GUT MICROBIOTA IN EARLY LIFE AND ITS IMPACT ON ALLERGIC DISEASES

Nicole Rutten

The young of elephants eat the feces of their mothers or other animals in the herd to obtain the bacteria required to properly digest vegetation found on their ecosystems. Probably this will also have a positive effect on their developing immune system.

Olifantenkalfjes eten de uitwerpselen van hun moeders of andere dieren in de kudde om aan de bacteriën te komen die nodig zijn voor het verteren van voedselbestandsdelen uit de omgeving. We veronderstellen dat dit tevens gunstig is voor de ontwikkeling van hun afweersysteem.

Gut microbiota in early life and its impact on allergic diseases

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De darmmicrobiota vroeg in het leven

en hun invloed op allergische aandoeningen

(met een samenvatting in het Nederlands)

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1

GENERAL INTRODUCTION

Introduction

The number of bacteria inhabiting the human body is at least ten times larger than the number of human body cells. The majority of these micro-organisms are found in the gastrointestinal tract, which comprises approximately 10^{14} bacteria.[1,2] This more than 1.5 kilogram weighing bacterial population, the gut microbiota, is referred to as a 'super organism' and a very important partner for its host. However, it is an 'acquired' organ, as intestine colonization starts right after birth and evolves as we grow. The microbiota constitutes an ecologically dynamic community that digests food ingredients and influences the development, maturation and regulation of the immune system. Moreover, the microbial ecosystem provides the host with valuable metabolic features that contribute to overall health.[3] In total, 1.000 to 1.150 bacterial species make up the gut microbiota and one individual harbors at least 160 different species.[4] Until the 1990s, knowledge of the gut microbiota was limited because conventional culture was the only technique to characterize its composition. However, it is estimated that at least 20 to 60% of the bacteria is uncultivable,[5] resulting in an underestimation of gut microbiota diversity. The introduction of culture-independent molecular and high-throughput approaches enabled phylogenetic investigations and quantification of the bacterial community, thereby refining the original perspective on composition and dynamic changes of the gut microbiota. These techniques moreover advanced our insights into the impact of host and environmental factors on bacterial community structure and dynamics.[6]

One of the most exciting scientific developments in recent years has been the understanding that the intestinal microbiota strongly has effect on human physiology, by elucidating the associations between gut microbiota, health and disease.[5,7,8] The number of publications on the human microbiota has been exponential in the last few years and continues to grow. The outburst of discoveries in the microbiome field in the course of the last five to six years has changed our perspective on human biology, both in terms of health and disease.

A co-existence arises from the beginning of life and comprises a continuously cooperation and conflict between the host and its microbiota, to maintain homeostatic balance. Factors that shape the gut microbiota in early life have not yet been satisfactorily elucidated. Moreover, the 'healthy microbiota' has not (yet) been characterized completely and knowledge on its development in healthy individuals still needs to be expanded before linking aberrant microbiota composition to disease. Modification of the intestinal microbiota may be a potential approach involved in governing this 'healthy' gut.

This thesis deals with the development of the intestinal microbiota in infancy. This includes the study of the effects of probiotics in early childhood on the composition and functionality of infant microbiota. Prior to pointing out the outline of the thesis, a brief overview of several aspects of the human microbiota will be given.

Origin and definitions

The potential association between the composition of human gut microbiota and the development of disease was proposed already by Metchnikoff in the early 20th century. He hypothesized that replacing or diminishing 'putrefactive' bacteria in the gut with lactic acid bacteria could normalize bowel health and prolong life.[9,10] Joshua Lederberg, a Nobel prize winning American molecular biologist, known for his work in microbial genetics, emphasized the importance of the intestinal bacterial population and introduced the overarching term 'microbiome', 'to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space'.[11] Nowadays the term 'microbiome' is used interchangeably with the term 'microbiota'.[12] The microbiome is the collective community of microbes that live inside and on the human body (bacteria, but also bacteriophages, fungi, protozoa and viruses), and their total genome capacity. The microbiota is the ecological community of commensal, symbiotic and pathogenic microorganisms that resides in a previously established environment.[7] This microbial community was previously termed the intestinal 'flora'. Description of the functionality of the gut microbiota can be done by investigating the collective assortment of metabolites present in a stool sample (the fecal metabolome), referred to as 'metabolomics'; by studying the total transcribed RNA, referred to as 'metatranscriptomics'; and by focusing on protein levels, called 'metaproteomics'. 'Metagenomics' (also known as environmental genomics or community genomics) is the study of the metagenome, i.e. the collective community of genomes from a particular ecosystem (in this case, the gut). When only genes are assessed, a marker gene study is performed.[5,13–15]

Classification and composition

The assumption that all life, microbes and large organisms have similar biochemistry evolution, builds on the ideas of the early evolutionists. Back in 1977, Carl Woese performed comparative studies of rRNA sequences and underpinned the basis for a universal phylogeny. He outlined a universal sequence-based tree of life, with three phylogenetic domains: *Archaea*, *Bacteria* and *Eucarya*.[16] Nowadays this three-domain model is grounded by further information on gene sequence and on biochemical correlations.[17] Over the past decades the rRNA gene sequences have been a major advancement for microbial identification. Close relatives within a population can be distinguished and the phylogenetic tree can be broken down into the taxonomic hierarchy as shown in Figure 1. When investigating changes in the microbial population, the level of assessment in the phylogenetic tree (simplified by the diagram in Figure 1) induces the degree of seen and unseen changes and level of (un)certainty.

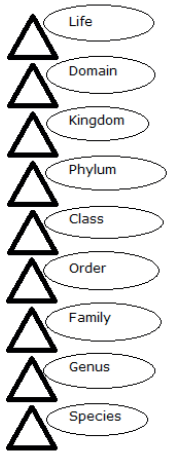

Figure 1. Diagram representing taxonomic hierarchy within the bacterial tree of life.

The adult human distal gut microbial community is typically dominated by two bacterial phyla (divisions): the *Firmicutes*, which are mainly represented by the genera *Clostridium*, *Faecalibacterium*, *Blautia*, *Ruminococcus*, and *Lactobacillus*, and the *Bacteroidetes* including *Bacteroides and Prevotella*, that constitute over 90% of the gut microbiota.[5,18] Other phyla such as *Proteobacteria (Gammaproteobacteria* with *Enterobacteriaceae)*, *Actinobacteria (Bifidobacterium)*, *Fusobacteria* and *Verrucomicrobia (Akkermansia)* are present in lower abundance but also have a significant influence on the total microbial configuration.[19]

Development of the intestinal microbiota

From birth onwards, the infant intestine increasingly becomes colonized with a wide variety of microorganisms. The classical early colonizers of the infant gut are facultative anaerobes of the phylum *Proteobacteria*, such as *E. coli* and other *Enterobacteriaceae*. These organisms pave the way for strictly anaerobic bacteria such as *Bifidobacterium*, *Clostridium*, and *Bacteroides*, and sometimes *Ruminococcus*, by depleting the initial available oxygen in a matter of days. The rapid colonization by commensal bacteria has great impact for the development of the (mucosal) immune response of the infant. It leads to a balanced development of the various components of the mucosal immune system and induces tolerance to those bacteria. The infant gastrointestinal community is characterized by low stability, limited bacterial richness and great inter-individual variation.[20] During the period prior to weaning the microbiome can display large shifts in the abundances of bacterial taxa.[21,22] Subsequently, the intestinal microbiota of the infant slowly develops and matures. The introduction of solid foods results in a shift towards bacterial groups characteristic of the adult microbiota. The microbial community is thought to reach an adult-like composition around three years of age.[7,21] Recent literature however indicates that the evolvement into adult-like microbiota may continue beyond that age.[23,24] Which bacteria should be represented (quality as well as quantity) in a 'healthy' or 'normal' intestinal microbiota has not been clearly defined yet.

