DOCTORAL THESES IN FOOD SCIENCES AT THE UNIVERSITY OF TURKU

Early Life Intestinal Microbiota in Health and in Atopic Eczema

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ABSTRACT

Decrease in microbial contacts in affluent societies is considered to lie behind the rise in allergic and other chronic inflammatory diseases during the last decades. Indeed, deviations in the intestinal microbiota composition and diversity have been associated with several diseases, such as atopic eczema. However, there is no consensus yet on what would constitute a beneficial or harmful microbiota. The aim of this thesis was to study the microbiota development in healthy infants and to characterize intestinal microbiota signatures associated with disease status and severity in infants with atopic eczema. The methodological aim was to compare and optimize methods for DNA extraction from fecal samples to be used in high-throughput microbiota analyses.

It was confirmed that the most critical step in successful microbial DNA extraction from fecal samples is the mechanical cell lysis procedure. Based on this finding, an efficient semi-automated extraction process was developed that can be scaled for use in high-throughput platforms such as phylogenetic microarray used in this series of studies. By analyzing a longitudinal mother-child cohort for 3 years it was observed that the microbiota development is a gradual process, where some bacterial groups reach the degree of adult-type pattern earlier than others. During the breast-feeding period, the microbiota appeared to be relatively simple, while major diversification was found to start during the weaning process. By the age of 3 years, the child's microbiota composition started to resemble that of an adult, but the bacterial diversity has still not reached the full diversity, indicating that the microbiota maturation extends beyond this age. In addition, at three years of age, the child's microbiota was more similar to mother's microbiota than to microbiota of non-related women.In infants with atopic eczema, a high total microbiota diversity and abundance of butyrate-producing bacteria was found to correlate with mild symptoms at 6 months. At 18 months, infants with mild eczema had significantly higher microbiota diversity and aberrant microbiota composition when compared to healthy controls at the same age.

In conclusion, the comprehensive phylogenetic microarray analysis of early life microbiota shows the synergetic effect of vertical transmission and shared environment on the intestinal microbiota development. By the age of three years, the compositional development of intestinal microbiota is close to adult level, but the microbiota diversification continues beyond this age. In addition, specific microbiota signatures are associated with the existence and severity of atopic eczema and intestinal microbiota seems to have a role in alleviating the symptoms of this disease.

TIIVISTELMÄ

Allergiset sairaudet ovat yleistyneet nopeasti viime vuosikymmeninä etenkin länsimaisissa hyvinvointivaltioissa. Vähentyneen mikrobialtistuksen varhaislapsuudessa uskotaan olevan yksi näille sairauksille altistava riskitekijä. Poikkeava suolistomikrobisto onkin yhdistetty kohonneeseen riskiin sairastua esimerkiksi atooppiseen ekseemaan. Toistaiseksi spesifisiä mikrobiston komponentteja, jotka toimisivat joko atopialta suojaavina tai sille altistavina tekijöinä, ei kuitenkaan ole pystytty tunnistamaan. Tämän väitöskirjatyön tavoitteena oli tutkia suolistomikrobiston kehittymistä terveillä lapsilla sekä määrittää atooppiseen ekseemaan ja sen vakavuuteen yhteydessä olevat suolistomikrobiston tunnusmerkit. Tavoitteena oli myös vertailla ja optimoida DNA-eristysmenetelmiä, jotka soveltuisivat laajojen näytemäärien käsittelyyn ja niiden analysointiin uusimmilla molekulaarisilla menetelmillä.

Osoitimme, että mikrobisolujen mekaaninen hajoittaminen on kriittisin vaihe onnistuneessa DNA -eristyksessä ulostenäytteistä. Tähän havaintoon perustuen kehitettiin tehokas puoliautomatisoitu DNA- eristysprosessi, jota voidaan hyödyntää korkean suoritustehon menetelmissä, kuten esimerkiksi tässä tutkimussarjassa käytetyssä fylogeneettisessä mikrosiruanalyysissä. Tässä tutkimuksessa osoitettiin, että lasten suolistomikrobiston kehittyminen on vaiheittainen prosessi, jossa tietyt bakteeriryhmät saavuttavat aikuisenkaltaisen tason aiemmin kuin muut bakteeriryhmät. Imeväisiässä lapsen suolistomikrobisto on hyvin yksinkertainen, ja bakteerilajien monimuotoisuus kasvaa voimakkaasti kun vieroitus äidinmaidosta aloitetaan. Kolmeen ikävuoteen mennessä lapsen suolistomikrobiston lajikoostumus on melko ”aikuismainen” ja hyvin samankaltainen kuin äidillä. Mikrobiston kehittyminen kuitenkin jatkuu, sillä sen monimuotoisuus (diversiteetti) on tässä iässä vielä merkittävästi alhaisempi kuin aikuisilla. Atooppista ekseemaa sairastavilla, kuuden kuukauden ikäisillä lapsilla bakteeriston korkeampi diversiteetti ja korkeampi butyraattia tuottavien bakteerien määrä oli yhteydessä lievempiin oireisiin. Toisaalta 18 kuukauden iässä lievää atooppista ekseemaa sairastavilla lapsilla oli korkeampi suolistomikrobiston diversiteetti ja erilainen bakteerilajisto kuin terveillä lapsilla. Tässä väitöskirjatyössä osoitettiin fylogeneettisen mikrosiruanalyysin avulla äidin suolistomikrobiston ja yhteisen elinympäristön vaikutus lapsen suolistomikrobiston kehittymiseen varhaislapsuudessa. Lisäksi määritettiin atooppiseen ekseemaan ja sen vakavuuteen yhteydessä olevat suolistomikrobiston tunnusmerkit ja osoitettiin, että suolistomikrobisto saattaa olla osallisena atooppisen ihottuman oireiden lievittymisessä.

LIST OF ABBREVIATIONS

dsRNA	Double stranded RNA
DC	Dendritic cell
EPS	Exopolysaccharide
GOS	Galacto-oligosaccharide
GPCR	G-protein coupled receptor
HDAC	Histone deacetylase
HITChip	Human Intestinal Tract chip
HMO	Human Milk Oligosaccharides
IEC	Intestinal Epithelial Cell
Ig	Immunoglobulin
IL	Interleukin
KF	KingFisher
LPS	Lipopolysaccharide
MAMP	Microbe-associated molecular pattern
MCPP	Monte Carlo Permutation Procedure
NEC	Necrotising enterocolitis
NeM	NucliSENS easyMAG
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
OTU	Operational Taxonomic Unit
PCA	Principal Component analysis
PCR	Polymerase chain reaction
PUL	Polysaccharide utilization loci
RBB	Repeated Bead Beating
RDA	Redundancy analysis
SCFA	Short-Chain Fatty Acid
SCORAD	SCORing Atopic Dermatitis
spp.	Species
subsp.	Subspecies
Th	Helper T cell
Treg	Regulatory T cell
TLR	Toll-Like Receptor
16S rRNA	Bacterial ribosomal 16S RNA molecule

LIST OF ORIGINAL PUBLICATIONS

I. Nylund L., Heilig H., Salminen S., de Vos WM. and Satokari R. Semi-automated extraction of microbial DNA from feces for qPCR and phylogenetic microarray analysis. J Microbiol Methods 2010; 83:231-235.

II. Nylund L., Grönlund M-M., Isolauri E., Salminen S., de Vos WM and Satokari R. High similarity between microbiota of three-year-old children and their mothers testify for vertical transmission and the impact of shared environment. *Submitted*

III. Nylund L., Satokari R., Nikkilä J., Rajilić-Stojanović M, Kalliomäki M, Isolauri E, Salminen S, de Vos WM. Microarray analysis reveals marked intestinal microbiota aberrancy in infants having eczema compared to healthy children in at-risk for atopic disease. BMC Microbiol. 2013; 13:12.

IV. Nylund L., Nermes M., Isolauri E., Salminen S., de Vos WM., Satokari R. Severity of atopic disease correlates with intestinal microbiota diversity and butyrate-producing bacteria. Allergy 2015; 70:241-244

ADDITIONAL PUBLICATION RELATED TO THE THESIS

The review of literature is partially based on the review article:

Nylund L., Satokari R., Salminen S. and de Vos WM. Intestinal microbiota during early life -impact on health and disease. Proc Nutr Soc 2014;73(4): 457-69.

1 INTRODUCTION

Although fetus is exposed to microbes and microbial structures already *in utero*, the major microbial colonization of the gastrointestinal tract starts at birth when an infant comes into contact with the microbes from the extrauterine environment. This initial exposure coincides with the ingestion of breast milk and its constituents, including oligosaccharides, immunoglobins and antimicrobial proteins, which facilitate the selective expansion of mutualistic microbes. This and other host factors are assumed to lead to a gradual compositional development and finally, to the establishment of a stable, individual-specific microbiota. The microbiota development process is affected by several factors including genetic background, mode of delivery, dietary pattern and medical procedures. These early colonization events and the establishing intestinal microbiota shape the immune system and have an effect on the development of variety of diseases later in life.

Atopic diseases are chronic, relapsing disorders usually starting at early childhood. During the last few decades, the incidences of these diseases (atopic eczema, asthma, allergic rhinoconjunctivitis and food allergy) have increased in epidemic proportions. In early life, the most common form of atopic disease is eczema, its prevalence being approximately 15 - 30 %, depending on the country studied (Deckers et al. 2012). According to the SCORAD (SCORing Atopic Dermatitis) assessment used to evaluate the severity of disease, the majority of patients are classified as “mild” (SCORAD <15), whereas 10-20 % of patients are “severe” (SCORAD >40) and this percentage seems to be higher in adult population with eczema (Mao et al. 2014).

The onset and severity of atopic diseases impair greatly the well-being and quality of life of patients and their family members (Alanne et al. 2011; Ricci et al. 2007). The pathogenesis of these diseases is thought to be a multifactorial process involving complex individual-specific interactions between microbial dysbiosis, genetic predisposition, impaired immune system and distinct environmental factors (Holloway et al. 2010; Hörmannsperger et al. 2012) (Figure 1).

Decrease in microbial contacts in affluent societies during last decades is considered to lie behind the increased prevalence of allergic and other chronic inflammatory diseases. Indeed, associations between the composition and diversity of intestinal microbiota and different diseases have been studied extensively and decreased total microbiota diversity has been linked to several disorders (de Vos et al. 2012). The reports on atopic eczema and microbiota in early life are partly contradictory, both decreased and increased microbiota diversity have been associated with the atopic eczema (Abrahamsson et al. 2012; Bisgaard et al. 2011; Forno et al. 2008; Gore et al. 2008; Stsepetova et al.

2007; Wang et al. 2008). Moreover, there is little overlap in the microbes that have been identified to be associated with atopic eczema in the different studies. This may be due to various factors such as low numbers of subjects, a great variation of ages of the studied infants and technical bias as the tools to characterize the microbiota are still developing. Hence, no consensus exists on what would constitute an "atopy-promoting" or "atopy-preventing" microbiota.

Previous studies have mainly addressed the microbiota composition preceding the development of atopic disease, although the age at the onset of atopic symptoms has been insufficiently clarified and the severity of eczema has not been considered (Abrahamsson et al. 2012; Bisgaard et al. 2011; Forno et al. 2008; Ismail et al. 2012; Kalliomäki et al. 2001a; Wang et al. 2008). Notably, the majority of these studies have been conducted by using traditional cultivation-based techniques or molecular techniques that only target a sub-set of the intestinal microbiota, thus limiting the detection of true microbiota diversity. In this study, we aimed to characterize the temporal changes in the diversity and community structure of early childhood intestinal microbiota and to identify specific microbiota signatures associated with eczema and its severity by using high-throughput phylogenetic microarray analysis.

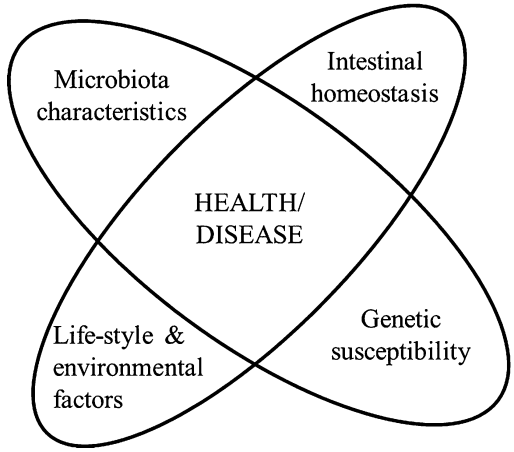

Figure 1. Factors affecting the microbiota development and subsequently the later life health.

2 REVIEW OF THE LITERATURE

2.1 General characteristics and functions of the intestinal microbiota

Microbial communities inhabit a variety of body surfaces of human host. The most diverse population resides in the human gastrointestinal tract, which is densely inhabited by up to 10^{14} microbial cells, which outnumbers human cells by 10:1 (Human Microbiome Project Consortium 2012; Qin et al. 2010). In addition, the total amount of microbial genes in the intestine exceeds the human genes approximately by a 150-fold (Human Microbiome Project Consortium 2012; Qin et al. 2010). The intestinal microbiota composition is characterized by a high inter-individual variation with 500-1000 distinct bacterial species constituting the gut microbiota of an individual (Jalanka-Tuovinen et al. 2011; Tap et al. 2009). Despite of the high individuality of human intestinal microbiota, conserved set of colonizers are shared among individuals (Jalanka-Tuovinen et al. 2011; Turnbaugh et al. 2009) and may be required for the proper functioning of the gastrointestinal tract. Indeed, intestinal microbiota is involved in several functions crucial for the human host such as education of the immune system and the metabolism of dietary carbohydrates indigestible by the human host. Moreover, the commensal microbial population produces essential bioactive metabolites such as vitamins, amino acids and short-chain fatty acids and provides colonization resistance against invading pathogens.

The vast majority of intestinal bacterial species colonizing the human intestine is strictly or facultatively anaerobic and has physiologically adjusted to the conditions residing in the gastrointestinal tract. The most dense microbial population reside in the large intestine with Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria being the most diverse and abundant phyla (Rajilić-Stojanović et al. 2014) (Figure 2). Actinobacteria, especially *Bifidobacterium* spp. are common inhabitants of the adult human intestine, comprising approximately 0.5-3 % of adult microbiota (Gura 2014; Jalanka-Tuovinen et al. 2011; Jost et al. 2014a). Since bifidobacteria are able to degrade complex oligosaccharides, these bacteria are notably abundant in breast-fed infants (up to 90 % of total microbiota) (Gura 2014; Yatsunenko et al. 2012).

In addition to bifidobacteria, also the growth of Bacteroidetes is supported by breast milk oligosaccharides (Marcobal et al. 2012) and these species are among the first colonizers of human infant intestine. In healthy adults, Bacteroidetes comprises 10-45 % of the total microbiota, shows high long-term stability (de Vos et al. 2012; Jalanka-Tuovinen et al. 2011; Rajilić-Stojanović et al. 2012) and

have been suggested to represent the phylogenetic core of the human intestinal tract (Tap et al. 2009).

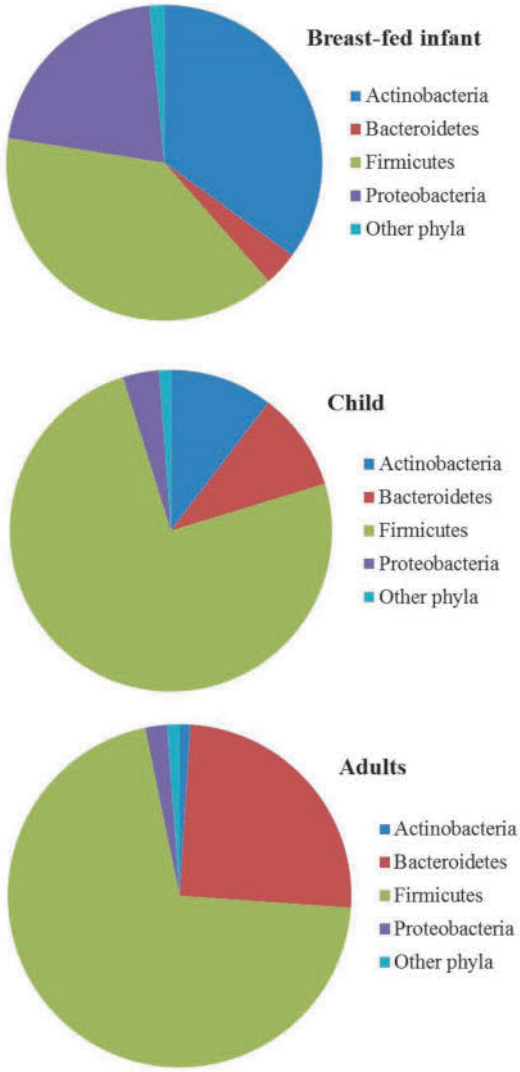

Figure 2. Typical phylum-level microbiota composition in breast-fed infant, child and adult (data derived from studies **II**, **III** and **IV**).

