with bifidobacteria may be beneficial in association with macrolide courses.

Based on the results, the following model emerges (Fig. 9). Long breastfeeding is associated with reduced risk of overweight, but the benefit is eliminated by antibiotic use in infancy. Long duration of breastfeeding, and LGG supplementation, protect against infections and thus prevent antibiotic use. Prenatal stress and antibiotic use in childhood alter the microbiota development, and the altered microbiota composition in infancy is associated with later weight development. Generally, this work suggests that promoting a natural microbiota development in childhood by breastfeeding, avoiding unnecessary antibiotics, and possibly probiotic use, may have long-term health benefits, particularly in terms of weight development.

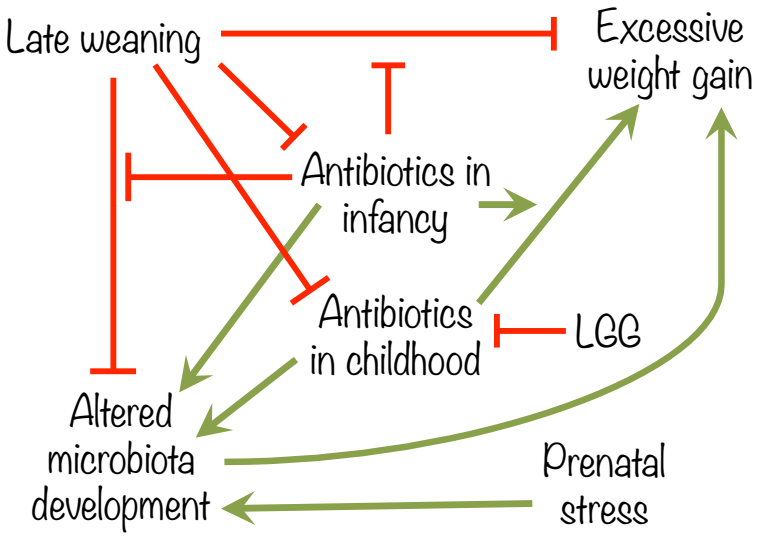

Figure 9. A tentative model based on the associations found in this thesis project. LGG = *Lactobacillus rhamnosus* GG supplementation. Red = preventing association, green = promoting association.

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