Recent literature suggests a broad core microbiota in adults with high variation in species, diversity and genetic functions between individuals, though the main microbial gene functions may be maintained in almost every individual.[25–27]

Genetic and environmental factors influencing development of the intestinal microbiota

A wide range of factors, genetic and environmental, can influence the diversity and composition of the intestinal microbiota and its establishment. In the first few hours of life, the mother's vaginal and fecal bacterial populations are usually the most important source of inoculum. Moreover, skin microbiota of parents and siblings, bacteria from breast milk, and bacteria from the environment are sources of strains colonizing the gut in early life.[2,28] Mode of delivery, gestational age and feeding mode especially affect the infant's microbiota composition.[28–30] Besides, prescription of antibiotics in early life and supplementation with prebiotics or probiotics can have a direct and major effect on the development of the intestinal microbiota.[30,31]

Generally, the mode of delivery is the most influencing factor for microbiota development. *Bifidobacterium* species, known as beneficial species for the host, dominate the relatively simply composited microbiota of full-term vaginally-delivered infants within the first to second week of life. Prominent genera are also *Lactobacillus*, *Prevotella*, *Escherichia* and *Bacteroides*, including a bacterial community that represents the mother's vaginal and intestinal microbiota.[32] Caesarean-delivered infants have a reduced number of Bifidobacteria; *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* are dominating bacterial species in their microbiota which shows less resemblance to their mother's, compared to vaginally delivered infants.[33] Another significant factor that influences microbiota composition is feeding type. The microbiota of breast-fed neonates is dominated by the genera *Bifidobacterium* and *Ruminococcus*. Formula-fed neonates have a more complex composition compared with breast-fed neonates and harbor a diverse microbiota including *Enterobacteriaceae*, *Enterococcus* and *Bacteroides*, as well as *Bifidobacterium* and *Atopobium*.[29,34,35]

Evidence is increasing that exposure to antibiotics in early life, which has become common in modern obstetric and neonatal practice, is associated with profound effects on the neonatal gut microbiota composition. In general, antibiotic treatment leads to a decrease in the microbial diversity.[36] Overgrowth of *Enterococci* and arrested growth of *Bifidobacterium* in term infants exposed to antibiotics in the first week of life have been described.[31,37,38] Antibiotic exposure at the beginning of life can modify the course of bacterial infections and may be associated with development of atopic disease.[39]

Information on the role of genetic factors affecting the composition of the intestinal microbiome comes from studies of monozygotic and dizygotic twins, and studies in humans where specific microbiota profiles are associated with subjects that have mutations at specific gene loci. Results reveal that the microbiome is shared among family members, as individuals from the same family (a twin and his co-twin, or twins and their mother) were shown to have a more similar bacterial community structure than unrelated individuals. Despite a wide array of shared microbial genes among individuals, an extensive, identifiable human 'core microbiome' at the gene level could be identified and deviations from this core were associated with different physiological states.[27] An increasing number of genes/molecules (e.g. major histocompatibility complex (MHC) II genes) can be associated with specific microbial compositions and detection and monitoring of the intestinal lumen by the intestinal immune system.[40]

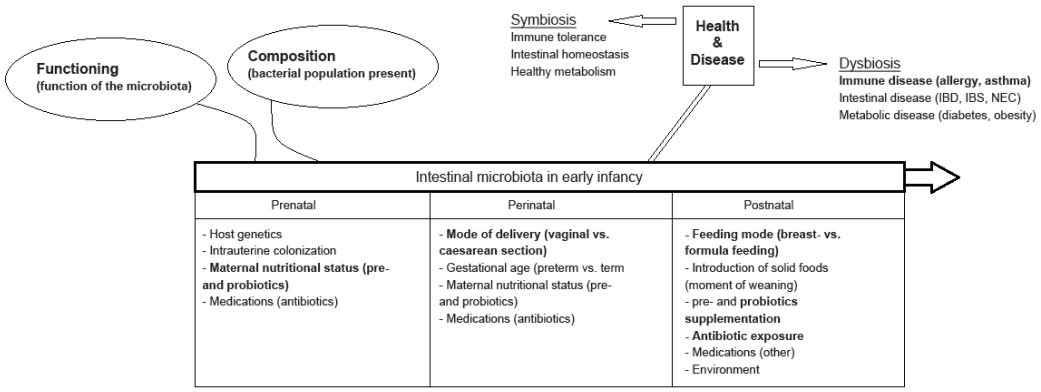

Figure 2. Factors influencing gut microbiota development in early infancy and conception of possible programming mechanisms by the intestinal microbiota.[12,41] Bold determinants are studied and/or described in this thesis.

Function of gut inhabitants and interaction with the host

Microbial colonization of the intestine is thought to play an important role in the postnatal development of the gastrointestinal, metabolic and immune systems, thereby influencing host metabolism and disease development.[12,14,41,42] Bacteria form a complex ecological community that can modulate the expression of genes involved in several important intestinal functions. The community influences normal physiology and susceptibility to disease through collective metabolic activities and interactions, including nutrient absorption, mucosal barrier strengthening, xenobiotic metabolism, angiogenesis and postnatal intestinal development. Other functions of gut bacteria are the involvement in the metabolism of otherwise indigestible carbohydrates and the production of essential metabolites such as vitamin K.[43,44]

Bacterial metabolites are suggested to mediate the communication between commensal microorganisms and the immune system by affecting the fragile balance between pro- and anti-inflammatory mechanisms. Human colonic bacteria ferment resistant starch and nonstarch polysaccharides (major components of dietary fiber) to short-chain fatty acids (SCFAs). Among the SCFAs, butyrate, that has multiple functions including anti-inflammatory properties, is important for a healthy gut physiology.[45] The essential gut immune homeostasis is preserved by regulating the differentiation and expansion of different subsets of T cells.[46] Recent data indicate that SCFAs, in particular butyrate and acetate, can directly promote regulatory T cell (Treg) differentiation in the colon of mice.[46,47] Treg cells are important for regulating and maintaining the balance of the various components and activities of the immune system, including limiting inflammatory responses in the intestine. It is difficult to correlate specific bacterial species and/or gene functions to microbial function. Molecular signals driving the process of generating anti-inflammatory Treg or pro-inflammatory T helper 17 (Th17) cells are not yet elucidated.[47]

Next to the involvement of SCFAs, evidence exists that the initial colonization influences the development of the immune system because intestinal bacteria are important in the development of gut-associated lymphoid tissues (GALT).[48] GALT enables the tissue to produce antibodies to pathogens that may enter the gut. When bacterial colonization is absent, GALT may not be developed.[48,49] Furthermore, it has been shown that bacteria play a role in the activation of toll-like receptors (TLRs) in the intestines. Through these TLRs (a class of proteins that recognize structurally conserved molecules derived from microbes), bacteria play a significant role in the regulation of the developing immune system.[48,50]

Modulation of intestinal microbiota: prebiotics and probiotics

If, as indicated above, the gut microbiota plays a crucial role in the physiological development of both the intestinal as well as immune system, disturbances in gut microbiota could be causally related to inflammatory and immune mediated diseases. The development of allergic diseases has indeed been linked to an altered gut microbiota composition, reduced microbial exposure and reduced bacterial diversity in childhood.[51–53] This hypothesis has been modified into the 'gut microbial deprivation hypothesis', with its emphasis on alterations of original gut microbiota during infancy.[54] As such, aberrancies in the microbial colonization patterns or distortion of the microbial ecology early in life might predispose the infant to diseases such as T-helper 2 (Th2) mediated diseases like allergy, wheezing and asthma, or auto-inflammatory T-helper 1 (Th1) diseases, like inflammatory bowel disease, diabetes and obesity.[41,52,55–60] In this context, the window of opportunity for influencing the composition of the intestinal microbiota and possibly modulate the development of allergic diseases, would be the neonatal period.