The most diverse bacterial phylum is Firmicutes which constitutes approximately 50-80 % of the microbiota in the intestine of healthy adults (Rajilić-Stojanović et al. 2014). The most abundant members of this phylum are bacteria belonging to the *Clostridium* clusters IV and XIVa (Rajilić-Stojanović et al. 2014). These clusters contain bacterial species such as *Faecalibacterium prausnitzii* and *Eubacterium rectale*, which are able to produce butyrate from

sugars and *Anaerostipes caccae* and *Eubacterium hallii*, which can also use lactate and acetate to produce this short chain fatty acid that is crucial for the maintenance of intestinal homeostasis and health (Smith et al. 2013). Proteobacteria are common although low abundant members of the healthy human intestine, comprising approximately 1-5 % of the total microbiota in adults (Tap et al. 2009). However, these bacteria are among the first colonizers of the newborn intestine, being the dominant group in early days of life (Wopereis et al. 2014) (Figure 2). *Akkermansia muciniphila* is the sole representative of the Verrucomicrobia, one of the less abundant phyla in the gastrointestinal tract. It is present at low level in early life, but grows out later to become an important resident of the intestinal mucosa, where it produces propionate, another important signaling molecule in the gut (Derrien et al. 2011; Everard et al. 2013; van Passel et al. 2011).

Although fetus is exposed to microbes and microbial structures already *in utero*, the major microbial colonization of the gut starts during and after birth, when the infant comes into a contact with mother's vaginal and intestinal microbiota and with the microbes from the surrounding environment. The early life microbiota development is a time-dependent process, where microbiota diversity and composition fluctuate in response to the nutritional behavior and major life events such as weaning, antibiotic treatments and starting at day-care (Wopereis et al. 2014).

Diversity is a measure of the variety of organisms present in a given community, e.g. in the human intestine. Microbiota diversity consists of two components: species richness (how many species present in a sample) and species evenness (the relative abundance of the species). Higher microbiota diversity is associated with more stable community structure that is resilient towards external disturbances. Indeed, once reached its full diversity and complexity, the intestinal microbiota of healthy individuals remains relatively stable for at least a decade (Rajilić-Stojanović et al. 2012). In elderly, the microbiota may destabilize again with the increasing age and is characterized by decreasing diversity and decline in the complexity of species community (Biagi et al. 2010; Claesson et al. 2011) (Figure 3).

Figure 3. Schematic presentation of the development of microbiota diversity from birth to old age. See text for an explanation.

2.2 Microbial interactions with the human host

A dynamic and complex relationship exists between the intestinal microbiota, the immune system and the intestinal epithelium. Intestinal epithelial cells (IECs) reside at the interface between the human host and commensal microbiota and are crucial for the maintenance of immune homeostasis (Artis 2008). These cells form a physical barrier, sense microbe-derived signals and regulate the microbiota by secreting cytokines and antimicrobial compounds. The expression of antimicrobial proteins and epithelial permeability and proliferation are regulated by immune cells via the secretion of cytokines (Artis 2008). In turn, the intestinal microbiota affects immune cell homeostasis and has a crucial role in maturation and education of both innate and adaptive immune system (Maynard et al. 2012). The maturation of gut-associated lymphoid tissues starts at birth and is strictly dependent on microbial signaling (Cerf-Bensussan et al. 2010). Microbial colonization is required also for the production of secretory IgA and differentiation of T helper 1 (Th1), Th2 and Th17 cells and the development of regulatory T cells in the gut (Kosiewicz et al. 2014)(Figure 4).

2.2.1 Recognition of microbes by the immune system

Intestinal epithelial cells have a central role in innate immune recognition of commensal bacteria via pattern-recognition receptors (PRR), including transmembrane Toll-like receptors (TLRs) and cytosolic nucleotide-binding oligomerization domain (NOD) –like receptors (NLRs).

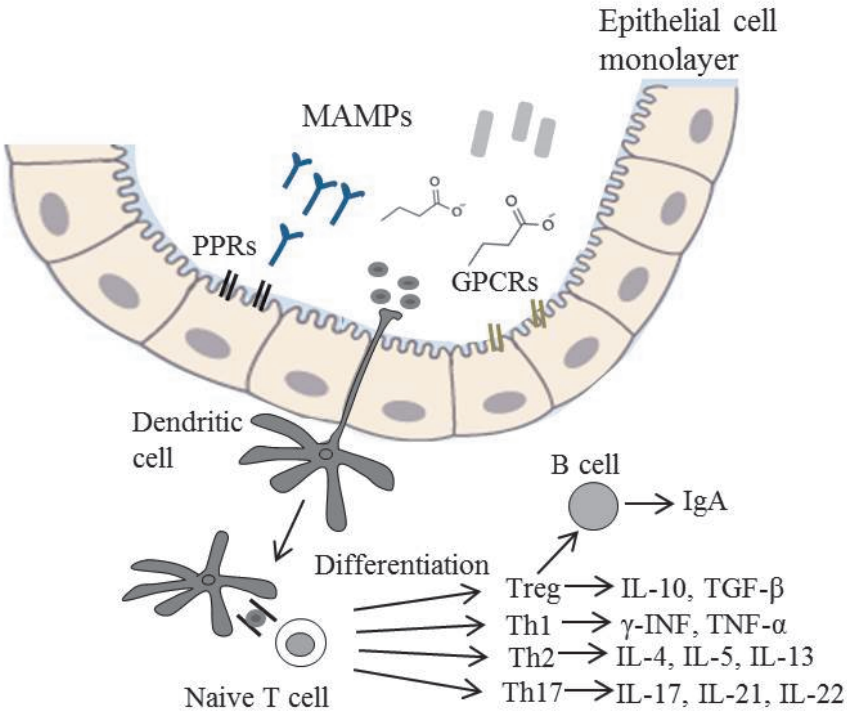

Figure 4. Effects of microbes and microbial compounds on immune cell homeostasis in the gut (modified from Kaukinen K. 2013).

These receptors recognize microbe-associated molecular patterns (MAMPs) which are widespread and conserved molecules often located on bacterial cell surface (van Baarlen et al. 2013). MAMPs include lipopolysaccharide (LPS) residing on the cell membrane of Gram-negative bacteria (recognized by TLR4), peptidoglycan (recognized by TLR2), bacterial flagellin (recognized by TLR5), lipoteichoic acid from Gram-positive bacteria (recognized by TLR1 and TLR6) and unmethylated CpG-motifs recognized by TLR9 and muramyl dipeptide (recognized by NOD2) (Table 1). Interaction between a MAMP and PRR leads to an induction of signaling cascades and activation of transcription factors such as nuclear factor kB (NF-kB). The induced signaling pathways depend on the type of interacting microbes and of the interacting host cell, leading into a molecular response against the detected microorganisms such as production of immunomodulatory cytokines and antimicrobial compounds (reviewed in (Kaiko et al. 2014; Sommer et al. 2014)).

Table 1. Main pattern recognition receptors and their targets.

Pattern recognition receptor (PPR)	Target organisms	Target structures/molecules
TLR2	Gram+ bacteria Mycobacteria Fungi	Peptidoglycan, lipoteichoic acid Lipoarabinomannan Zymosan
TLR2 & TLR1	Gram- bacteria, mycoplasma	Triacyl lipopeptides
TLR2 & TLR6	Gram+ bacteria, mycoplasma	Diacetylated lipopeptides
TLR3	Viruses	dsRNA
TLR4	Gram- bacteria	Lipopolysaccharide (LPS)
TLR5	Bacteria	Flagellin
TLR7 & TLR8	Viruses	ssRNA
TLR9	Bacteria and DNA viruses	unmethylated CpG –motifs of DNA
TLR10	Listeria, Influenza A	Unknown
NLR1	Bacteria	Specific peptidoglycan fragment
NLR 2	Bacteria	Muramyl dipeptide from peptidoglycan

TLR= Toll -like receptor, NLR = NOD -like receptor

PRRs also activate dendritic cells (DC), inducing them to produce cytokines and express cell-surface signals and to migrate to the lymph nodes where they present the pathogen-derived antigens, together with PRR-induced signals to T cells. Two distinct subsets of dendritic cells have been described in humans. Myeloid DCs express Toll-like receptor TLR2 and produce IL-12 in response to the bacterial and viral stimuli, whereas plasmacytoid DCs express TLR7 and TLR9 and release large quantities of type 1 interferons during the outcome of antiviral immune responses (Sasai et al. 2013). The activation of dendritic cells by PRRs result in T-cell activation and in the case of T helper (CD4+) cells, differentiation into one of several types of effector T helper (Th) cell (Medzhitov 2001). Th cells are classified into Th1, Th2 or Th17 subsets, defined by their cytokine patterns (Jutel et al. 2011). Intestinal microbiota shapes the immune system by regulating this differentiation and is also able to induce the differentiation of regulatory T (Treg) cells in the intestine (Furusawa et al. 2013). Tregs are crucial in the maintenance of intestinal homeostasis, the establishment of controlled immune responses and the inhibition of allergen-specific effector cells (Jutel et al. 2011). Further, Tregs induce and maintain the immunoglobulin

A (IgA) –producing plasma cells in the intestine. The main role of Treg regulated secretory IgA seems to be the establishment and maintenance of homeostasis with the commensal microbiota (Cong et al. 2009).

2.2.2 Microbial metabolites and immune system

In addition to microbial cell components, also their metabolites are able to interfere with the cells of intestinal epithelium and to modulate the signaling pathways through PPRs. Short-chain fatty acids (SCFAs) are produced by the breakdown of dietary fiber in the colon by commensal microbes and the types and amounts of SCFAs produced in the intestine are determined by the amount of carbohydrates consumed and the composition of microbiota (Smith et al. 2013; Tremaroli et al. 2012). The most abundant SCFAs in the intestine are acetate, propionate and butyrate. These SCFAs, especially butyrate, can regulate homeostasis of Tregs in the colon (Smith et al. 2013) and down-regulate host inflammatory responses (Maslowski et al. 2009). Further, butyrate has been proposed to reinforce gut defense barrier by increasing the production of mucins (Burger-van Paassen et al. 2009) and to decrease the intestinal permeability by up-regulating the expression of tight junction proteins (Burger-van Paassen et al. 2009; Ma et al. 2012; Wang et al. 2012). In addition, butyrate serves as an important energy source for the intestinal epithelial cells. Recently, the potential of SCFAs to regulate the host energy metabolism was reported (De Vadder et al. 2014). Moreover, butyrate is able to modulate the gene expression of colonic epithelial cells (Davie 2003). These effects are mediated by specific G-protein coupled receptors (GPCR) for SCFAs (Gpr41, Gpr43, Gpr109a and Olfr78) and by the inhibition of histone deacetylase (HDAC) (Natarajan et al. 2014; Samuel et al. 2008) (Figure 5).

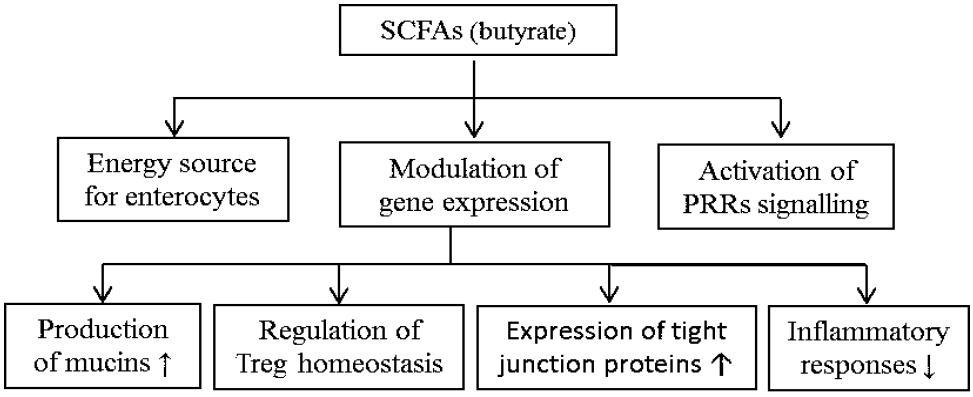

Figure 5. The effects of short-chain fatty acids (SCFAs), especially butyrate, on the maintenance of intestinal homeostasis.

2.2.3 Dysregulation of immune system

The dysregulation of T-cell responses and the imbalance between various Th cell subsets leads to chronic, typically relapsing and remitting diseases such as allergies, celiac disease and inflammatory bowel diseases (Maynard et al. 2012) (Figure 6). In prenatal period, immune responses are physiologically of the Th2 –type as a consequence of the immunologic balance prevailing *in utero* to protect the developing fetus (Amirchaghmaghi et al. 2013; Koga et al. 2014)) and it is predominant at newborn infants. Microbial derived signals are needed for normalizing the Th1/Th2 balance through the induction of Th1, Th17 and Treg cells and Th2 –skewed immunity decreases during the first months of life in healthy infants (Hörmannsperger et al. 2012; West et al. 2015). Decreased Th1/Th2 ratio is involved in the pathogenesis of atopic diseases and Th2 responses increase with age in infants with atopy (West et al. 2015). In contrast, Th1 –type immunity response is prevailing in autoimmune diseases (Izcue et al. 2009) (Figure 6).

Taken together, the cross-talk between microbes, intestinal epithelial cells and immune system is needed for the complete development, functional maturation and maintenance of intestinal homeostasis (Maynard et al. 2012). The aim of this cross-talk is to maintain the balance between tolerance towards commensal microbiota and the rapid identification and elimination of infection causing pathogens.

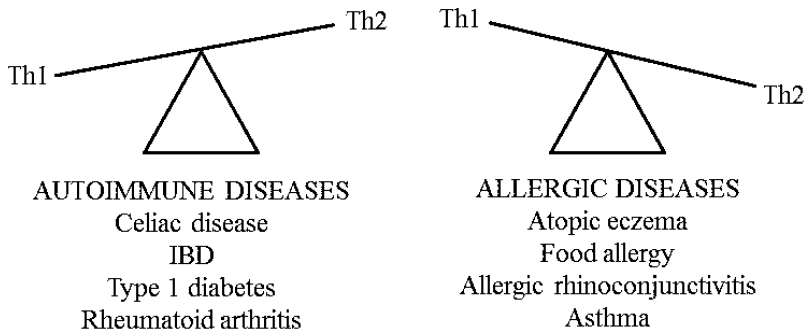

Figure 6. T helper cell imbalances in autoimmune and allergic diseases.

2.3 Importance of intestinal microbiota in human health and disease

It is assumed that aberrant bacterial diversity and relative abundances of specific microbial taxa lead to functional imbalance where the mutualistic relationship between the host and his microbes is disturbed. Deviations in the microbiota composition has been associated with several local and systematic diseases such as atopic diseases, obesity, celiac disease and inflammatory bowel diseases (Table 2) and it may contribute to the pathogenesis and/or clinical manifestation of these diseases (de Vos et al. 2012; Walker et al. 2013). However, there is no consensus yet on defining a healthy microbiota. The lack of knowledge of the microbiota composition before the onset of the disease makes it difficult to understand the role of specific microbes affecting positively or negatively to the disease process. In addition, human studies tend to be correlative and thus evidence supporting the causality between specific bacteria and the pathogenesis of certain diseases are challenging to obtain. Thus far, the causality has been shown only in recurrent *Clostridium difficile* –infection, where replacement of a microbiota with low diversity and networks by fecal microbiota transplantation with a healthy donor has proven to be an efficient therapy for recovering the normal microbiota composition and function (Fuentes et al. 2014; Mattila et al. 2012; van Nood et al. 2013). Another causal relation has been suggested for metabolic syndrome where fecal transplantation from a healthy donor resulted in the improvement of insulin sensitivity, while this did not occur when the own microbiota was transplanted (autologous transplantation) (Vrieze et al. 2012).