There is increasing evidence that that modulation of the infant microbiota can restore ecological balance.[61,62] This modulation is mainly done by supplementation of pre- and probiotics. A prebiotic is defined as 'a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health'.[63] The term 'probiotic' describes microorganisms which exert health benefits beyond basic nutrition, and probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[64] The most widely used probiotic bacteria belong to either the *Lactobacillus* or the *Bifidobacterium* genera. Various studies have investigated the potential of probiotics, both for primary prevention as well as treatment for immune-mediated diseases. Results however are inconsistent,[65–68] which may be related to the strain-specific effects of probiotic bacteria and/or considerable variations in study design, doses administered and duration of probiotic supply. One of the potential mechanisms by which probiotic bacteria regulate the development of the mucosal immune system is stimulation of the differentiation of naive T-cells towards Th1 or Treg cells, thereby shifting the balance between Th1 and Th2 cells. However, the precise biological mechanisms involved in successful clinical outcome of probiotic supplementation are still unknown. Future studies should address host and microbiota interactions and identify optimal timing and duration of pre- and/or probiotic supplementation as strategy for prevention of immune-mediated diseases.

To summarize: the human infant develops an initial microbiota as it becomes colonized with a wide variety of microorganisms directly after birth. The neonatal period comprises a critical period for its development, during which early programming occurs and the immature immune system matures. The long-term composition and function of the newborn's gut microbiome is programmed during this period, thereby laying the foundation for future health and influencing the risk of developing disease later in life.[69,70] The development of the gut microbiota continues

during the first years of life and its composition is considered to resemble the adult gastrointestinal tract by the age of 3.[24] There is increasing evidence that that modulation of the infant microbiota can restore the ecological balance of the microbiota.

Outline of the thesis

PART I: INTESTINAL MICROBIOTA IN INFANCY

For the identification of intestinal bacterial species, culture and biochemical typing were the gold standards for many years. Culture-independent (molecular) approaches however have changed considerably and subsequently refined the original perspective on composition and dynamic changes of gut-microbiota. The majority of these techniques are based on sequence analysis of the 16S rRNA gene, which is highly conserved between bacterial species, but varies in a manner that allows species identification.[5] In **Chapter 2** we investigated the dynamics of the fecal microbiota of breast-fed infants and formula-fed infants during the first 3 months of life at 16 consecutive time points. Microbial signatures of microbiota composition were analyzed by using three different molecular/ high-throughput techniques.

Since evidence is increasing that exposure to antibiotics in early life is associated with profound effects on the gut microbiome and various disorders later in life, we performed an observational cohort study to investigate the potential clinical and microbial consequences of empiric antibiotic use in early life and to deepen our understanding of this complex ecosystem and its role in human health and disease. **Chapter 3** describes the protocol of the so-called INCA-study (INtestinal microbiota Composition after Antibiotic treatment in early life). **Chapter 4** focuses on the results of this study with respect to the microbiota composition during the first 3 months of life, between infants exposed to antibiotics in early life, compared to controls. Besides the effect of antibiotic treatment on mainly species of the *Bacteroidetes* phylum, at one week of age infants could be clustered into *Bacteroidetes*-dominant or *Firmicutes-Actinobacteria-Fusobacteria-Verrucomicrobia* (FAFV) dominant microbiota, regardless of antibiotic treatment. Subsequently, this separation into two clusters, termed settler types, was further explored. **Chapter 5** discusses the microbial composition of healthy infants included in the INCA study, by exploring the settler types and relating the infant's microbiota to the composition of maternal microbiota and to the type of feeding.

PART II: EFFECTS OF USING PROBIOTICS IN EARLY CHILDHOOD

Modification of the intestinal microbiota by administration of probiotic bacteria is a potential approach to prevent allergic disease. A previously conducted randomized controlled trial in the Netherlands (PandA-study) showed that this way of primary prevention, in which children at high-risk for atopic disease received probiotic supplementation or placebo, led to significantly less children developing eczema compared to controls at the age of three months.[71] **Chapter 6** describes the long-term clinical effects of this study on perinatal administration of selected probiotic strains on the prevalence of asthma, allergic rhinitis and eczema at the age of 6 years.

In **chapter 7** the long-term effects of added probiotics on the composition and diversity of gut microbiota in the PandA-study population are discussed. Probiotics are suggested to exert their effects by influencing the activity of the resident microbiota. **Chapter 8** is a first step to assess gut metabolic activity of the microbial population as it describes the metabolomics of fecal extracts in three-month-old children included in the PandA-study. **Chapter 9** discusses screening of a panel of 19 different probiotic strains for their ability to modulate the *in vitro* differentiation of T-helper lymphocytes. This may be a first step in designing a multispecies probiotic preparation for the management of children with atopic dermatitis in the future. Not only an aberrant microbiota composition of newborns has been associated with clinical consequences later in life, also manipulation of the maternal microbiota through the use of probiotics may have subsequent consequences for the health of their offspring. In **chapter 10**, characteristics of mothers who use probiotics during pregnancy are described, as well as the effects of maternal use of probiotics on the offspring's health in an unselected population. The main findings described in this thesis will be discussed with regard to clinical consequences and future investigations in the closing **Chapter 11**, followed by a summary.

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PART I - INTESTINAL MICROBIOTA IN INFANCY

2

INTESTINAL COLONIZATION PATTERNS IN BREAST-FED AND FORMULA-FED INFANTS DURING THE FIRST 3 MONTHS OF LIFE REVEAL SEQUENTIAL MICROBIOTA SIGNATURES

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Abstract

Background The establishment of the gut microbiota during the first weeks of life in infants is a highly dynamic process dependent upon extrinsic as well as intrinsic factors.

Methods The dynamics of the fecal microbiota of four breast-fed infants and four formula-fed infants during the first 3 months of life, at 16 consecutive time points, were characterized. Microbial signatures of microbiota composition were analyzed by a combination of 454 barcoded-pyrosequencing, fluorescent in situ hybridization with microscopy (FISH-MS), and quantitative PCR (qPCR).

Results Low abundance of potential pathogens was detected by pyrosequencing and confirmed by qPCR. Individuality was the major driver of microbiota composition (P=0.002) and was significantly more pronounced in breast-fed infants than in formula-fed infants. A time-resolved developmental signature could be distinguished in the microbial colonization patterns during the first 3 months of life, characterized by sequential colonization by i) intrauterine/vaginal birth derived taxa in meconium samples, ii) skin derived taxa (including the typical early colonizers such as *Streptococcus* and Enterobacteriaceae), iii) domination of Bifidobacteriaceae, and iv) the first appearance of adult like taxa, particularly species associated with *Blautia*, Eggerthella, Ruminococcacea, and including the pathobiont *Clostridium difficile*. Incidence and dominance of skin and human breast milk derived microbes were increased in the gut microbiome of breast-fed infants compared to formula-fed infants.

Conclusion The microbiota profiling approaches reported in this study consistently indicate that microbiota development of breast- and formula-fed infants proceeds according to similar developmental stages with distinct microbial marker species.

Background

The neonatal intestine is considered to become colonized with the first microbes immediately after birth,[1] although recent studies have suggested that *in utero* the environment may not be completely sterile.[2,3] Directly after birth, the infant intestine is colonized by a succession of a variety of microorganisms. The development of the intestinal microbiota is highly variable during the first period of life due to initial low stability, limited bacterial richness and low diversity of the ecosystem that is establishing.[4,5] This dynamic process differs considerably between individuals dependent upon multiple extrinsic factors including type of nutrition, mode of delivery, use of antimicrobials, and gestational age.[6-8] With regard to development of organ systems of the infant, the colonizing microorganisms play not only a key role in driving post-natal maturation of the infant gut, but also development of the mucosal immune system.[9-13] As such, deviating microbial colonization patterns or distortion of the microbial ecology early in life, which may be caused by nutritional changes, pathogen challenges, or antibiotic use, may elicit deviating immune development processes that potentially cause long-lasting effects on the host organism, including susceptibility for a variety of developmental disorders and diseases.[14-16] A broad range of molecular and culture-independent techniques is now available for identifying and enumerating specific commensal and potential pathogenic populations present in the intestinal microbiota.[17,18] In recent years, amplicon based sequencing of the ribosomal RNA gene has enabled the analysis of hundreds of samples from different origins at high phylogenetic resolution and much greater depth than previously possible. A very recent study by Bäckhed *et al*.(2015), applying shotgun metagenomics, revealed that cessation of breast-feeding was one of the key factors driving maturation of the gut microbiome into an adult-like composition and functionally encoded capacity.[19] In this study, the studied time-frame was restricted to the first 3 months of life, but frequency of sampling was high, allowing for in depth phylogenetic reconstruction of the *de novo*-colonization of the newborn when fed a milk-based diet only. While using only a relatively small population of infants, 4 breast-fed and 4 formula-fed infants, different molecular technologies were applied (i.e. 16S profiling, FISH-MS targeting specific phylogenetic groups and qPCR targeting specific pathogens) to assess the impact of time, diet and individuality on the early-life microbiota colonization pattern, pathogen carriage and their interactions in the first 3 months of life.

Materials and methods

Subjects and study design

For this three-month longitudinal study, ten newborns were recruited, encompassing two times five infants that were exclusively breast- or formula-fed respectively. These healthy infants were all born after 37 or more weeks of gestation and all vaginally delivered at the St. Antonius Hospital Nieuwegein, the Netherlands. Each infant stayed in the hospital for a maximum of 24 hours after delivery. Signed informed consent was obtained from each infant's parents. The St. Antonius Hospital local Ethics Committee approved the study.

Formula-fed infants were allowed to receive the parents' choice of formula-feeding, and the brand was recorded. All parents decided on the same brand of formula, containing a blend of prebiotics. All children were vaccinated according to the National Vaccination Schedule and the specific dates were documented during the study. Parents were instructed to keep a diary recording key events in the categories of illness, medication and dietary change.

Fecal sample collection

Infant fecal samples were collected by the parents at specific time points during the first 3 months of life; every other day during the first two weeks of life, and after that, every week until the infant was 3 months old (17 samples in total).

Parents were provided with collection devices and diapers (Pampers, Procter & Gamble) for the complete study period. Latter approach was used to ensure minimal variation in faeces absorption by the diaper and unambiguous collection of fecal samples. After immediate collection, parents stored the fecal samples in their home freezers (at -20°C), and the samples were subsequently transferred and stored in the hospital freezers until the end of the collection period of the study. Finally, all samples were transported on dry ice to NIZO Food Research (Ede, the Netherlands) and stored at -20ºC until further processing. Each transportation step of the fecal samples was carried out in frozen state, strictly preventing intermediate thawing of the samples. Batches of 48 samples were thawed overnight on ice at 4ºC after which different aliquots were prepared for below described assays.

Pyrosequencing analysis

DNA Extraction

DNA isolation from faeces was performed as previously described.[20,21] Briefly, after bead-beating, DNA was purified by maximal 3 consecutive phenol-chloroform extractions (until a lucid solution was obtained), followed by isopropanol precipitation of the DNA. The resulting pellets were washed with 70% (v/v) ethanol, and dissolved in 100 ml Tris EDTA buffer by overnight incubation at 4 °C. DNA samples were stored at -20ºC until further processing.

Library preparation for 16S rRNA pyrosequencing

For the preparation of the amplicon pool for pyrosequencing, the following universal primers were applied for amplification of the V3-V6 region of the 16S rRNA gene: a) forward primer, 5'-CCATCTCATCCCTGCGTGTCTCCGACTAGNNNNNNACTCCTACGGGAGGCAGCAG-3' (NNNNNN designates the sample-specific six-base barcode used to tag each PCR product); b) reverse primer, 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGCRRCACGAGCTGACGAC-3'. PCR amplification mixture contained: 1 µL fecal DNA, 1 µL bar-coded forward primer, 15 µL master mix (1 µL KOD Hot Start DNA Polymerase (1 U/µL; Novagen, Madison, WI, USA), 5 µL KOD-buffer (10×), 3 µL MgSO4 (25 mM), 5 µL dNTP mix (2 mM each), 1 µL (10 µM) of reverse primer) and 33 µL sterile water (total volume 50 µL). PCR conditions were: 95°C for 2 minutes followed by 35 cycles of 95°C for 20 s, 55°C for 10 s, and 70°C for 15 s. The approximately 750 bp PCR amplicon was subsequently purified using the MSB Spin PCRapace kit (Invitek) and the concentration was checked with a Nanodrop 1000 spectrophotometer (Thermo Scientific). A composite sample for pyrosequencing was prepared by pooling 200 ng of these purified PCR products of each sample. The pooled sample was electrophoresed on a 1% agarose gel and the approximately 800 bp band was excised and extracted from the agarose gel with the MinElute Gel Extraction kit (Qiagen, Venlo, The Netherlands). The concentration of the gel-extracted amplicon was determined and 50 µl (concentration 14.5 ng/µl purified PCR product) was submitted for pyrosequencing of the V3-V4 region of the 16S rRNA gene on the 454 Life Sciences GS-FLX platform using Titanium sequencing chemistry at GATC-Biotech, Germany.

16S rRNA gene sequence analysis

Pyrosequencing data were analyzed with a workflow based on the Quantitative Insights Into Microbial Ecology (QIIME) v1.2,[22] using settings as recommended in the QIIME 1.2 tutorial, with the following exceptions: reads were filtered for chimeric sequences using Chimera Slayer,[23] and OTU clustering was performed with settings as recommended in the QIIME newsletter of December 17th 2010 (http://qiime.wordpress.com/2010/12/17/new-default-parameters-for-uclust-otu-pickers/) using an identity threshold of 97%. Diversity metrics were calculated as implemented in QIIME 1.2. To determine the degree of diversity shared between two communities (beta diversity) the UniFrac metric was employed.[24] UniFrac distances are based on the fraction of branch length shared between two communities within a phylogenetic tree constructed

from the 16S rRNA gene sequences. With unweighted UniFrac, only the presence or absence of lineages is considered (community membership). With weighted UniFrac, branch lengths are weighted based on the relative abundances of lineages within communities (community structure). Additional data handling was performed using in-house (NIZO) developed Python and Perl scripts.