Table 2. Some diseases associated with microbiota aberrations.

Atopic eczema/allergies	Celiac disease
Colic crying	Diabetes (type I and II)
Necrotizing enterocolitis (NEC)	Obesity
Clostridium difficile infection	Metabolic syndrome
Inflammatory bowel diseases (IBD)	Atherosclerosis
Colorectal cancer	Autism

2.4 Development of healthy microbiota

The initial colonization process of the infant gastrointestinal tract forms the basis for the subsequent microbiota and immune response development, thus greatly affecting the later life health and predisposition to the development of several diseases.

2.4.1 Prenatal exposure to microbes and microbial compounds

This chapter has been published in Nylund L et al. Intestinal microbiota during early life –impact on health and disease. Proceedings of the Nutrition Society 2014 Jun 5:1-13.

Traditionally the fetus has been thought to be microbiologically sterile before birth. The presence of microbes in the amniotic fluid and placenta has mainly been associated with preterm deliveries due to maternal intrauterine infections and other pathological conditions (DiGiulio et al. 2008; Pararas et al. 2006; Wang et al. 2013) and the presence of bacterial DNA in amniotic fluid has been associated with lower gestational age and with low mean birth weight (DiGiulio et al. 2008; DiGiulio et al. 2010). However, recent studies utilizing molecular methods have shown that DNA of non-pathogenic bacteria can be detected in placenta and amniotic fluid samples in normal conditions (Rautava et al. 2012; Satokari et al. 2009). Hence, ingestion of amniotic fluid continuously during pregnancy exposes the fetus to bacteria and/or microbe-associated molecular patterns (MAMPs). The exact mechanism(s) of bacterial entry into the intrauterine environment remains elusive. However, ascension from the vagina, by retrograde spread from abdominal cavity, hematogeneously through placenta and contamination during medical procedures (such as amniocentesis) has been suggested as potential routes (Goldenberg et al. 2008). Also MAMPs can induce the immuno-stimulatory effects, for example via the stimulation of Toll-Like Receptors (TLRs), without the need for microbial cells to enter the amniotic cavity. This is supported by the study by Rautava and coworkers where the presence of bacterial DNA, indicative for the presence of other MAMPs too, in placenta and amniotic fluid was associated with the induction of expression profiles of TLRs, especially TLR2 and TLR5 in fetal intestine (Rautava et al. 2012).

Further, the expression of different TLRs, including TLR9, has been shown to change during the maturation of gut epithelial cells (Gribar et al. 2009; Nanthakumar et al. 2011). TLR9 recognizes unmethylated CpG motifs in bacterial DNA and its signaling maintains the gut epithelial homeostasis by improving the barrier functions and by inducing tolerance towards other MAMPs (Kant et al. 2013). *In utero* the intestinal expression of TLR9 of mouse embryos decreases from day 14 to 18 and then increases again during the postnatal period (Gribar et al. 2009). On the other hand, bacterial lipopolysaccharide (LPS) recognizing TLR4 follows an opposite expression pattern i.e. its expression decreases towards the end of pregnancy (Gribar et al. 2009). Thus, it appears that a full-term newborn is programmed to receive TLR9 stimulation, which will improve the tolerance towards commensal bacteria. Consistently with this,

necrotising enterocolitis (NEC) in preterm infants has been associated with decreased TLR9 and increased TLR4 expression of the intestinal epithelium (Gribar et al. 2009). Remarkably, TLR9 activation via CpG-DNA supplementation significantly reduced NEC severity (Gribar et al. 2009), suggesting that microbiota rich in CpG -motifs but poor in TLR4 ligands (such as lipopolysaccharide (LPS) produced by Gram-negative bacteria) could be optimal for the prevention or alleviation of NEC. In this respect human breast milk, which supports the growth of bifidobacteria, appears to be a "superfood" for the newborns. Bifidobacteria are organisms with high-G+C% content genomes that are especially rich in TLR9 –stimulating CpG-motifs (Kant et al. 2013). A number of strains of commercially produced lactobacilli have also been found to be rich in CpG-DNA (Kant et al. 2013) and probiotic interventions have shown some promising results in the prevention and alleviation of NEC (Downard et al. 2012). In a mouse model, TLR9 signaling was indeed observed to be an essential mediator of anti-inflammatory effects of probiotics (Rachmilewitz et al. 2004). Furthermore, DNA of *Bifidobacterium* and *Lactobacillus* spp., both rich in CpG motifs, have been found in human placenta (Satokari et al. 2009). Thus, it seems that prenatal exposure to MAMPs is an important step in programming the development of gut epithelium and immune system already *in utero*.

2.4.2 Microbiota development after birth

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Meconium is the very first fecal specimen produced by the infant after birth. It consists mainly of amniotic fluid but includes also mucus, intestinal epithelial cells and concentrate of metabolites such as bile acids and pancreatic secretions (Kumagai et al. 2007). Several reports have described meconium microbiota composition providing further evidence for the suggestion that microbiological colonization may begin already *in utero* (Dominguez-Bello et al. 2010; Jiménez et al. 2008; Moles et al. 2013). However, most of the studies analyzing meconium bacteria have utilized 16S rRNA gene based methods and thus it remains to be assessed whether the presence of bacterial DNA in these samples is an indication of the real low level prenatal colonization or not.

Bacteria belonging to four major bacterial phyla in the intestine, Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria, are already detectable in the meconium. The predominant cultured bacteria seem to be bacilli within the Firmicutes phylum such as enterococci and staphylococci, or certain Proteobacteria such as *Esherichia coli, Klebsiella* and *Enterobacter* spp. (Dominguez-Bello et al. 2010; Favier et al. 2002; Gosalbes et al. 2013; Hu et al.

2013; Jiménez et al. 2008; Moles et al. 2013). This is in agreement with the reports that these facultative anaerobes are present in feces of healthy newborn infants (Adlerberth et al. 2009; Palmer et al. 2007; Scheepers et al. 2014). In addition, *Enterococcus* spp. are commonly present, approximately 40 % and 50 % of infants colonized at day 3, respectively (Adlerberth et al. 2009; Favier et al. 2002).

Following the colonization of facultative bacteria, anaerobic bacteria appear in the infant feces within the first weeks of life, decreasing the abundance of facultative anaerobes and thus introducing a shift in microbiota community structure (Avershina et al. 2014). It should be noted, however, that this shift may represent an outgrowth of specific groups of bacteria and does not preclude the fact that their colonization might have already occurred at low level. Especially the abundance of bifidobacteria increases rapidly from approximately 3.5 – 10 % in meconium (de Weerth et al. 2013; Jiménez et al. 2008; Moles et al. 2013) to 50-70 % and even up to 90 % in the feces of breast-fed infants at one month and 3 months of age, respectively (Bezirtzoglou et al. 2011; Fallani et al. 2010; Roger et al. 2010a; Turroni et al. 2012). However, large inter-individual variations are characteristic for infant microbiota and the abundance of bifidobacteria varies from 5 to 100 % in breast-fed infants (Roger et al. 2010a). Considering formula-fed infants, bifidobacteria (determined by fluorescent in situ hybridization (FISH)) may form a minor part of the microbiota, constituting approximately 25 % of the total microbiota (Roger et al. 2010a). In addition to the individual variation, the bifidobacterial abundance seems to vary greatly according to the geographic origin; infants from (northern) European countries harbor in general high numbers of bifidobacteria (Fallani et al. 2011; Roger et al. 2010a; Turroni et al. 2012), while these bacteria are less predominant in Asian and American infants (Fan et al. 2014; Koenig et al. 2011; Palmer et al. 2007). This observation can be mainly explained by demographic differences and by differences in the rate and duration of breast-feeding between the countries and potentially also the differences in use of antibiotics.

After the introduction of solid foods and weaning the relative abundance of bifidobacteria decreases gradually being approximately 60 % at 4 months, 25 % at 6 months and 10 % at 2 years (Avershina et al. 2014; Ringel-Kulka et al. 2013). Simultaneously, the relative abundances of lactobacilli decrease, whereas bacteria predominant in adult microbiota, such as Bacteroidetes and bacteria belonging to the *Clostridium* clusters XIVa and IV increase (Koenig et al. 2011; Ringel-Kulka et al. 2013; Roger et al. 2010a). However, the early life microbiota composition is characterized by high inter-individual (Avershina et al. 2014; Avershina et al. 2013). The bacterial abundances in healthy infant microbiota vary greatly in a subject-wise manner and fluctuate further in response to the changes in different life events such as antibiotic treatments and introduction of

solid foods (Koenig et al. 2011; Palmer et al. 2007). During and after weaning major changes occur in microbiota diversity and composition, this transitional phase being more pronounced in breast-fed than in formula-fed infants (Roger et al. 2010a). The succession of *Bacteroides* spp. and bacteria belonging to the *Clostridium* clusters XIVa and IV proceeds rapidly while the relative proportion of bifidobacteria decreases (Koenig et al. 2011; Roger et al. 2010a).

Previously, it has been suggested that the microbiota diversity and composition stabilize and reach the level of adult microbiota within the first year or two (Mackie et al. 1999; Palmer et al. 2007; Yatsunenko et al. 2012) . However, recent studies have shown that microbiota maturation will continue longer (Agans et al. 2011; Ringel-Kulka et al. 2013). Interestingly, the establishment of bacteria belonging to *Clostridium* cluster XIVa at a level similar to adults has been observed already in young children (1-4 years) (Ringel-Kulka et al. 2013), while other bacterial groups still remain at low-level abundance. This indicates that the microbiota development is a gradual process, where some bacterial groups may reach the degree of stabilization earlier than others. However, considering the major physiological changes taking place in the human body within the childhood and adolescence it may be argued that the development of the intestinal microbiota continues throughout this time period and is not finished until the human host reaches adulthood. This is supported by the first studies on adolescent microbiota which reported significant differences between the microbiota composition of adolescent children (11-18 years) and adults (Agans et al. 2011). The most striking difference was the almost two-fold higher abundance of bifidobacteria in adolescent subjects (9 % vs 5.5 % of total microbiota, respectively) (Agans et al. 2011) .

2.5 Factors affecting the microbiota development

Different factors affecting the early life microbiota development and consequently the later life health are summarized in Figure 7.

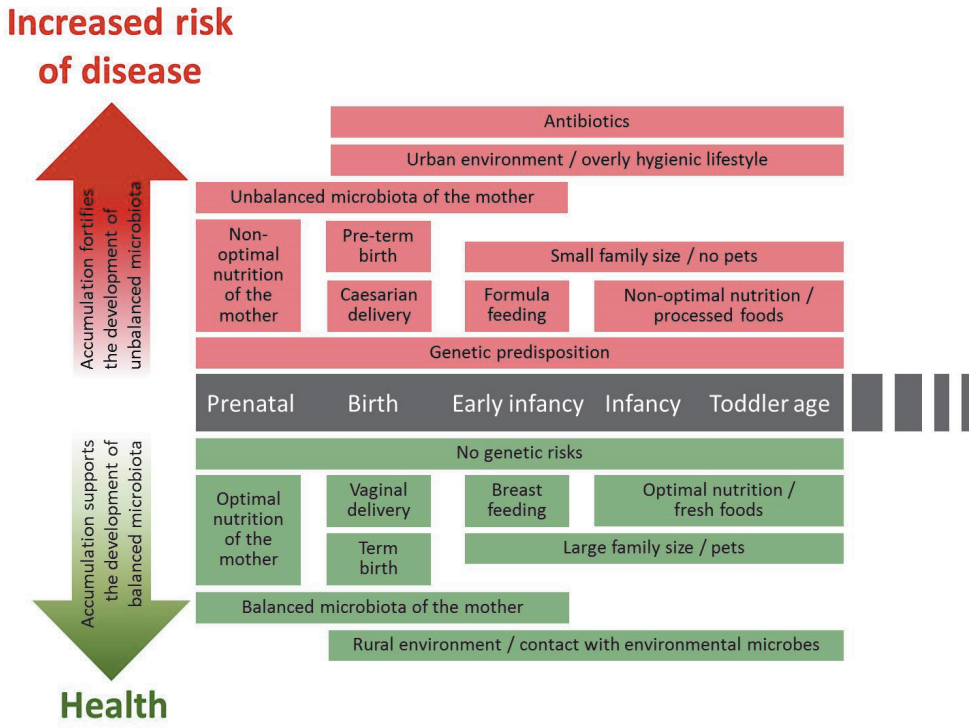

Figure 7. Modern life-style factors associated with the development of intestinal microbiota and later life health.

2.5.1 Maternal intestinal microbiota

Maternal microbiota serves as a first inoculum for the infant microbiota, thus having a crucial role in the microbiota colonization process and in the postnatal immune development. The intestinal microbiota of the pregnant mothers changes during pregnancy and is characterized by a decreased diversity (Koren et al. 2012). Further, the remodeling of microbiota composition over the course of pregnancy has been reported, with a decrease in the abundance of *Faecalibacterium* spp. and an increase in Proteobacteria, *Streptococcus* spp. and Actinobacteria (Jost et al. 2014a; Koren et al. 2012). It can be speculated that the intestinal microbiota composition of mothers may change towards a structure that is beneficial for the infant, since the bacteria increased in late pregnancy are the predominant ones in the feces of newborn infants and have an increased capacity

to harvest energy to the host (Koren et al. 2012). Similarities between the microbiotas of child and his/her mother increase with the age of the children, indicating the importance of shared environment and diet on shaping the microbiota composition (Koren et al. 2012). In addition, maternal microbiota shapes the specificities of secretory IgA secreted to breast-milk (Maynard et al. 2012). Transfer of these maternal sIgA to the infant promotes the establishment of regulatory immune system and supports the mutualistic relationship with the commensal microbiota (Maynard et al. 2012).

The vertical transmission of specific bacterial species or strains from mother to infant has been shown in studies utilizing conventional culturing methods (Keski-Nisula et al. 2013; Makino et al. 2013) and with molecular methods (Takahashi et al. 2010), whereas comprehensive studies considering the transmission of whole microbial community are scarce. The strongest evidence exists for the vertical transmission of lactobacilli and bifidobacteria strains from mother to her infant (Gueimonde et al. 2006; Makino et al. 2011; Makino et al. 2013). Moreover, each family appears to harbor their own unique set of these species (Makino et al. 2013). In addition, the mother-derived *Bifidobacterium* strains that were shared between mother-infant pairs showed higher growth in the presence of galacto-oligosaccharides (GOS) and at a higher redox potential than bifidobacteria originating from other sources (Takahashi et al. 2010). This indicates the ability of these strains to utilize breast- milk GOS as an energy source and the high adaptation to the intestinal conditions of newborn infant. Moreover, vaginally born infants have been shown to share significantly more similar proportion of 16S rRNA gene sequence types with their own mother than with other mothers, whereas no significant sequence overlapping was observed in infants born via Caesarean section (C-section) and their mothers (Jakobsson et al. 2013).

2.5.2 Breast -milk

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Human milk oligosaccharides (HMO) have an essential role in the promotion of the development of normal physiology of intestine and immune system in infants. Human milk contains a complex mixture of oligosaccharides, their exact composition varying according to different extrinsic and intrinsic factors. These factors include the genetic background of the mother, maternal health status, diet, secretor status and Lewis blood group type (Albrecht et al. 2011; Bode 2012; Thurl et al. 2010). Oligosaccharide molecules participate in the maintenance of a healthy gut microbiota of the infant in three ways. 1) They block the colonization

of pathogenic bacteria by acting as receptor analogs and binding to the bacterial surface, thus preventing the pathogens from binding to their target oligosaccharides on the epithelial cell surface (Zivkovic et al. 2011). 2) They act as prebiotic substrates promoting the growth of beneficial bacteria, notably bifidobacteria, concurrently preventing the adherence of potentially harmful bacteria via colonization resistance (Bode 2012). 3) They have also been suggested to stimulate intestinal epithelial cells, lymphocyte cytokine production and leukocyte rolling and adhesion (Bode 2012).

HMOs are the third most abundant component in human milk after lactose and lipids and the abundance of oligosaccharides is more than ten times higher in human than in cow's milk (5-16 g/l vs 0.03-0.06 g/l, respectively) (Kunz et al. 2000). However, human infants lack the extensive set of enzymes needed for the digestion of glycan residues of human milk oligosaccharides. Thus, these molecules are both non-nutritive and non-digestible, necessitating they have some other biological function which entitles their presence in human breast milk (Zivkovic et al. 2011). Indeed, since HMOs pass undigested to the lower part of the intestinal tract, they can be consumed by the specific members of infant gut microbiota (Marcobal et al. 2012). Since a wide repertoire of enzymes are needed for the degradation and utilization of the intricate structures of both human milk oligosaccharides and plant polysaccharides, such processes most likely involve several different commensal bacteria acting synergistically.