FISH Microscopy

A sample was also processed and stored for analysis of microbiota using FISH.[25] Briefly, samples were diluted 1:10 (w/v) in PBS (NaCl (8 g/l), KCl (0·2 g/l), Na2HPO4·2H2O (1·44 g/l), KH2PO4 (0·24 g/l), pH 7·4) and fixed in 4% paraformaldehyde in PBS for at least 4 h. Washed cells were resuspended in PBS–ethanol solution (1:1, v/v) and stored at -80°C prior to the present analysis. Fluorescent in situ hybridization for quantification of the bacteria in these fecal samples, multiple slides with 1 cm^2 wells were prepared for cell counting. Per well, 10 microliter of diluted sample were spread. After drying, the cells were fixed to the glass surface with 96% ethanol for 10 min. In the present study hybridization was performed with an extended set of 16S rRNA-targeted probes (summarized in Supplementary Table 2). The probe set used for bacterial groups covers approximately 88% of the total number of bacteria which hybridize with the EUB338 probe in healthy volunteers (20). The probes were manufactured by Eurogentec (Seraing, Belgium) and were labelled at the 5′ end with either fluorescein isothiocyanate (FITC) or Cy3. The samples were hybridized overnight at 50°C in hybridization buffer (0·9 M-NaCl, 20mM-2-amino-2-hydroxymethylpropane- 1,3-diol (Tris)-HCl (pH 7·2), 0·1% SDS (w/v)) containing 9 ng labelled probe per slide. The slides were washed for 20 min in wash buffer (0·9 M-NaCl, 20mM-Tris-HCl (pH 7·2)), rinsed briefly in Milli-Q water and dried using compressed air. Total cells were enumerated after staining with 40,6-diamidino-2-phenylindole (DAPI). Slides were mounted in Vectashield (Vector Labs, Burlingame, CA, USA) to minimize fading of the fluorescent signal. The fluorescent cells in the samples were counted automatically with a Leicaw DMRA2 epifluorescence microscope using a modified version of the Leicaw Q-winw software (Leica, Wetzlar, Germany). The detection limit used with this method was set at 10^6 cells/g faeces.

qPCR

Real-time detection was performed on DNA samples targeting the following set of potential pathogens that were detected by pyrosequencing: *Clostridium difficile*, *Clostridium perfringens*, *Haemophilus parainfluenzae*, *Klebsiella pneumonia* and *Streptococcus pneumonia*. To compare the relative bacterial abundance between samples, total bacterial count was also determined for each sample. The target organisms, the genes amplified, the primers and probes sequences as well as PCR conditions and the relevant literature references are summarized in Supplementary Table 2. Amplification and detection were performed using the ABIPRISM 7300-PCR sequence detection system (Applied Biosystems. Foster City, CA), detecting the fluorescent products in the last step of each amplification cycle. After amplification, melting curve analysis was employed to establish PCR-product specificity in case of SYBR Green based detection. Positive and negative controls (other bacterial species) were included in each qPCR run. Gene copy numbers per gram of faeces were extrapolated for each sample, using positive-control template DNA and standard curves generated in triplicate and linear C_t-value regression in serial 10-fold template dilutions.

Statistical analysis

Clinical characteristics of the infants and potential differences between groups were assessed by Mann-Whitney-U test. All analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Principal Coordinate Analyses (PCoA), Principal Component Analysis (PCA) and Redundancy analysis (RDA) was performed using CANOCO for Windows 5.0 (Microcomputer Power, USA) according to the manufacturer's instructions[26] where the UNIFRAC distances (PCoA) and the relative abundance of the 168 identified species through 16S sequencing and the 7 phylogenetic groups targeted by FISH-MS were used as responsive variables (PCA and RDA). RDA is the canonical form of PCA analyses and is a multivariate linear regression model where several response parameters are related to the same set of environmental (explanatory) variables. Partial RDA was employed to analyze the effect of time and individual after removal of variance attributable to diet (always), individual, and time, respectively, on microbiota composition. Statistical significance was assessed by the Monte Carlo permutation procedure (MCPP) with 499 random permutations under the full model. Machine learning algorithms (Random Forest) were used to predict to what extent each taxon can predict type of feeding received.

Results

Subjects

Five breast-fed and five formula-fed infants were initially enrolled. All infants were of Caucasian origin and were born during the winter season. After sample collection was completed, two infants (one breast-fed and one formula-fed infant) were excluded a priori from further analysis because of too many missing samples and/or collection of insufficient fecal material. The clinical characteristics of the eight infants included in the analysis are shown in Supplementary Table 3. Baseline characteristics at time of recruitment were not significantly different between the two groups.

General bacterial population dynamics during the first months of life

Most children from birth onwards and irrespective of type of feeding developed a gut microbiota characterized by sequential colonization by intrauterine/vaginal birth derived first colonizers, followed by skin derived taxa and then are rapidly dominated by bifidobacteria (around 3 weeks of age) (Figure 1A). In a heat map representing all detectable taxa at the genus level, the relative abundance of other genera showed variation over a 4-5 log unit range. Among the early life colonizers were members of the *Streptococcus* genus and Proteobacteria phylum, which both decreased at later stages of the period of evaluation (Figure 1A).

Classification of the 16S sequences down to the species level revealed the presence of potential pathogens of the gastrointestinal tract, namely *C. perfringens, C. difficile*, but also of the upper orogastrointestinal and respiratory tracts, namely *H. influenzae*, *K. pneumonia* and *S. pneumoniae*. Their identity and presence was confirmed by a targeted qPCR approach (Figure 1B) and in contrast with the pyrosequencing based approach, more positive samples were detected due to lower detection limit of qPCR. All infants had at least one fecal sample with detectable levels of *S. pneumoniae* and *H. parainfluenza*, though at very low relative abundance, during the first 3 months of life. During these episodes of carriage there were no concurrent reports of upper respiratory illness by their parents. The parents of infant BF3 reported a cold on day 13, which coincided with the detection of *K. pneumoniae* from day 14-56. This microorganism was not detected in any of the other infants. High levels of *C. perfringens* were detected from day 9 onwards in BF1. Detectable levels of this microorganism during day 9-42 co-occurred with increased and decreased dominance of typical adult-like taxa belonging to the Lachnospiraceae and Bacteroidaceae families (Figure 1B), respectively. *C. difficile* was detected in the majority of infants (62,5%), and typically emerged in the second to third month of life.

In order to characterize the development of microbiota composition during early life, multivariate statistical analyses were performed to identify the impact of the individual, time and diet on the overall community development. PCA based on the relative abundance of species revealed that the afore mentioned variables accounted for 59,5% of the variation in microbiota composition observed, and microbiota signatures clustered predominantly by individual and were less

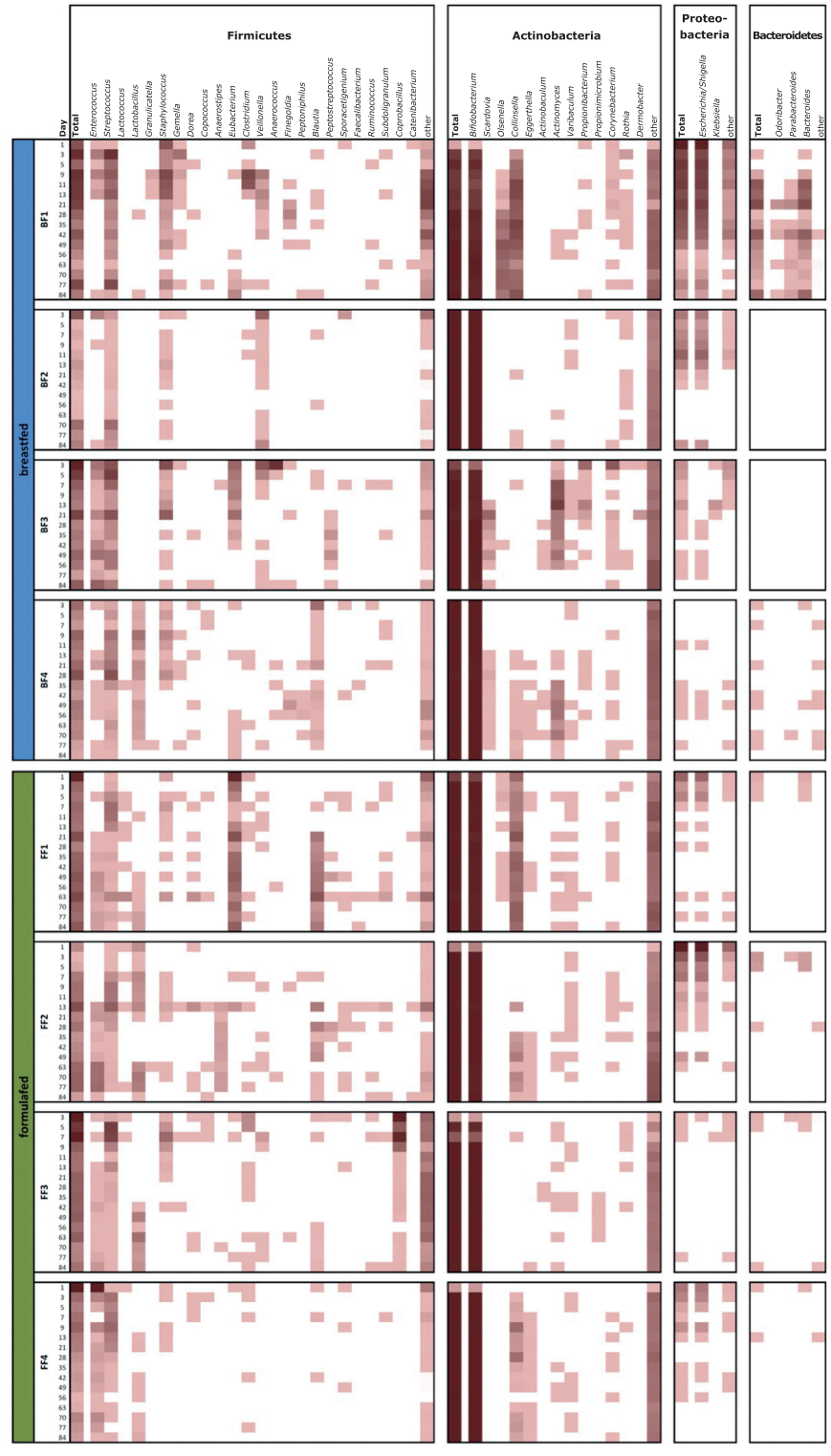


Figure 1A. Heat map representation of all detected taxa at the genus level from 1 day to 3 months of age, of 4 breast- and 4 formula fed infants. Values are represented as log10 values of the relative abundance.

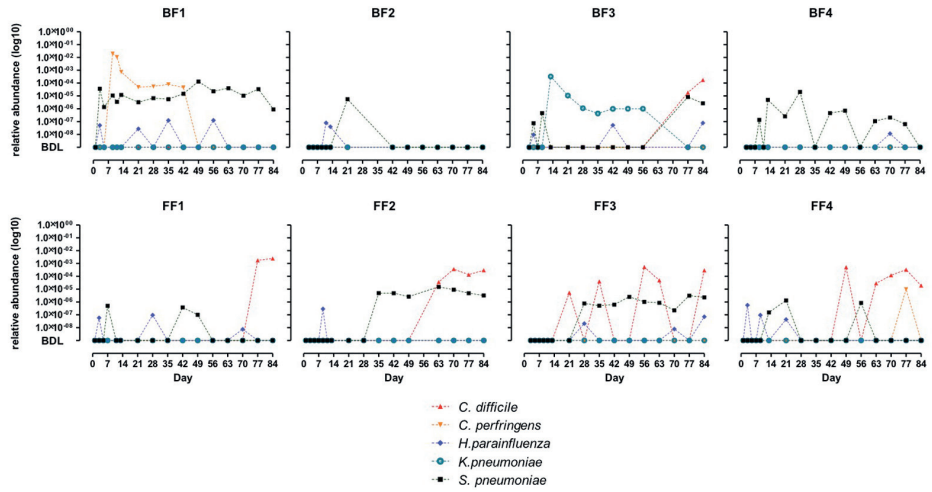


Figure 1B. Relative abundance of potential pathogens of the orogastrointestinal and respiratory tract as detected by qPCR in the faeces of breast and formula-fed infants from 1 day till 3 months.
Relative abundance was calculated as a fraction of the calculated copy numbers for total bacterial counts (16S universal primers) and targeted pathogens numbers below 100 copy number per ul of DNA was considered to be below detection threshold for all qPCR performed.

dependent on feeding type or sampling time, although the latter variables also contributed to microbiota composition clustering (Figure 2). Importantly, the centroids of the sampling time points during the first 21 days were clearly distinct from each other compared to the later sampling points that also clustered more closely together (Figure 2). The latter finding implies that the most dynamic developmental changes in gut microbiota composition take place during the first 21 days of the assessed time-frame (see also Figure 4A).

The importance of individuality as a major driver of microbiota composition was confirmed by a partial Regularized Discriminant Analysis (RDA), revealing that individuality alone, after removing the effects of feeding and time, explained 46.5% of the partial variation observed (Figure 3A). The significance of this difference was validated by the Monte Carlo permutation test (p=0.002). Microbiota composition of one of the breast-fed infants (BF1) was most distinct compared to all other samples, which was dependent on the substantially higher abundance of taxa belonging to the Bacteroidaceae, Lachnospiraceae and Enterobacteriaceae families, which might potentially reflect a dysbiotic community state related to the *C. perfringens* colonization observed in the first weeks of life. *Bifidobacterium* is an important contributing genus to the separation of the different individuals. Presence and absence of the identified members (phylotypes/species) were features that were strong drivers of the observed individuality.