Only two bacterial genera, *Bifidobacterium* and *Bacteroides,* have been described to have the capability for milk oligosaccharide utilization (Marcobal et al. 2010; Yu et al. 2013). Bifidobacteria, such as *B. longum* subsp. *infantis* and *B. bifidum*, typically abundant in infant microbiota, harbour a complex set of genes specifically involved in HMO utilization (Sela et al. 2012). The *B. longum* subsp. *infantis* genome harbors entire gene clusters controlling the expression of glycosidases, membrane-spanning transporters and other proteins dedicated to human milk oligosaccharide utilization (Sela et al. 2008; Sela et al. 2012) and is the only bacterial species able to digest all HMO structures (Sela et al. 2014; Underwood et al. 2015). In contrast, *B. longum* subsp. *longum*, which is more abundant in adult microbiota, is unable to use diverse HMOs, but have the capability to utilize short chain oligosaccharides (Sela et al. 2012) . However, HMOs have reported to up-regulate the expression of several pathways in *B. longum* subsp. *longum* such as genes involved in carbohydrate degradation and cell adherence (Gonzalez et al. 2008). Possibly, *B. longum* subsp. *longum* relies on cross-feeding with other bacteria, which first degrade complex polysaccharides to shorter units and thereby can also use HMOs as a nutrient source.

Bacteroides spp. genomes harbour a specific gene cluster termed polysaccharide utilization loci (PUL), enabling a wide range of saccharolytic

ability (Marcobal et al. 2012; Martens et al. 2011). For example *B. thetaiotaomicron* can degrade more than a dozen different types of glycans (Marcobal et al. 2012), most likely also HMOs. In addition, *in vitro* utilization of HMOs by *B. fragilis* and *B. vulgatus* has been reported (Marcobal et al. 2012). Consistently, *B. fragilis* and *B. vulgatus* are the predominant *Bacteroides* spp. found in breast-fed infants (Tannock et al. 2013). The abundance of bacterial groups, which have restricted capacity to utilize different polysaccharide compounds, are likely to fluctuate more in response to the type of incoming carbohydrates, whereas bacteria with a wide glycan-degrading capability may have a competitive advantage in the gut. *Bacteroides* spp. are among the first groups colonizing the gut (Palmer et al. 2007; Penders et al. 2006), increasing further after the introduction of solid food and weaning (Koenig et al. 2011; Roger et al. 2010a), and are part of the common core microbiota in adults (Huse et al. 2012; Qin et al. 2010; Rajilić-Stojanović et al. 2009). In addition, the ability of *Bacteroides* spp. to switch substrate specificity in response to the changing ingestion of nutrients indicates that they are adapted to the symbiotic life with human host and are permanent colonizers of the gut.

Human milk is also a source of bacteria to the infant and together with the bacterial transfer during birth they constitute the essential pioneer colonizers of the neonatal gut. The presence of commensal microbes in breast milk of healthy mothers was first acknowledged only a decade ago (Martin et al. 2003) and since then, more than 200 different species belonging to 50 different genera have been described in human milk (Hunt et al. 2011). The predominant bacteria observed in human milk samples are Bacilli, such as *Streptococcus* spp. and *Staphylococcus* spp. (Hunt et al. 2011; Jost et al. 2013). In addition, *Bifidobacterium* spp. are present and Bacteroidetes and specific clostridia such as butyrate-producing bacteria *Faecalibacterium* and *Roseburia* spp. have been detected (Hunt et al. 2011; Jost et al. 2013; Martin et al. 2009) . As the latter bacterial species are only found in the intestine, this strongly points to a fecal origin. The bacterial composition of breast milk may vary depending on the genetic background, maternal dietary habits and demographic differences between the mothers. For example, European mothers commonly harbour *Lactobacillus* and *Bifidobacterium* spp. in their breast milk, whereas these bacteria were rarely detected in mothers from the USA. However, it can be speculated that methodological drawbacks in DNA extraction may have impacted these results (Jost et al. 2013; Ward et al. 2013). Further, the mode of delivery has been shown to affect the milk microbiota composition (Cabrera-Rubio et al. 2012; Khodayar-Pardo et al. 2014; Simsek et al. 2014). Milk samples from mothers who delivered their infants vaginally contained more *Leuconostocaceae* and less *Carnobacteriaceae* (Cabrera-Rubio et al. 2012) and bifidobacteria

(Khodayar-Pardo et al. 2014) than milk samples from mothers who had gone through an elective C-section.

Maternal health status seems to have a major effect on the milk microbiota composition. For example, milk microbiota of overweight mothers differs from that of normal weight mothers (Cabrera-Rubio et al. 2012; Collado et al. 2012). The bacterial composition of breast milk seems to be stable at intra-individual level over time, while representing a great inter-individual variation. This suggests that human milk microbiota is highly personalized, in a manner similar to intestinal microbiota (Costello et al. 2009; Human Microbiome Project Consortium 2012; Ursell et al. 2012).

Recently, the existence of a ”core” milk microbiota has been suggested (Hunt et al. 2011). The milk core microbiota consisted of nine OTUs (operational taxonomic unit), corresponding to *Staphylococcus*, *Streptococcus* (Firmicutes), *Corynebacterium*, *Propionibacterium* (Actinobacteria) and *Serratia*, *Pseudomonas*, *Ralstonia*, *Sphingomonas* and *Bradyrhizobiaceae* (Proteobacteria), constituting approximately half of the total bacterial community. It is noteworthy that many of the core microbiota genera are typically found from the skin and it seems likely that some part of the breast milk microbiota originates from the skin. Another origin of bacteria in human milk may be the intestinal tract of the mother. While fecal contamination is a likely avenue, it also has been suggested that intestinal bacteria could transfer within the phagocytosing cells from the gut to human milk via entero-mammary circulation of immune cells (Grönlund et al. 2007). Interestingly, *Bifidobacterium breve* is one of the most commonly detected bifidobacterial species in human milk samples (Alp et al. 2010; Boesten et al. 2011; Martin et al. 2009; Roger et al. 2010b; Solis et al. 2010; Turroni et al. 2011) and it produces exopolysaccharide (EPS), which masks other surface antigens and presents an ability to remain immunologically “silent” (Fanning et al. 2012). The production of EPS seems to be important for the persistence of *B. breve* in the gut (Fanning et al. 2012). Speculatively, EPS may also play a role in the survival of this bacterium within immune cells, enabling its transfer via the entero-mammary circulation route. Moreover, *B. breve* and other bifidobacteria are known to produce specific pili that are assumed to play a role in colonization (O'Connell Motherway et al. 2011). While fecal and skin contamination and selection by the milk environment are ecologically plausible routes, other origins of bacteria in human milk cannot be excluded. Whatever the route, human milk bacteria should be considered as an important source of bacteria for breast-fed infants.

2.5.3 Other factors affecting the microbiota development

Other factors affecting the early life microbiota development are presented in Table 3.

Table 3. Environmental factors affecting the microbiota development in children.

Environmental factor	Effect on intestinal microbiota in children	References
Mode of delivery	Vaginal bacteria are prevalent in vaginally born neonates (lactobacilli and *Prevotella* spp.) vs. skin bacteria prevail in neonates born via CS *(Staphylococcus*, *Propionibacterium* and *Corynebacterium* spp.)	Dominguez-Bello et al. 2010; Goldani et al. 2011
	Delayed colonization and decreased abundance of bifidobacteria and Bacteroidetes in infants born via CS	Biasucci et al. 2010; Jakobsson et al. 2013
	High diversity in infants born via emergency CS vs. very low diversity in infants born via elective CS	Azad et al. 2013
Gestational age	Very low microbial diversity, delayed bacterial colonization, especially that of anaerobic bacteria in pre-term infants	Rouge et al. 2010; Barrett et al. 2013; Greenwood et al. 2014; La Rosa et al. 2014; Moles et al. 2013; Normann et al. 2013
Geographical factors: country, its climate and environment, hygienic conditions, typical dietary habits and other cultural issues	Bifidobacterium spp. highly dominant in infants from (northern) European countries, whereas less predominant in Asian and American infants	Fallani et al. 2011; Roger et al. 2010a; Turroni et al. 2012; Fan et al. 2013; Koenig et al. 2011; Palmer et al. 2007
Antibiotic treatment(s)	Decreased microbiota diversity	Dethlefsen et al. 2011; Fouhy et al. 2012
	Higher proportion of Proteobacteria and reduction of bifidobacteria, lactobacilli and Bacteroidetes	Fouhy et al. 2012; Fallani et al. 2010; Hussey et al. 2011; Mangin et al. 2012; Rea et al. 2011; Tanaka et al. 2009a

CS =Caesarean section

2.6 Perturbations in the microbiota development and its implications on health

Exposure to the microbes from natural environment is thought to be crucial for the normal microbiota development and the optimal maturation of immune system. Modern life style factors such as hygienic conditions, reduced contact with animals and extended use of broad spectrum antibiotics lead to a decreased exposure to these microbes. Since sufficient microbial exposure is needed for the development of balanced microbiota community and consequently, for the optimal maturation of immune system, any factors causing deviations in this process may have a long-lasting impact on later life health. For example, early life antibiotic treatment(s) have been associated with the increased risk for health problems later in life such as the risk for celiac disease development (Mårild et al. 2013), allergic diseases (Foliaki et al. 2009) and the increased risk of obesity at school age (Ajslev et al. 2011). Interestingly, maternal antibiotic use during pregnancy have been associated with an increased risk of cow's milk allergy, asthma, eczema and hay fever in their infants (McKeever et al. 2002; Metsälä et al. 2013), highlighting the importance of balanced maternal microbiota in providing a beneficial inoculum for the infant microbiota development and subsequently health later in life.

2.6.1 Atopic diseases

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Atopic diseases are chronic and relapsing disorders usually starting in early childhood. Atopy has been characterized as a genetic disposition to develop an allergic reaction and produce elevated levels of immunoglobulin E (IgE) upon exposure to an environmental antigen (Bieber 2010). Atopic diseases include eczema (atopic dermatitis), allergic rhinitis (hay fever), allergic conjunctivitis and allergic asthma. In early life, the most common form of atopic disease is eczema, its prevalence being approximately 15-30 % depending on the country studied (Deckers et al. 2012). During the last decades, associations between the composition of intestinal microbiota and atopic diseases have been studied intensively.

Previous studies have mainly addressed the microbiota composition preceding the development of atopic disease, although the age at the onset of atopic symptoms has been insufficiently clarified and the severity of eczema has not been considered. Reduced diversity at early life (i.e. at 1 week, 1 month or 4 months of age) has been associated with an increased risk of developing atopy or

allergic disease (Abrahamsson et al. 2012; Bisgaard et al. 2011; Forno et al. 2008; Ismail et al. 2012; Kalliomäki et al. 2001a; Wang et al. 2008) (Table 4). However, after 1 year of age the total microbiota diversity in children either developing or having eczema is comparable or even higher than that of healthy children (Abrahamsson et al. 2012). Notably, the majority of these studies have been conducted by using traditional cultivation-based techniques or molecular techniques that only target a sub-set of the intestinal microbiota, thus limiting the detection of true microbiota diversity.

The results on specific bacteria either increasing or decreasing the risk of developing atopic diseases or associated with their onset are still conflicting (Gore et al. 2008; Johansson et al. 2011; Mah et al. 2007; Penders et al. 2006; Sepp et al. 2005; Stsepetova et al. 2007). Aberrancies in bifidobacterial community have been associated with children with atopic diseases, most often characterized by either reduced total abundance or shifts in species community (Gore et al. 2008; Mah et al. 2007; Sepp et al. 2005; Stsepetova et al. 2007). Further, decreased amounts of *Bacteroides* spp. and increased amounts of specific Firmicutes such as *Staphylococcus aureus* and different clostridial groups, have been associated with the development and onset of allergic diseases (Abrahamsson et al. 2012; Bisgaard et al. 2011; Björksten et al. 2001; Sjögren et al. 2009a; Storrø et al. 2011; Thompson-Chagoyan et al. 2011). Interestingly, both *Bifidobacterium* spp. and *Bacteroides* spp. have been reported to have anti-inflammatory properties via their ability to direct the cellular and physical maturation of the developing immune system (Chiclowski et al. 2012; Hooper et al. 2001; Pagnini et al. 2010). For example, polysaccharide A from *B. fragilis* is able to direct the development of CD4+ T cells, thus inducing the differentiation of Th1 lineage and correction of the Th1/Th2 imbalance (Mazmanian et al. 2005). Further, this polysaccharide has been shown to promote immunologic tolerance through induction of regulatory T (Treg) cells, resulting in the suppression of IL-17 responses (Round et al. 2011). Moreover, both *Bifidobacterium* and *Bacteroides* spp. have high frequency of immunostimulatory CpG motifs in their genomes, thus being rich in TLR9 ligands (Kant et al. 2013). TLR9 stimulation is known to both enhance epithelial integrity and direct immune responses towards Th1 type (reviewed in Kant et al 2013 (Kant et al. 2013)). These effects may be diminished in allergic subjects, who have reduced numbers of *Bifidobacterium* and *Bacteroides* spp.

In recent studies, increased levels of IL-17 have been associated with asthma (Alyasin et al. 2013; Ramirez-Velazquez et al. 2013). Furthermore, one of the most important defense mechanisms in the epithelial barrier is IgA, which is present at high concentrations in the intestinal mucus layer (Brandtzaeg 2009). Low levels of IgA predisposes infant to increased binding of antigens to mucosal membrane, to increased mucosal leakiness and an increased uptake of dietary

antigens (Johansen et al. 1999). Low levels of IgA have also been associated with increased risk for the development of IgE-mediated allergic diseases in children (Kukkonen et al. 2010). Further, it has been suggested that high numbers of *Clostridium* spp. may be associated with degradation of antigen-specific IgA, which could debilitate the immature gut barrier (Pärtty et al. 2013). The protective role of specific bacteria and their compounds against atopy and allergic diseases is further supported by several clinical studies reporting the effects of probiotic strains on the alleviation of allergic symptoms even when the probiotics failed to modify the microbiota composition or diversity (Elazab et al. 2013; Nermes et al. 2011). These effects can be related to the probiotic effects on the hosts' immunological functions such as improvement of the barrier function and increasing allergen-specific IgA levels, which are essential for the development of tolerance and can be considered as a marker for immune maturation (Di Mauro et al. 2013; Kukkonen et al. 2010; Mantis et al. 2011; Nermes et al. 2011; Rautava et al. 2006; Rosenfeldt et al. 2004). Further, probiotics have been suggested to have immunomodulatory impacts that affect the Th1/Th2 balance such as stimulation of Th1 type immune responses, induction of apoptosis of Th2 cells and induction of Treg and dendritic cells (Kant et al. 2013; Kwon et al. 2010; Lyons et al. 2010; Marschan et al. 2008; Rautava et al. 2006; Torii et al. 2011; West et al. 2009).