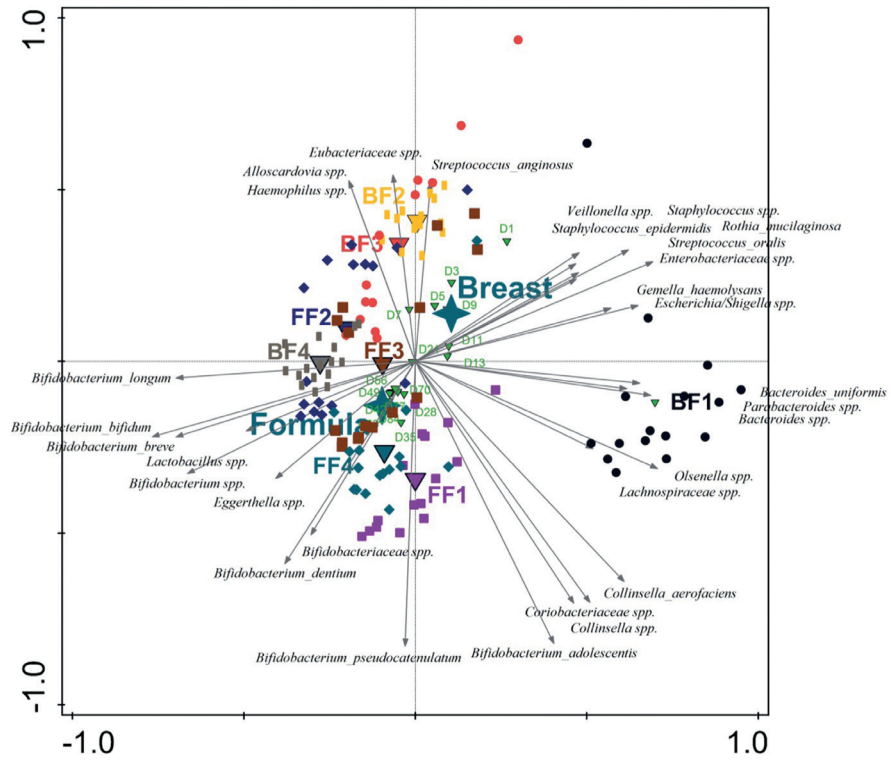


Figure 2. Principal component analysis based on species level abundance of the fecal microbiota composition of breast and formula-fed infants from day 1 to 3 months of age.
Nominal environmental variables (infant: BF1-4 and FF1-4, time point: day 1- day 84, diet: breast- and formula feeding) are indicated by down triangles and stars, respectively).

Unsupervised clustering using Principal Coordinates Analysis (PCoA) of unweighted UniFrac distance matrices revealed that individuality was more pronounced in breast-fed infants compared to formula-fed infants (Figure 3B). This higher degree of individuality in the breast-fed group was consistently detected during the first 3 months of life (Figure 3C). Notably, individuality was also the most important, and the only significant parameter explaining 28,0% of the variation in microbiota composition as assessed by FISH-MS (Supplementary Figure 1).

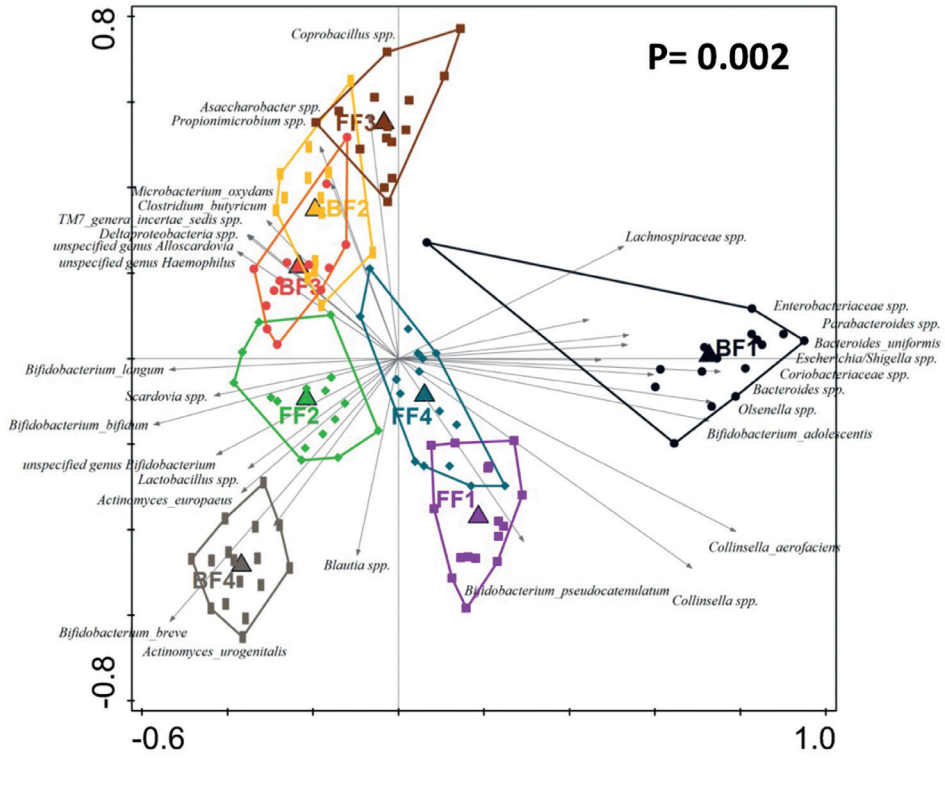


Figure 3A. Triplot of partial RDA (redundancy analyses) based on the relative abundance of detected species of the variable individual after removing the effects of time and type of feeding.

Constrained explanatory variables are indicated by triangles: BF1-4 represents infants being breast-fed and FF1-4 represents infants being formula-fed. The arrows indicate the 30 species which had the highest amount of variability in their values explained by the canonical axes. Upper right shows p-value of Monte Carlo Permutation test.

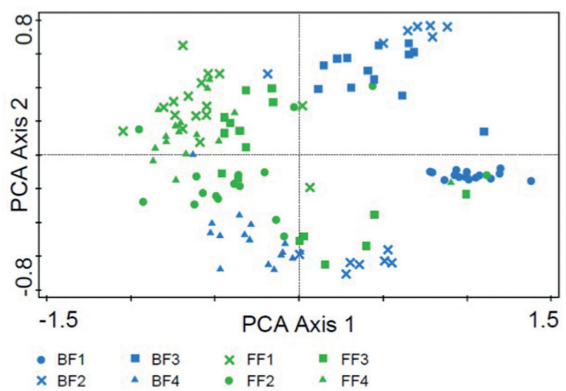


Figure 3B. PCoA of unweighted UniFrac interindividual distances of breast- (BF) and formula-fed (FF) infants.

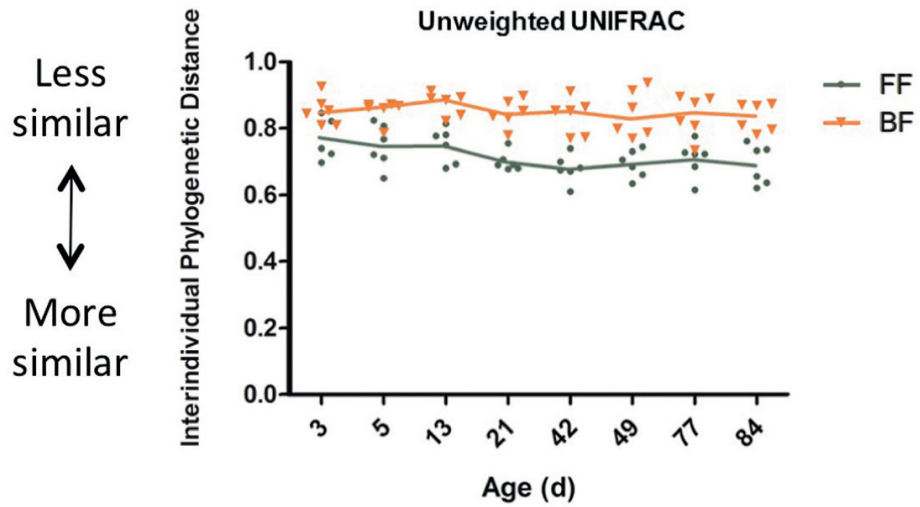


Figure 3C. Unweighted UniFrac interindividual distances of breast- (BF) and formula-fed (FF) infants from day 1 till 3 months of age. X-axis: age, in days