Table 4. Summary of studies analyzing the associations between intestinal microbiota diversity and the risk of developing atopic eczema

Reference	Study population	Fecal samples analyzed	Follow-up	Method used	Main findings
Abrahamsson et al. 2012	Infants with IgE – associated eczema (n=20) and healthy controls (n=20)	1 week, 1 month and 12 months	2 years	454-pyrosequencing	Infants with IgE –associated eczema had lower diversity of total microbiota and Bacteroidetes at 1 month and lower diversity of Proteobacteria at 12 months of age.
Bisgaard et al. 2011	Children who developed atopic eczema (n=127), allergic rhinitis (n=28) or asthma (n=27) and healthy controls (n=71)	1 and 12 months	6 years	PCR-DGGE and conventional culturing	Reduced bacterial diversity at 1 and 12 months of age was associated with increased risk of allergic sensitization (SPT+ or specific IgE) and allergic rhinitis but not atopic eczema
Forno et al. 2008	Infants with eczema (n=9) and healthy controls (n=12)	1 and 4 months	6 months	PCR-DGGE	Infants with eczema had lower diversity at 1 and 4 months.
Ismail et al. 2012	Infants who developed eczema (n=33) and healthy controls (n=65)	1 week	12 months	T-RFLP	Microbial diversity at 1 week of age lower in infants who had eczema at 12 months of age.
Kalliomäki et al. 2001a	Infants who developed atopic eczema (n=22) and healthy controls (n=54).	3 weeks and 3 months	2 years	FISH	Children with atopic eczema had more clostridia and tendency to have lower bifidobacteria
Wang et al. 2008	Infants who developed atopic eczema (n=15) and healthy controls (n=20).	1 week	18 months	T-RFLP and TTGE	Infants with atopic eczema had reduced diversity

PCR-DGGE = PCR combined with Denaturing Gradient Gel Electrophoresis, T-RFLP = Terminal Restriction Fragment Length Polymorphism, FISH = Fluorescence In Situ Hybridization, TTGE = Temporal Temperature Gradient Gel Electrophoresis

2.7 Studying the early life microbiota –special challenges

Although the intestinal microbiota of infants is dominated by fewer bacterial groups than that of adults, the inter-individual variability is significantly higher than in adults (Yatsunenko et al. 2012). Microbiota in children under 3 years of age fluctuates substantially and is extremely prone to environmental factors (Koren et al. 2012). Especially during and after weaning process, the income of new substrates promotes the selection of different microbial species (Arrieta et al. 2014; Fallani et al. 2011). In addition, the conditions of the gastrointestinal tract changes along with the introduction of a variety of non-digestible carbohydrates other than those present in breast-milk (Fallani et al. 2011). Furthermore, the behavior of infants encourage the significant exposure to microbes from their environment, since they are constantly introducing hands, feet, toys etc. to their mouth (Arrieta et al. 2014). In addition, they often suffer from infectious diseases. Thus, when the microbial community structure is constantly fluctuating and changing in an individual-specific manner, it is a great challenge to assess the effect of different factors such as antibiotic treatment or intervention procedures on this development process.

2.8 Overview of high-throughput microbiota analysis

Most of the bacterial species colonizing the human intestine are strictly or facultatively anaerobic and have physiologically adjusted to the conditions residing in the gastrointestinal tract. These conditions are difficult to mimic in laboratory conditions. In spite of the fact that over 1000 species have been cultured from the human intestinal tract (Rajilić-Stojanović et al. 2014), an important fraction (estimated to be approximately 70 %) of the intestinal microbes have not been cultured or identified yet (Fraher et al. 2012). Therefore, the true diversity of intestinal microbiota has remained incompletely covered by using traditional cultivation strategies or molecular methods with low resolution, whereas the rapid development of culture-independent, high-throughput molecular applications during the recent years has enabled the deep and comprehensive analyses of total microbiota diversity. These high-throughput techniques are based on either direct sequencing of nucleic acids in the sample or on their detection by using high-density oligonucleotide arrays.

Phylogenetic microarray is a high-throughput platform designed for the simultaneous detection of thousands of DNA or RNA sequences. Further, it allows the concurrent analysis of numerous samples, making microarrays a fast, cheap and user-friendly technology. As other molecular methods for microbiota

community analysis, this technology is most often based on sequences of variable regions of the 16S rRNA gene, denoting that the sequence knowledge is a prerequisite for the array design. Phylogenetic microarrays have been shown to have higher sensitivity than high-throughput sequencing strategies (Claesson et al. 2009; Tottey et al. 2013) and to detect bacterial DNA as low as 0.00025 % of the sample (Paliy et al. 2009). The major challenge in this technology is the possibility of cross-hybridizations, i.e. that probes hybridize also to highly similar non-target sequences (Fraher et al. 2012). Several phylogenetic microarrays targeting the human intestinal microbiota have been developed (Centanni et al. 2013; Paliy et al. 2009; Rajilić-Stojanović et al. 2009; Tottey et al. 2013), the Human Intestinal Tract Chip (HITChip) being one of the most intensively used. The HITChip enables rapid and sensitive profiling of the microbiota diversity and allow the relative quantification of all bacterial groups at different taxonomic levels simultaneously (Rajilić-Stojanović et al. 2009) (Table 5). The so-called next-generation sequencing methods deliver sequences from 16S rRNA gene amplicons or from total community DNA. Direct sequencing of partial 16S rRNA gene amplicons ('massively parallel sequencing') is able to sequence massive amount of sequences at the same time in same reaction. Due to the sequencing of short reads (typically around 100 bp), it is possible to increase the amount of sequences analyzed, thus enabling also the detection of low abundant bacteria (reviewed in (Fraher et al. 2012)). This method gives phylogenetic and quantitative information and enables detection of unknown bacteria. Massively parallel sequencing is fast but requires intense computational data analysis that includes the removal of chimeric sequences (Fraher et al. 2012). One overlooked issue is the generation of errors as is illustrated by the need for developing a low error 16S rRNA amplicon sequencing method to be able to monitor the microbiota of subjects over time (difficulties in identifying subjects based on their fecal microbiota (Faith et al. 2013), while this was done easily with the HITChip (Rajilić-Stojanović et al. 2012).

Table 5. Different taxonomic levels and representative bacteria covered by the phylogenetic microarray HITChip

	Phylum-like level (Level 1)	Genus-like level (Level 2)	Phylotype–level (Level 3)
	Actinobacteria	*Bifibobacterium*	*B. longum* *B. breve*
	Bacteroidetes	*Bacteroides fragilis* et rel. *Bacteroides vulgatus* et rel. *Prevotella melaninogenica* et rel.	*B. fragilis* *B. vulgatus* *P. intermedia*
FIRMICUTES	Bacilli	*Staphylococcus* *Lactobacillus plantarum* et rel. *Streptococcus bovis* et rel. *Enterococcus*	*S. aureus* *L. rhamnosus* *S. pyogenes* *E. faecalis*
	Clostridium cluster IV	*Clostridium leptum* et rel. *Faecalibacterium prausnitzii* et rel. *Ruminococcus bromii* et rel.	*C. leptum* *F. prausnitzii* *R. bromii*
	Clostridium cluster IX	*Veillonella*	*V. parvula*
	Clostridium cluster XI	*Clostridium difficile* et rel.	*C. difficile*
	Clostridium cluster XIVa	*Anaerostipes caccae* et rel. *Coprococcus eutactus* et rel. *Ruminococcus obeum* et rel.	*A. caccae* *C. eutactus* *R. obeum*
	Proteobacteria	*Escherichia coli* et rel. *Enterobacter aerogenes* et rel.	*E. coli* *Citrobacter werkmanii*
	Verrucomicrobia	*Akkermansia*	*A. muciniphila*

Metagenomics of microbiota involves sequencing of whole community DNA, which is done by massive parallel sequencing of the mixed DNA sample (reviewed in (Fraher et al. 2012)). DNA is randomly fragmented and sequenced, and overlapping sequences are then reconstructed to assemble them into a continuous sequence (Fraher et al. 2012). The main advantage is the determination of large amounts of sequences from total DNA, thereby avoiding PCR bias. This technique has proven to be very powerful since it obtains information both on diversity of microbial population (microbiota analysis) and their genetic capacity (microbiome analysis) (Tasse et al. 2010; Walker et al. 2014). The limitation is the need for intense computational data analysis and relatively high costs and limited depth, as there are around 1000 times more coding genes than 16S rRNA genes in a prokaryotic genome.

While advances in molecular microbiological techniques allow a high-throughput analysis of the microbiota, several problems are related to inappropriate use of these 16S rRNA gene sequence -based technologies (Table 6). Sufficient pretreatment of fecal samples such as sample collection procedures and storage conditions, efficient nucleic acid extraction and unbiased PCR amplification are crucial in order to cover the full microbial diversity (Maukonen et al. 2012). In addition, identification of sequences based on very short reads is not always reliable (Werner et al. 2012). Consequently, quantitative PCR (qPCR) and other molecular techniques targeting specific taxonomic groups are still relevant in verifying the results obtained by using more sophisticated methods described above. Quantitative PCR is one of the most accurate molecular methods currently available for microbiota analysis. It allows also the detection of the low abundant members of the intestinal microbiota, which may be missed by high-throughput techniques (Werner et al. 2012).

Despite the powerful molecular techniques for microbiota characterization, culturing is still utmost valuable as it is a necessary step for the detailed physiological and biochemical identification of individual isolates. Experimental characterization of isolates and their metabolic activities are needed to expand the reference databases used for the annotation of metagenomic sequence data, because currently a significant proportion of obtained data is lacking a close match in the reference databases (Le Chatelier et al. 2013; Walker et al. 2014). The recent development of advanced, high-throughput culturing has proven to be a powerful tool in the discovery of the unknown members of human intestine (Dubourg et al. 2013; Lagier et al. 2012; Pfleiderer et al. 2013)). This ‘microbial culturomics’ employs a large set of growth media and a wide variety of culturing conditions and is combined with 16S rRNA gene sequencing and mass spectrometry for the identification of obtained colonies (Lagier et al. 2012). In future, high-throughput cultivation of new species in combination with metagenomics will give us new insights into microbiota composition and function (Rajilić-Stojanović et al. 2014).

Table 6. Outline of the steps and main challenges involved in sequence-based microbiota analysis (modified from Tyler et al. 2014).

Step	Critical point	Reference
Sample collection and storage	Preserving DNA quality (rapid freezing, use of preservatives)	Bahl et al. 2012; Maukonen et al. 2012
Nucleic acid extraction	Efficient cell lysis Prevention of DNA smearing/shearing	Santiago et al. 2014
Amplification of 16S rRNA gene and HT-analysis (microarrays, sequencing)	Selection of targeted variable region Primer design Minimizing the amplification biases Minimizing cross-hybridizations Sufficient sequencing depth & read length Sequencing errors	Kim et al. 2011 van den Bogert et al. 2011 Sim et al. 2012 Claesson et al. 2009 Schloss et al. 2011b
Data analysis	Quality filtering Data normalization Sequence identification (quality of reference database)	Haas et al. 2011 Schloss et al. 2011a
Statistical analysis and interpretation of findings	Inter-individual variation Temporal variation (esp. pediatric studies)	Yatsunenko et al. 2012

3 AIMS OF THE STUDY

The main purpose of the present series was to analyze the presence of specific groups of bacteria that relate to the health status in babies. The specific aims were:

1. To compare and optimize DNA extraction to facilitate fecal DNA extraction in large cohort studies (**I**).
2. To characterize the long-term effects of maternal microbiota on infant intestinal microbiota composition and development (**II**).
3. To characterize the diversity and temporal changes of intestinal microbiota in early childhood (**II, III, IV**)
4. To identify intestinal microbiota characterisitcs and specific bacterial groups associated with eczema and its severity (**III, IV**).

4 MATERIALS AND METHODS

4.1 Subjects and study design

4.1.1 Subject characteristics

Baseline characteristics of study subjects (studies **II-IV**) are summarized in Table 7.

Table 7. Summary of baseline characteristics of subjects from studies **II-IV**.

	Study **II**		Study **III**	Study **IV**
	Children	Mothers		
Number of subjects	10	10	19	39
Diagnosed atopic disease	0	10 (100 %)	15 (44 %)	28 (72 %)
Fecal samples analyzed	1, 3, 6, 12, 24 and 36 months of age	3rd trimester of pregnancy, 1 and 36 mo postpartum	6 and 18 months of age	All infants 6 months of age. Additional sample at 9 months of age from subjects with more severe disease
Birth weeks of gestation	39.3 (1.5)	NA	39.2 (1.3)	39.3 (1.5)
Caesarean section	0	NA	0	5 (13 %)
Male gender	5 (50 %)	NA	16 (47 %)	20 (51 %)
Birth weight, g	3589 (339)	NA	3638 (416)	3530 (439)
Has older sibling(s)	5 (50 %)	NA	12 (35 %)	23 (59 %)
Probiotic group	0	0	15 (44 %)	19 (49 %)

Values are mean (SD) or n (%). NA = not applicable

4.1.2 DNA extraction protocols for high-throughput analyses

Samples from four infants from study **IV** were used to evaluate the effect of DNA extraction procedures on high-throughput analyses (**I**).

4.1.3 Microbiota characteristics of mother and her infant (II)

The study population for study **II** comprised 10 healthy mother-infant pairs from a prospective allergy prevention study at Turku University Central Hospital, Finland (Identifier: NCT00167700, section 3) (Grönlund et al. 2011). This trial involved families where mother had clinical symptoms of atopy with positive skin-prick test(s) (total n=80). All infants were vaginally delivered after full-term pregnancy (≥ 37 weeks), they had normal birth weight (≥ 2500 g) and were partially or exclusively breast-fed at least until the age of 6 months. Additional inclusion criterion for the study **II** was the availability of fecal samples taken at the ages of 1, 3, 6, 12, 24 and 36 months. In addition, the availability of fecal samples from mothers taken at third trimester of pregnancy (two months before expected delivery) and 1 month and 36 months postpartum were used as inclusion criterion. All subjects in study **II** belonged to placebo group and the mothers received Pro Natal matrix without prebiotics or probiotics two months before and two months after delivery.

4.1.4 Microbiota diversity and composition in healthy infants and infants with mild eczema (III)

Subjects of study **III** represent a sub-population from a prospective follow-up trial at Turku University Central Hospital, Finland (Identifier: NCT00167700, section 1) (Kalliomäki et al. 2001b). Mothers participating in the follow-up trial were randomized to receive capsules containing either placebo or 1 x 10^{10} colony-forming units of *Lactobacillus rhamnosus* GG (ATCC 53103) daily for 2-4 weeks before expected delivery. The intervention continued 6 months postnatally. The capsule contents were consumed by mothers during the exclusive breastfeeding, otherwise infants received the agents. The occurrence of atopic eczema was diagnosed by the age of 2 years by typical skin lesions found in children and chronic relapsing course. This last criterion was fulfilled if the child had had eczema for 1 month or longer at the 24-month study visit and on at least one previous visit (at ages 3, 6, 12, 18 months). Further inclusion criterion for study **III** were vaginal delivery after full-term pregnancy (≥ 37 weeks), normal birth weight (≥ 2500 g) and the availability of fecal samples taken at the ages of 6 and/or 18 months. In addition, all infants were exclusively or partially breast-fed for at least four months. Based on these criteria, 15 children with eczema and 19 healthy

controls (Figure 8) from the original study population (n= 132) (Kalliomäki et al. 2001b) were included in study **III**.

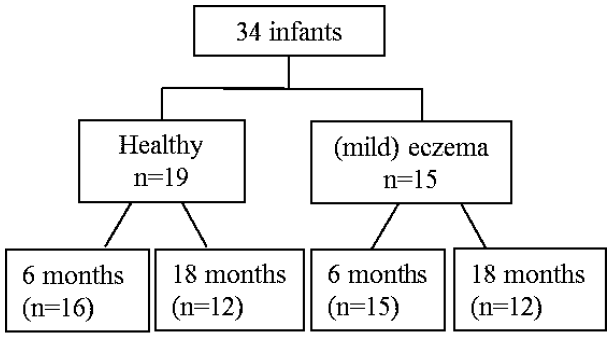

Figure 8. Study **III** design

4.1.5 Microbiota signatures and severity of eczema (IV)

Subjects for this study were selected from two prospective follow-up trials at Turku University Central Hospital, Finland (Identifiers: NCT00167700, section 1 and NCT01148667) (Kalliomäki et al. 2001b; Nermes et al. 2011). Infants with moderate/severe atopic eczema represent a sub-population from a probiotic intervention study where infants were randomized in a double-blind design to receive extensively hydrolyzed casein formula supplemented with or without *Lactobacillus rhamnosus* GG (ATCC 53103). The duration of intervention was 3 months and fecal samples were collected at the study entry and at the end of the intervention. The extent and severity of atopic eczema was assessed using the SCORAD index (Kunz et al. 1997; Nermes et al. 2011). The severity of atopic eczema was classified in three categories: mild (SCORAD <15), moderate (15<SCORAD >40) and severe eczema (SCORAD >40) (Kunz et al. 1997). Five infants were born by caesarean section and 33 were delivered vaginally. All infants were born full-term and were at least partially breast-fed before the study entry. In addition, they all received solid food(s) at the time of fecal sample collection. Infants with other diseases except atopic eczema were excluded. Additional inclusion criterion for the present study was the availability of two fecal samples taken at baseline and after the intervention. Based on these criteria, 16 children from the original study population (n= 37) (Nermes et al. 2011) were included (Figure 9). Healthy infants (n=11), infants with mild atopic eczema (n=10) and two infants with moderate atopic eczema are a sub-population from another follow-up trial (Kalliomäki et al. 2001b). The selection criteria and other characteristics of these children were as described in study **III**.

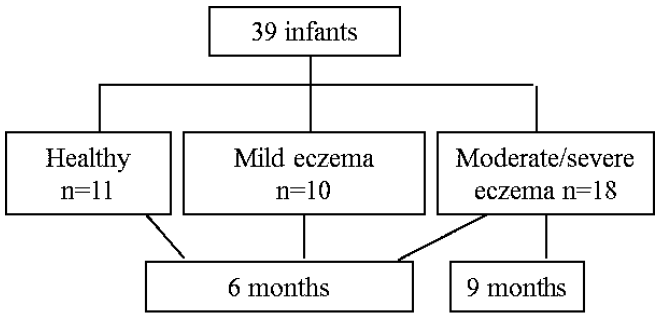

Figure 9. Study **IV** design

4.2 Methods

4.2.1 Fecal samples (I-IV)

The fecal samples were taken for the analysis from mothers two months before delivery and 36 months postpartum (**II**) and from children at the age of 1 (**II**), 3 (**II**), 6 (**I**, **II, III, IV**), 9 (**IV**), 12 (**II**), 18 (**III**), 24 (**II**) and 36 (**II**) months. The samples were aliquoted and frozen immediately after collection and stored in -70 °C until further processing.

4.2.2 DNA extraction (I-IV)

The DNA extraction procedures are presented in Figure 10.

4.2.2.1 Pre-treatment of fecal samples by repeated beat-beating (I- IV).

Fecal samples were melted on ice and 0.25 g (0.18 – 0.29 g) of feces was weighted into a 2 ml screw-cap tube and mixed with 1 ml of lysis buffer, four glass-beads (3.0 mm, Lenz Laborglas GmbH & Co., Germany) and 0.5 g of zirconia beads (0.1 mm, Biospec Products, Inc., USA) and homogenized with two rounds of bead-beating with FastPrep-24 (FP120-230, Bio 101 ThermoSavant, Holbrook, NY). Treatment time was 3 min in both bead-beating rounds. Lysate fraction obtained from the first homogenization step was removed before performing the second round of bead-beating. The lysis buffers used in the RBB-step are described below in section of the corresponding extraction protocol. After RBB-step, the subsequent DNA purification was done by using protocol(s) described below.

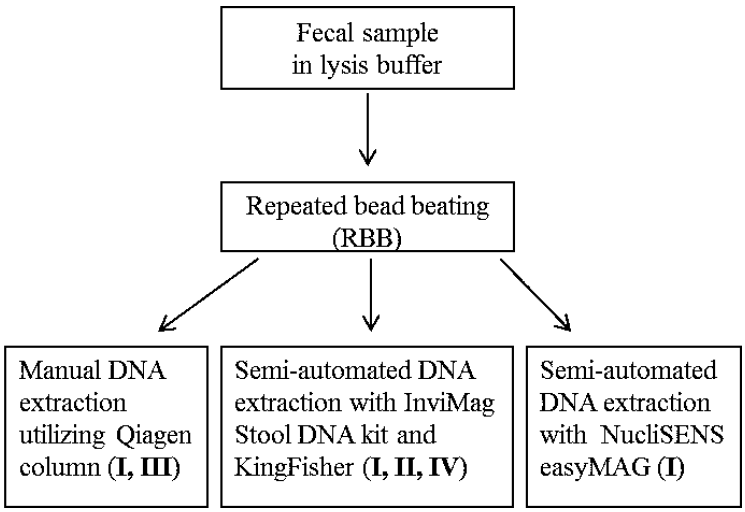

Figure 10. DNA extraction protocols used in the studies.

4.2.2.2 Manual DNA extraction utilizing the Qiagen column (I, III).

The lysis buffer used in the RBB-step was 4% (w/v) sodium dodecyl sulphate (SDS), 50 mM Tris-HCl, 500 mM NaCl and 50 mM EDTA. The RBB-step was followed by DNA precipitation with ammonium acetate and isopropanol. DNA was further purified by RNase A and proteinase K treatments and by using the purification columns and buffers from the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

4.2.2.3 Semi-automated DNA extraction by using KingFisher (I, II, IV)

The commercially available InviMag® Stool DNA kit (Invitek GmbH, Berlin, Germany) was used for the extraction of DNA in the KingFisher magnetic particle processor (Thermo Electron, Vantaa, Finland). KingFisher makes use of magnetic beads as a solid carrier of nucleic acids (or other substances) and performs magnetic bead-based protocols in an automated fashion in a 96-well plate format.

The repeated bead beating -step was performed with lysis buffer P of the kit and after the bead-beating the samples were centrifuged at +4 °C for 5 min at full speed and the supernatant was collected. DNA was extracted by following the kit manufacturer's instructions for bacterial DNA extraction involving proteinase K treatment and subsequent purification by using the KingFisher device. The KingFisher protocol included binding of nucleic acids on magnetic beads, five washing steps and elution.

4.2.2.4 Semi-automated DNA extraction by using NucliSENS easyMAG (I)

In the RBB-step NucliSENS easyMAG Lysis Buffer (BioMérieux, Marcy l'Etoile, France) was used. Following the bead-beating, the samples were centrifuged at +4 °C for 5 min at full speed and the supernatant was collected. DNA was extracted by using the NucliSENS easyMAG bio-robot with the standard reagents and protocols provided by the manufacturer (BioMérieux, Marcy l'Etoile, France). The easyMAG system performs nucleic acid purification with silica magnetic beads, which mediate purification of nucleic acids by binding them and allowing other substances to be washed away.

4.2.3 Measurement of DNA concentration (I-IV)

DNA yield was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE). The DNA extractions were stored at -20 °C until use.

4.2.4 16S rRNA gene microarray analysis (I-IV)

The composition of total microbiota was assessed by using the phylogenetic Human Intestinal Tract chip (HITChip) (Rajilić-Stojanović et al. 2009), manufactured by Agilent Technologies. The array contains duplicate-spotted 3699 unique oligonucleotide probes targeting the V1 and V6 hypervariable regions of the 16S rRNA gene and covering over 1100 intestinal bacterial phylotypes.

For analysis by the HITChip (Figure 11), the entire 16S rRNA encoding gene was amplified from fecal DNA using the primers that enabled the incorporation of T7 promoter sequence (Rajilić-Stojanović et al. 2009). PCR product was transcribed to RNA and labelled with Cy3 or Cy5. Labelled RNA was fragmented and two samples, each carrying a different dye, were hybridized to the microarrays. The arrays were washed and scanned and data were extracted and normalized as described below. All samples were performed in at least two independent hybridizations until satisfactory reproducibility was achieved (>96%) as assessed by Pearson's product moment correlation.

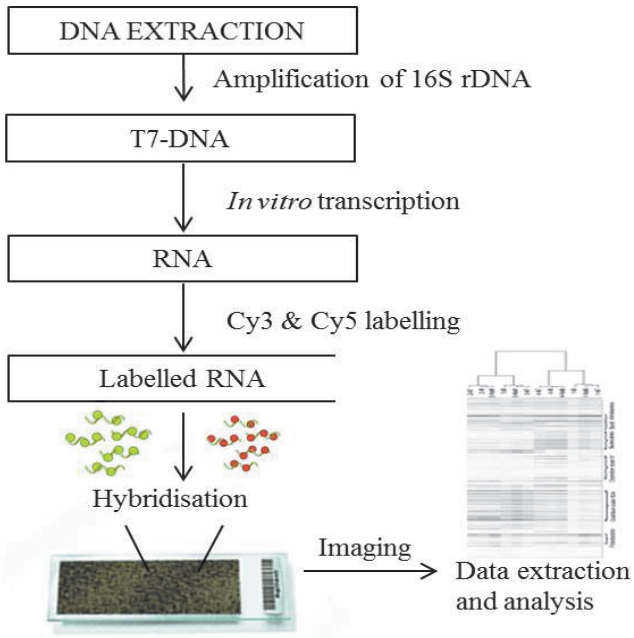

Figure 11. The Human Intestinal Tract Chip (HITChip) work flow

4.2.5 Microarray data analysis (I-IV)

Data was extracted from microarray images by using the Agilent Feature Extraction software (www.agilent.com). Data normalization and the further microarray analysis were performed using a set of R-based scripts (www.r-project.org/) (**I-IV**) (Salojärvi et al. 2014) in combination with a custom designed relational database running under the MySQL database management system (www.mysql.com) (**I, III**) (Rajilić-Stojanović et al. 2009).

In the HITChip data, the cut-off values for positive responding probes were calculated as described before (Rajilić-Stojanović et al. 2009). The HITChip allows the analysis at three phylogenetic levels: phylum-like level (level 1), genus-like level (level 2) and phylotype level (species-like, level 3). The hybridization profiles of each sample provide identification and a relative quantification of the phylotypes present in a sample. The analyzed values were summary values on phylum-like and genus-like level, obtained by summing the intensities from all the probes assigned to the respective phylum-like or genus-like phylogenetic groups. Microbiota composition is expressed as relative abundances, which refers to how common a phyla or genus-like group is relative to other groups in intestinal community (**II-IV**).

The diversity of the microbial community assessed by the HITChip (**I-IV**) was expressed as Simpson's reciprocal index of diversity (1/D) by using the probe-level data. This index was calculated using the equation D = ∑ Pi^2 where Pi is the proportion of i^{th} taxon. The proportion of each taxon was calculated as a proportion of each probe signal compared to the total signal (Rajilić-Stojanović et al. 2009). Simpson's reciprocal index of diversity takes into account the number of microbiota members as well as their abundance. A higher index value indicates a more diverse microbial community.

4.2.6 Quantitative PCR (III)

Accuracy of HITChip analysis on phylotype level was shown in study III by using strategic quantitative PCRs (qPCR) to analyze the composition of bifidobacterial community in fecal samples by using genus and species-specific primers. All qPCR reactions were carried out in an Applied Biosystems 7300 Fast Real-Time PCR System in a 96-well format and by using SYBR Green chemistry (SYBR Green PCR Master Mix, Applied Biosystems, USA). The PCR reactions and thermocycling conditions were as reported earlier (Gueimonde et al. 2004; Rinne et al. 2005). The fluorescence products were detected in the last step of each cycle and a melting curve analysis was made after amplification. The bacterial concentration in each sample was calculated by comparing the Ct values to those obtained from standard curves. A standard curve was made from serial dilutions of DNA isolated from each pure culture of the different reference strains. Samples were analyzed in duplicate in at least two independent runs. The primers, their specificities and reference strains have been published previously (Nylund et al. 2013).

4.2.7 Statistical analyses.

Statistical analyses of both HITChip and qPCR data were carried out with log-transformed data. In qPCR data, non-detected values were imputed with the half of the theoretical detection limit (**II**). In microarray analysis, genus-like level data was used in correlation analysis (**IV**) and in the RDA (**II, III, IV**) and PCA (**II**) clustering. In principal component analysis (PCA), any variable that best explains the variance in the data set is searched, followed by the transformation of data in a way that the greatest variance comes to lie on the first axis. Relative abundances are presented as medians with interquartile ranges (**II, III**) or as mean with standard deviation values (**I-IV**). The overall statistical differences between the groups were determined by using Student's t-test or One-way ANOVA, and subsequent contrast tests (**I, III, IV**). Microbiota development was assessed using ANOVA for repeated measurements (**II**) Hierarchical cluster analysis, computed with Pearson

correlation distance metric was used to compare the microbiota profiles obtained with the HITChip (**I**). Correlations between diversity indexes, bacterial groups and SCORAD values were calculated using Pearson's coefficient (r) (**IV**).

Redundancy analysis (RDA) was performed by using the multivariate statistical analysis software Canoco version 4.5 (**III**) or 5 (**II, IV**) for Windows (Microcomputer Power, Ithaca, USA). RDA plot shows bacterial groups principally contributing to the difference between the groups of subjects. The Monte Carlo Permutation Procedure from Canoco program was used to assess the statistical significance of the variation in large datasets. Statistical analyses were performed by using IBM SPSS Statistics versions 18 (**I**) and 21 (**II, IV**) and by using R (www.r-project.org/) (**III**) and a P value < 0.05 was considered statistically significant.

4.2.8 Ethical aspects

The study protocols of original interventions were approved by the Ethics Committee of the Hospital District of South-Western Finland and subjects were enrolled in the study after written informed consent was obtained from the parents. Clinical trials are registered at ClinicalTrials.gov (Identification codes: NCT00167700 (**II, III, IV)** and NCT01148667 (**IV**)).

5 RESULTS

5.1 Improvement of DNA extraction protocols for high-throughput analyses (I)

5.1.1 The yield and purity of nucleic acids obtained with different extraction methods.

The yield of total nucleic acids (both DNA and RNA) obtained with the manual method utilizing the Qiagen kit was ten-fold lower than that derived from the semi-automated methods. The KingFisher with InviMag® Stool DNA kit (KF) -method provided nucleic acids with best purity as assessed by the ratio of optical density (OD) at 260 nm to OD at 280 nm. The NucliSENS easyMAG (NeM) -method yielded in less pure nucleic acids as compared to the other two methods most likely because it does not involve a proteinase step. All three extraction methods generated DNA that was sufficiently pure for subsequent molecular analyses.

5.1.2 Analysis of microbiota diversity and composition by phylogenetic microarray profiling.

All three extraction methods gave comparable microbiota profiles as assessed by the microbiota composition analysis based on the HITChip hybridization signals. The average hybridization signals at the phylum (level 1) and genus-like levels (level 2) were comparable with all methods used. In addition, the diversity indices of analyzed samples varied from 54 to 88 and the indices were comparable between the profiles obtained by different extraction methods.

5.2 Postpartum changes in microbiota composition of mothers (II)

Maternal microbiota composition showed only minor changes when samples taken during the third trimester of pregnancy (T3) were compared to those obtained 1 and 36 months postpartum ($p=0.38$). At the phylum-level comparison, a significantly decreased Bacteroidetes abundance was observed at late pregnancy when compared to 36 months postpartum sample (10.93 % ± 11.26 % vs 23.12 % ± 15.68 %, respectively, $p=0.048$). In addition, there was a tendency towards a decreased microbiota diversity in late pregnancy when compared to 36 months postpartum samples (132.2 ± 53.7 and 168.55 ± 32.5, respectively, $p=0.07$).

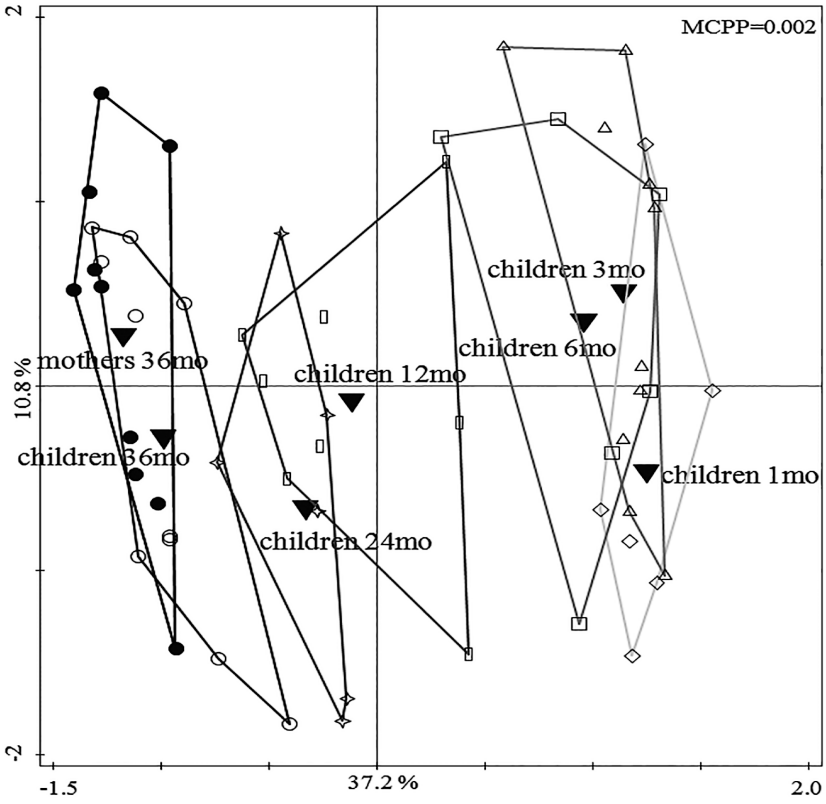

Figure 12. Principal component analysis plot showing the microbiota development towards an adult-type pattern from 1 month to 36 months of age.

5.3 Temporal microbiota development in early childhood (II, III, IV) and the effect of maternal microbiota (II)

During the first 3 years of life, the microbiota community gradually develops towards an adult-type pattern (Figure 12). During the breast-feeding period, the microbiota composition is extremely infant-specific and similarity to an adult-type community structure is low (Pearson correlation <0.16). The microbiota community of breast-fed infant is simple and dominated by a few bacterial phyla, mainly Actinobacteria, Proteobacteria and Bacilli, each constituting approximately 30 % of total microbiota in 1 month old infants. During breast-feeding, the microbiota diversity and composition are relatively stable, while major diversification of microbiota community starts during weaning process, when the microbiota develops rapidly towards a toddler-specific composition. This transitional period is characterized by increasing Bacteroidetes and *Clostridium* clusters and decreasing Actinobacteria and

Proteobacteria. Although the total microbiota composition is close to that of an adult at the age of 3 years (Figure 12), the diversity is still significantly lower than in mothers (112.5 ± 40.0 and 168.6 ± 32.5, respectively (p=0.003)). At 3 years of age the total microbiota similarity between mother and her child is approximately 0.73, being significantly higher than the microbiota similarity of child and non-relative mother's (Pearson correlations 0.73 ± 0.06 and 0.66 ± 0.09, p=0.02) (Figure 13).

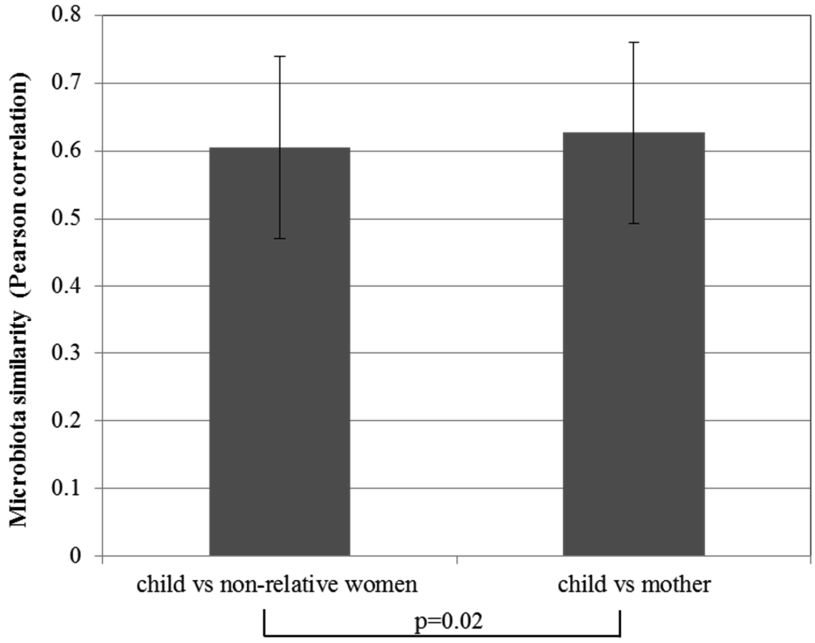

Figure 13. Similarity of the total microbiota profiles between 3 years old children and their mothers at the same time point. Similarity was assessed by Pearson correlations between the HITChip microbiota profiles.

However, each phylum develops towards an adult-like pattern in an individualistic way and with discordant timing (Figure 14). Bacteroidetes, Bacilli and *Clostridium* cluster XIVa have relatively high similarity to mother's microbiota already at 1 year of age, whereas Actinobacteria, Proteobacteria and *Clostridium* cluster IV increase in similarity much later after 2 years of age.

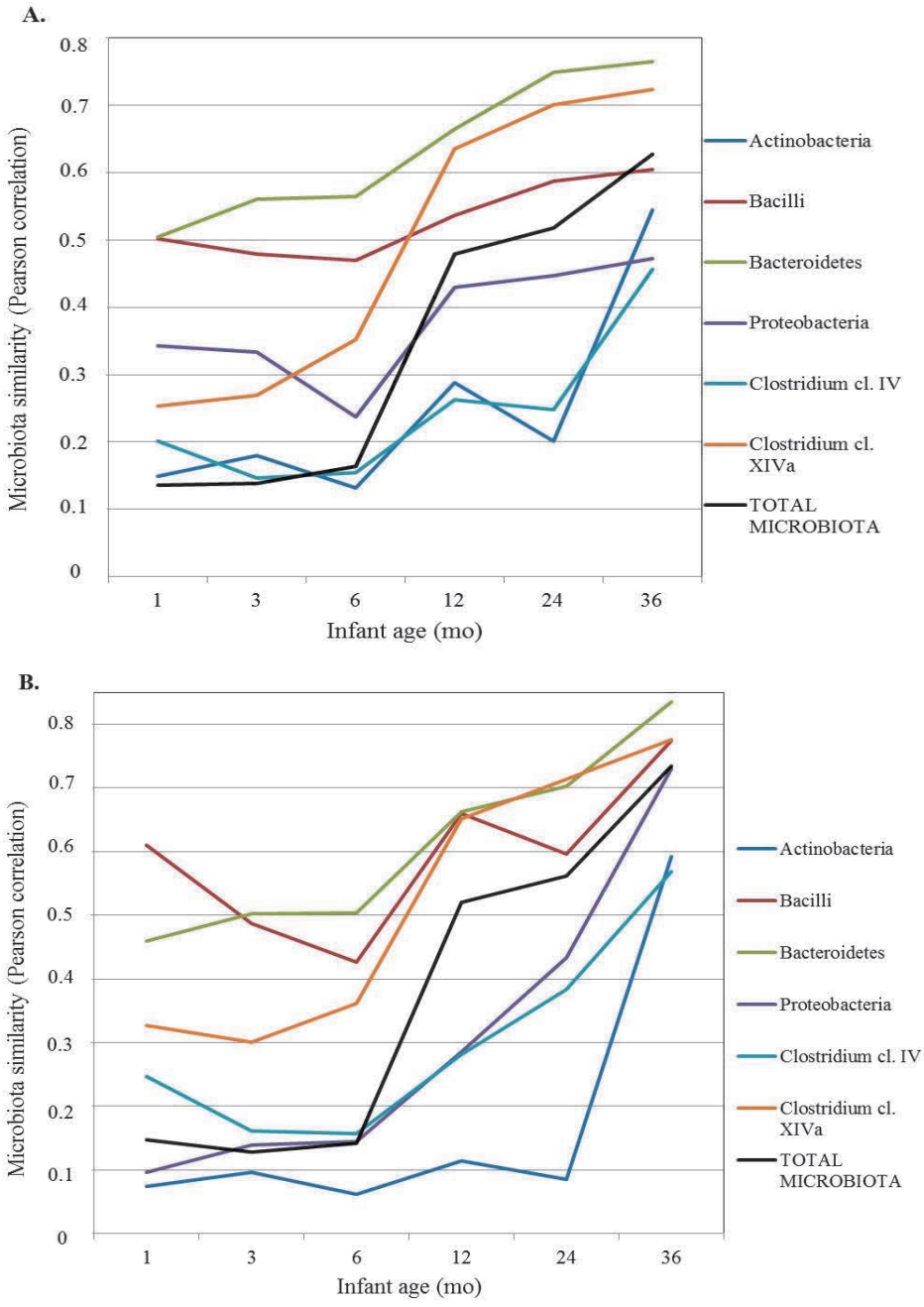

Figure 14. The similarities of infants´ phylum-level microbiota to mothers´ third trimester (A) and 3 years postpartum (B) samples.

5.4 Microbiota signatures associated with eczema and its severity (III, IV)

At the age of 6 months, both microbiota composition (MCPP, p=0.35) and diversity (p=0.2) were comparable in children with mild eczema and healthy controls (**III, IV**). In contrast, infants with more severe eczema had significantly decreased microbiota diversity when compared to healthy controls (1.5-fold difference, p=0.03) and to infants with mild eczema (2-fold difference, p=0.001). Furthermore, the severity of eczema correlated inversely with microbiota diversity (r=-0.54, p=0.002) and with the abundance of butyrate-producing bacteria (r=-0.52, p=0.005). Infants with mild eczema (SCORAD <15) had 1.6-fold higher diversity index values (p=0.002) and two-fold higher abundance of butyrate-producing bacteria when compared to the infants with more severe eczema (SCORAD 15—45.5) (p=0.03). In addition, the severity of eczema was the most discriminating factor in the clustering of infants based on the microbiota profiles as assessed by RDA analysis (MCPP, p=0.03, Figure 15).

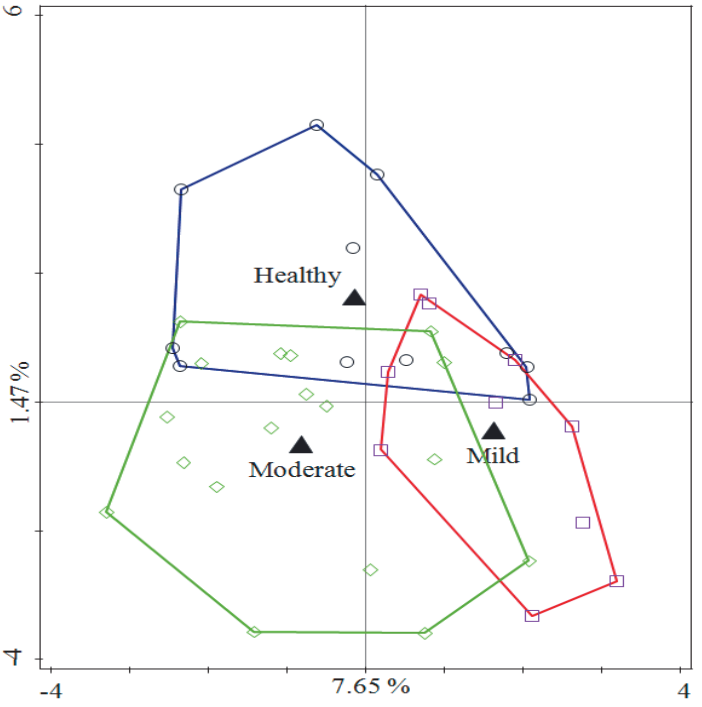

Figure 15. The clustering of microbiota profiles by eczema severity at the age of 6 months as assessed by redundancy analysis (RDA).

At 18 months, microbiota profiles were compared between infants with mild eczema and healthy controls. Significantly distinct microbiota profiles were observed between the health groups as assessed by MCPP (p=0.01) and a total of 9.1 % of the variation within the dataset could be related to the health status of the infants. Infants with mild eczema had a significantly more diverse total microbiota when compared to healthy controls (p=0.03). The most remarkable difference at phylum-level composition was observed in the Bacteroidetes, which were 3-fold more abundant in healthy children (p= 0.01). The effect of Bacteroidetes is also clearly shown in the RDA plot (Figure 16). In contrast to the Bacteroidetes, specific bacterial groups from the most abundant groups of the Firmicutes phylum, *Clostridium* clusters IV and XIVa were significantly more abundant in children with mild eczema (**III**).

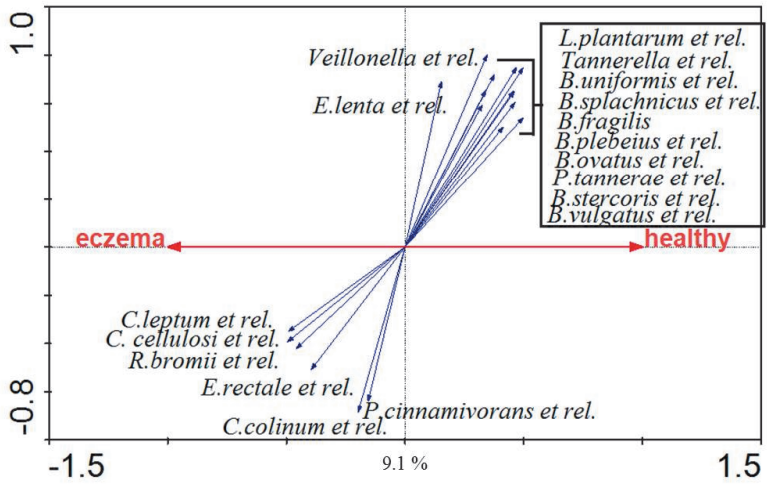

Figure 16. RDA plot of the microbiota composition of healthy infants and infants with (mild) eczema at 18 months of age.

5.5 Changes in microbiota signatures related to the improvement of eczema symptoms (IV)

The alleviation of eczema symptoms and microbiota changes within the more severe eczema group (SCORAD >15, n=15) was analyzed. These infants participated in the probiotic intervention study where they were randomized in a double-blind design to receive extensively hydrolyzed casein formula supplemented with or without *Lactobacillus rhamnosus* GG (ATCC 53103). Microbiota composition showed significant development within the study period as assessed by RDA analysis (MCPP, P= 0.002), but there were no differences in the microbiota composition between the intervention groups (RDA and MCPP, p=0.54). During the intervention, SCORAD values decreased ($p<0.001$) and microbiota diversity increased ($P<0.001$) in all infants. The decrease in SCORAD values correlated with the increase in bacteria related to *Coprococcus eutactus* (r= -0.59, p=0.02), the predominating butyrate-producing bacteria (Figure 17).

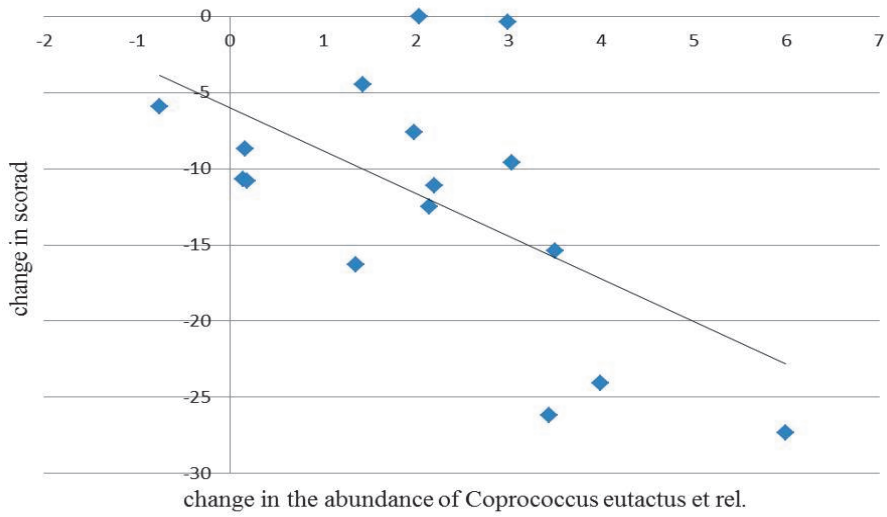

Figure 17. The decrease in SCORAD values correlates with the increase in bacteria related to the butyrate-producer *Coprococcus eutactus*.

5.6 Influence of probiotic supplementation on microbiota composition (III, IV)

When comparing the levels of HITChip signals between children from the placebo group and those who or whose mothers had received *L. rhamnosus* GG supplementation, no statistically significant differences in microbiota diversity or composition were observed at the age of 6 months (**III, IV**). However, the supplementation with *L. rhamnosus* GG showed effects on three genus-like (level 2) bacterial groups at the age of 18 months i.e. a year after the cessation of the probiotic supplementation. The children that had received *L. rhamnosus* GG had higher levels of the butyrate-producing groups related to *Anaerostipes caccae*(LGG 2.89 ± 2.13 % and placebo 1.18 ± 0.91 % of the total microbiota, p=0.03) and *Eubacterium ventriosum*(LGG 0.17 ± 0.11 % and placebo 0.11 ± 0.07 of the total microbiota, p=0.04) than those of placebo group. Moreover, the placebo group children had higher levels of bacteria related to *Clostridium difficile* at 18 months of age as compared to the probiotic group children (1.19 ± 0.85 % and 0.78 ± 0.60 %, respectively, p=0.047) (**III**).

6 DISCUSSION

6.1 Importance of DNA extraction in molecular based microbiota analysis

While advances in molecular microbiological methods allow a high-throughput analysis of the microbiota, the laborious manual nucleic acid extraction from fecal and other clinical samples remains a bottleneck in clinical trials with large number of samples. In addition, when selecting the protocol(s) to be used it should be noted that even small differences in experimental techniques at different steps of molecular based microbiota analysis may affect the structure of obtained community and lead to contradictory results from seemingly similar studies (Tyler et al. 2014).

A combination of mechanical and chemical disruption is typically used to lyse the microbial cells prior to DNA purification and analysis by using molecular techniques. The relative efficiency of disrupting different organisms is highly dependent on the cell wall structure of the microbial cell and the selection of a specific technique (Maukonen et al. 2012; Rosewarne et al. 2011; Salonen et al. 2010; Tyler et al. 2014; Zoetendal et al. 2001). Together with previous observations, our results show that the most critical step in successful microbial DNA extraction from fecal samples is the mechanical cell lysis by bead-beating, which enables the detection of highest bacterial numbers and full coverage of microbial diversity (Study **I**, de Boer et al. 2010; Maukonen et al. 2012; Salonen et al. 2010). However, a too rigorous lysis protocol will shear the DNA of easily disrupted Gram-negative bacterial cells while being too mild for the hard-to-lyse Gram-positive bacteria. In repeated bead-beating, the physical damage of DNA is prevented by employing two successive rounds of bead-beating. After the first round of mechanical lysis the lysate is drawn off before performing the second beating step in order to lyse the remaining intact cells (Salonen et al. 2010).

The protocol used to purify the obtained nucleic acids after mechanical cell lysis has only a minor effect on the yield and microbial community composition as assessed by high-throughput phylogenetic microarray (**I**). We showed that the automated purification methods are efficient for microbial DNA extraction from fecal samples after the mechanical disruption of cells. In addition, the size of the extracted DNA was shown to be compatible with high-throughput analysis systems such as next-generation sequencing and phylogenetic microarrays. Importantly, the semi-automated extraction methods evaluated in study **I** could be performed in half of time required for the reference manual protocol and the hands-free time allows the next set of

samples to be pre-treated by bead-beating for DNA extraction, thereby significantly speeding up the DNA extraction from large numbers of samples.

6.2 Maternal microbiota during and after pregnancy

By analysing the fecal samples taken at 3rd trimester of pregnancy and early lactation (1 month postpartum), we detected only minor differences in the maternal microbiota composition , which is in line with the previous results (Jost et al. 2014a; Koren et al. 2012). The intestinal microbiota of the mothers-to-be are reported to shift over the course of pregnancy, being characterized by a decreased diversity and compositional changes occurring in late pregnancy (Collado et al. 2008; Koren et al. 2012). These changes in intestinal microbiota community coincide with the appearance of bacteria in milk ducts and both of these processes seem to be driven by hormonal signaling (Fernandez et al. 2013). However, we observed the increase in relative abundance of Bacteroidetes and slightly increased diversity at 3 years after delivery (**II**). These results show, for the first time long-term perinatal microbiota changes, which may reflect the microbiota recovery from the physiological demands of the pregnancy and lactation.

6.3 Microbiota development and the effect of maternal microbiota

During and after the birth process, microbes from the mother and surrounding environment colonize the gastrointestinal tract of the infant. By 1 month, the rapid initial acquisition of microbes after birth is thought to be over and to have developed into a breast milk adapted composition that will remain relatively stable during the exclusive breastfeeding (Hesla et al. 2014; Jost et al. 2014b; Roger et al. 2010a). All the infants analyzed in this series of studies were breast-fed at least until the age of 4 months and indeed, no major changes were detected in the microbiota community structure until the introduction of solid foods (**II**). Our results showed that the microbiota compositions of breast-fed infants are characterized by low diversity and the dominance of a few bacterial groups. This infant-specificity was highlighted by the extremely low similarity to maternal microbiota (Pearson correlation <0.16) during the first 6 months of life (**II**).

In a longitudinal analysis of 10 mother-child pairs we showed that at 3 years of age, the child´s microbiota is more similar to his/her own mother's microbiota than to the non-relative women's microbiota (**II**). This may indicate that the mother continues to share her microbiota with the child even after birth.

It has been suggested that during the breast-feeding, human milk acts as an intermediary between the maternal intestinal microbiota and the infant (Newburg et al. 2014). In addition, microbial components and metabolites such as short chain fatty acids are transported across the placenta and secreted into breast milk, providing an additional point of interaction between maternal microbiota and infant immune system (Thorburn et al. 2014). Thus, the breast-milk may represent a post-natally extended bond between mother and her infant, providing one mechanism by which microbial communities tend to cluster in family members and are more similar in twins (Maynard et al. 2012; Tims et al. 2013).

Although the mother serves as the main source of bacteria in early life due to the constant and intimate contact with her infant during delivery and lactation, the lack of long-term maintenance of maternal microbes in the infant gut has been suggested (Vaishampayan et al. 2010). Recently, the direct impact of family members in general on the microbial colonization process have been highlighted (Song et al. 2013; Yatsunenko et al. 2012). In study II, this became evident after the cessation of exclusive breast-feeding, when an infant comes into contact with novel sources of microbes and to nutrients other than those present in breast milk (**II**). Introduction of solid foods leads to a rapid compositional development, when microbiota is prone to the influence of external factors such as microbial diversity in surrounding environment (Hanski et al. 2012), dietary preferences and other lifestyle factors (Dominguez-Bello et al. 2010; Song et al. 2013). Interestingly, in a recent study, the microbiota composition of teenagers was found to be as similar to their father's as to their mother's microbiota, although the number of studied subjects was relatively low (Yatsunenko et al. 2012). In addition, the skin (Song et al. 2013) and fecal (Yatsunenko et al. 2012) microbiota of cohabiting couples were more similar to each other's than to adults from different families (Song et al. 2013), further highlighting the influence of shared household on microbiota composition. This is supported by our finding that microbiota of 3 years old children was more similar to own mother's microbiota than to microbiotas of nonrelative women (**II**).

Even though the major diversification of microbiota takes place during the weaning period, its development continues even after the transition to consume full adult-type diet. Despite the highly significant increase of microbiota diversity with age, the HITChip diversity indexes at 3 years of age were observed to be relatively low (~110) when compared to the indexes (150-200) detected in healthy adults (**II**). Previously, it has been suggested that by the age of 2 to 3 years the microbiota resembles that of an adult (Koenig et al. 2011; Louis et al. 2009; Palmer et al. 2007). However, our results obtained by a deep and longitudinal analysis indicate that the microbiota maturation continues at

least until the age of 3 years and most likely even further, because the bacterial diversity has still not reached the diversity of an adult person. This is supported by recent cross-sectional studies where significantly lower diversity indexes at 4 years old children (Ringel-Kulka et al. 2013) and differences in the microbiota composition of adolescent children aged 11-18 years (Agans et al. 2011) were reported when compared to healthy adults. Considering the major physiological changes occurring within childhood and adolescence it may be speculated that the microbiota development is not finished until the human host reaches adulthood.

The microbiota development is a gradual process, where some phyla reach the level of maturation earlier than the others (**II**). Notably, Bacteroidetes was found to be more similar to mother's microbiota than the microbiota in general in all time points analyzed in study **II**. Together with previous studies, our finding suggests that anaerobic species may become dominant early in the development process (Jost et al. 2014b; Ringel-Kulka 2013.)

To conclude, although the maternal inoculum forms the basis of microbiota composition, other factors involved in the microbiota development process such as environment, diet, medications and host related factors create a selection pressure and thus greatly affect the formation of individual –specific adult microbiota (Kerr et al. 2014).

6.4 Microbiota signatures in infants with eczema and correlation with the severity of symptoms

In newborn infant, the immune system and mucosal epithelium of the intestine are anatomically and functionally immature and the barrier function is still developing (Rouwet et al. 2002). At birth, intestinal permeability is high while declining rapidly after birth in the process called gut closure (Drozdowski et al. 2010). This maturation process is tightly regulated by growth factors, hormones, breast milk, mucus layer composition and commensal microbiota to promote infant growth and to avoid severe diseases (Drozdowski et al. 2010). Increased permeability can have beneficial effects in early life such as uptake of nutrients and development of systemic tolerance (van Elburg et al. 2003). However, in subjects developing atopic eczema this increased permeability extends beyond neonatal period and the persistent loss of epithelial integrity is suggested to be the main mechanism leading to atopic eczema (De Benedetto et al. 2011; Faura Tellez et al. 2014; Hon et al. 2013).

In study **IV**, milder symptoms of eczema were found to correlate with the higher abundance of butyrate –producing bacteria which coincided with the higher microbiota diversity. Previous studies have mainly reported associations

between reduced diversity in early life and the development of atopic eczema. However, in these studies the age at the onset of atopic symptoms has not been clarified and the severity of eczema has not been considered. If subjects with different severity of disease are analysed as a one group, it may result in biased conclusions on the associations between microbiota signatures and atopic eczema. Moreover, the majority of these studies have been conducted by molecular techniques with incomprehensive throughput capacity, thus limiting the detection of true microbiota diversity.

The total microbiota of children with mild eczema was found to become more diverse than the microbiota of healthy controls by 18 months of age (**III**). High diversity of the intestinal microbiota in childhood is considered to be beneficial, since decreased microbiota diversity has been associated with increased risk of allergic diseases. In addition, the pathogenesis of atopic diseases is associated with an impaired gut barrier function and increased intestinal permeability and gastrointestinal symptoms are common among the patients (Rosenfeldt et al. 2004). It seems that a certain level of diversity of microbiota in the early infancy is essential for modulation of the expression of genes involved in the normal pattern of intestinal development such as postnatal intestinal maturation and maintenance of mucosal barrier (Hooper et al. 2010; Sjögren et al. 2009b). This repeated exposure to a variety of bacterial antigens potentially enhances the development of immune regulation through inhibition of responses to inappropriate targets, such as gut contents and allergens (Munyaka et al. 2014). However, it has been shown that only a few bacterial species may contribute to the differences in microbiota diversity (Le Chatelier et al. 2013). Interestingly, butyrate-producing bacteria *Faecalibacterium prausnitzii* (*Clostridium* cluster IV) and *Coprococcus eutactus* (Clostridium cluster XIVa) were shown to significantly distinguish between individuals with high and low bacterial richness (Le Chatelier et al. 2013).

Butyrate is the main energy source for colonocytes and it also has numerous other functions which are crucial for the host physiology (Schilderink et al. 2013). One of the primary functions of butyrate is its ability to enhance the intestinal barrier function by promoting mucin production, by up-regulating the expression of tight-junction proteins and by inhibiting pro-inflammatory signaling pathways such as nuclear factor kappa B (NF-κB) activation (Lakhdari et al. 2011). SCFAs may also support the intestinal integrity by promoting the IgA secretion by B cells (Ishikawa and Nanjo 2009) and they contribute to the gut motility and wound healing (Tremaroli et al. 2012). Moreover, butyrate promotes the proliferation and activation of regulatory T cell (Treg) in the colon (Furusawa et al. 2013) and was shown to increase the capacity of Tregs to suppress the proliferation of effector CD4+ T cells in a

mouse model (Smith et al. 2013). This may be an important function through which the butyrate is able to alleviate the eczema symptoms, since Tregs have an important role in the early life immune programming and in shaping the immune system toward tolerant state. In a recent study by Ismail and coworkers (Ismail et al. 2014), deficient Treg responses to microbial stimuli at birth were associated with the development of eczema by the age of 1 year. Moreover, intestinal epithelial cells exposed to TLR9 ligand *Bifidobacterium breve* m16-V were shown to induce Treg and Th1 polarization which reduced the severity of eczema symptoms in infants with moderate or severe eczema (de Kivit et al. 2013).

Higher levels of bacteria related to the butyrate-producing *Anaerostipes caccae* and *Eubacterium ventriosum* were observed in 18 months old infants who or whose mothers had received the *L. rhamnosus* GG supplementation (**III**). Although the number of subjects in this study was insufficient for a more comprehensive analysis on the effects of probiotic supplementation on microbiota composition, this is result is of interest, since *Lactobacillus* and *Bifidobacterium* strains produce acetate and lactate as their carbohydrate breakdown products. Acetate and lactate then serve as substrates in metabolic cross-feeding interactions between intestinal bacteria and are subsequently metabolized to butyrate and other SCFAs (De Vuyst et al. 2011). These specific groups of bacteria may be affected by changes in daily carbohydrate intakes. For example, consumption of inulin or resistant starch increases the levels of bifidobacteria and subsequently the levels of butyrate-producing *Faecalibacterium prausnitzii* or *Ruminococcus bromii* and *Eubacterium rectale*, respectively (Ramirez-Farias et al. 2009; Walker et al. 2011). Thus, supporting the growth of butyrate-producing bacteria may have an important role in preventing and alleviating eczema symptoms in early childhood (**IV**). Further comparison between healthy children and children with mild eczema was carried out at the age of 18 months (**III**). A decreased relative abundance of bacteria belonging to Bacteroidetes was observed in children with mild eczema at 18 months of age when compared to healthy children. Previous studies have reported an association between decreased amounts *Bacteroides* spp. and the development of atopy and increased risk for atopic sensitization (Abrahamsson et al. 2012; Björksten et al. 2001; Storrø et al. 2011). *Bacteroides* spp. are specialized in the breakdown of complex plant polysaccharides (Xu et al. 2003) and their abundance has been associated with increased short-chain fatty acid concentrations in the infant gut after introduction of first solid foods (Koenig et al. 2011). Furthermore, *B. fragilis* polysaccharide has been shown to direct the cellular and physical maturation of the developing immune system in mice model (Mazmanian et al. 2005) by inducing the differentiation of Th1 lineage and thus correcting the Th1/Th2 imbalance (Mazmanian et al. 2005). In

addition, a decrease in the levels of Bacteroidetes was observed in mice deficient in production of active alpha -defensins (Salzman et al. 2010). Furthermore, decreased alpha-defensin levels and increased beta-defensin levels have been associated with increased risk of developing atopy (Savilahti et al. 2012). Altogether, the significance of *Bacteroides* spp. in the development and maintenance of healthy infant gut and balanced mucosal immunity needs to be considered in future studies.

7 SUMMARY AND FUTURE ASPECT

The microbiota development is a gradual process, which begins during early phases of life. During the succession of microbes some bacterial groups reach the degree of adult-type pattern earlier than others. The development and diversification of the intestinal microbiota is likely to continue throughout childhood and adolescence and may not be fully completed until the human host reaches adulthood. The course of microbiota succession is affected by both life-style factors and medical practices that direct the intestinal colonisation and have an impact on health later in life. These pre- and postnatal exposures to microbes, microbial structures and their metabolites are crucial for the maturation and education of immune system. Unlike our own genome, our intestinal microbiota can be modified by a variety of foods, food components, pharmaceutical treatments and other practices targeting microbiota composition, stability and activity. Due to its plastic nature and susceptibility towards external modulations, the intestinal microbiota modification potentially provides new complementary therapeutic approach to alleviate symptoms of a variety of diseases. However, the microbial species composition does not necessarily reflect the functional profile in the intestine (Arrieta 2014) and thus, possible mechanisms explaining the observed associations between differences in microbial communities and specific disease states are difficult to determine. While 16S rRNA gene analysis of the microbial communities gives a survey of the bacteria present, it does not provide any functional information. In future, the decreasing costs of metagenomic sequencing will make it a more feasible tool for microbiome analysis in clinical studies with large amounts of samples. This will allow the definition of the composition and essential functional capability of the microbiomes associated with a normal physiological condition, as well as with different disease processes. However, the host-microbiota interactions are complex and most likely individual-specific thus, it is unlikely that “one-model-fits-all” mechanistic explanations of the disease etiologies exist. The exploration of these interactions will provide new patient-specific strategies to be developed as an era of personalized medicine is emerging.

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APPENDIX: ORIGINAL PUBLICATIONS

I. Reprinted from *Journal of Microbiological Methods* 2010, 83, 231-235 with permission from Elsevier Limited.

II. Submitted manuscript.

III. Reprinted from *BMC Microbiology* 2013, 13, 12 with permission from BioMed Central.

IV. Reprinted from *Allergy* 2015, 70, 241-244 with permission from John Wiley and Sons.

ADDITIONAL PUBLICATION RELATED TO THESIS

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