To identify microbiota signatures that represent significant developmental progression of the microbiota composition over time, the effects of individuality and feeding-type were removed (partial RDA) and enabled the recognition of time-resolved, early life microbiota developmental trajectories with high significance (Figure 4A; p=0.002). Within these trajectories, microbiota compositional transitions can be recognized on basis of discriminatory taxa, being intrauterine/vaginal birth derived first colonizers (day 1 to 3), such as *Lactobacillus crispatus, Corynebacterium pseudogenitalium, Sphingomonadales spp., Pelomonas, Dyella, Enterococcaceae, Ochrobactrum* and *Howardella*.[27] These first colonizers were followed by the cumulative colonization of typical early life colonizers such as Enterobacteriaceae, *Streptococcus* and skin derived taxa, like *Staphylococcus* during the period of day 3 to day 21, which in turn is followed by an increasing domination of Bifidobacteriaceae (day 21 to 35) for the remainder of the sampling period and the appearance of lowly-abundant adult like taxa (e.g. *Eggerthella, Ruminococcaceae spp. and Blautia*) at around 3 months of age. Separate analyses of breast- and formula fed infants (Figure 4 B and C, respectively) revealed that the effect of time is a more dominating factor as compared to diet, although the time-resolved developmental progression pattern was more homogeneous in the formula-fed infants (explained variation is 18.4%, p=0.002). This suggests that microbiome development of the breast-fed infants is more individually determined (explained variation is 7.0%, p= 0.02, Figure 3B). Although the time-resolved developmental signature is very comparable between breast- and formula-fed infants, several diet-associated discriminating signature taxa are characteristic for the subsequent microbiota-transitions observed. *Streptococcus* and *Enterococcus* species were discriminatory signature taxa associated with formula feeding in the first weeks and second

to third month of life, respectively. Next to these taxa, random Forest analyses revealed that among the top 20 microbial classifiers for distinguishing the feeding regime, 5 skin-associated genera (*Staphylococcus*, *Actinomyces*, *Propionibacterium*, *Corynebacterium* and *Gemella sp.*, ranked by importance) were present. Additionally, the prevalence and dominance of typical skin colonizers, that were selected on basis of previously identified skin-associated taxa,[28] was more pronounced and more persistent during the different microbiota developmental stages in breast-fed infants (Supplementary Figure 2A). Next to these skin-associated microbial groups, Random Forest analysis also identified the relative abundance of several *Bifidobacterium* species as a classifier for the discrimination of the infants' microbiota in relation to the feeding regime. In particular, a phylotype most closely related to *Bifidobacterium dentium* was identified as being present in higher relative abundance in formula-fed infants compared to those that were breast-fed (Supplementary Figure 2B).

4A

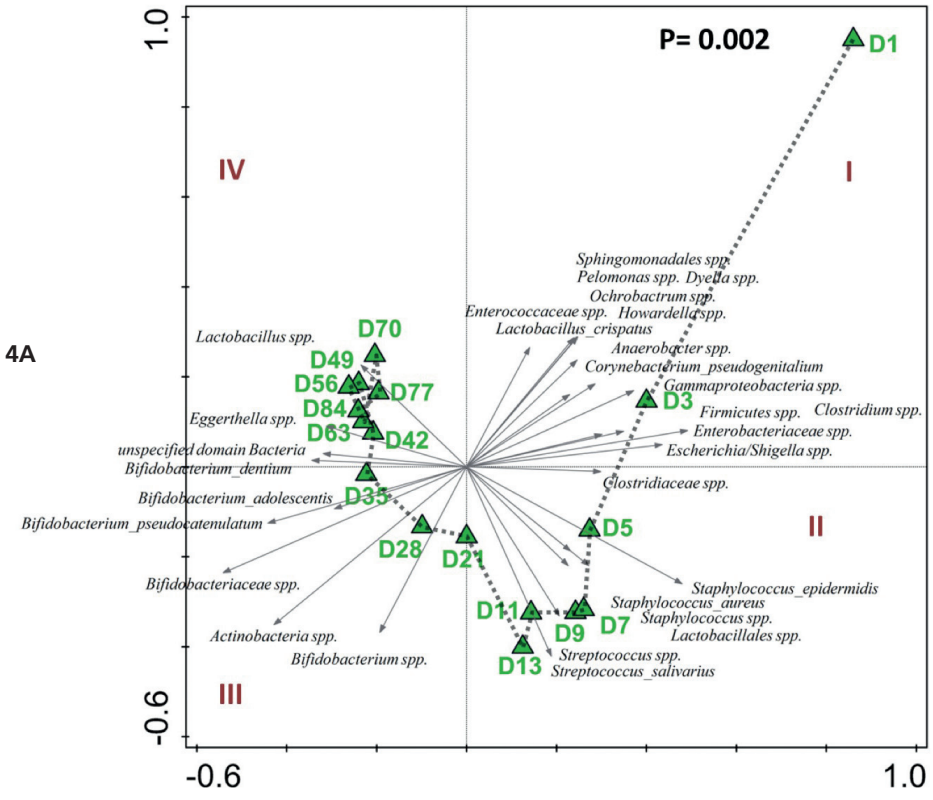
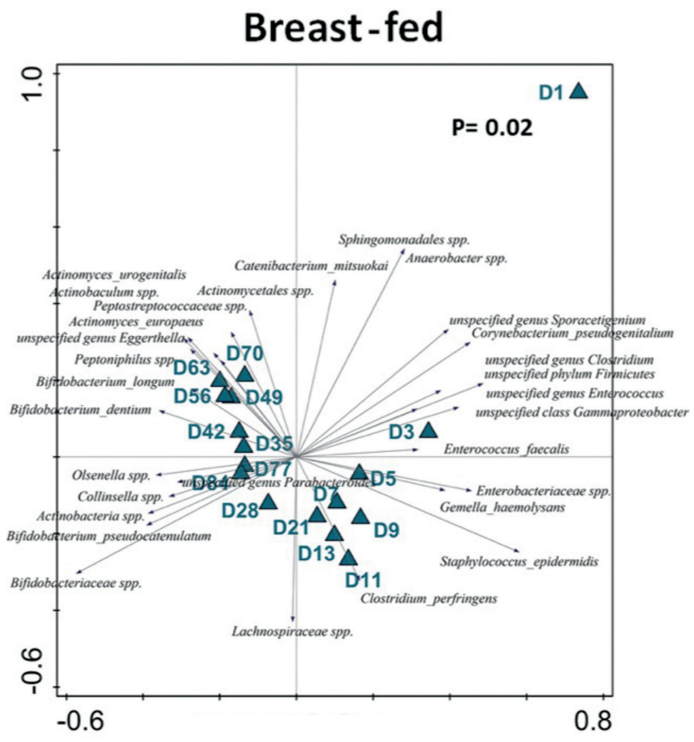


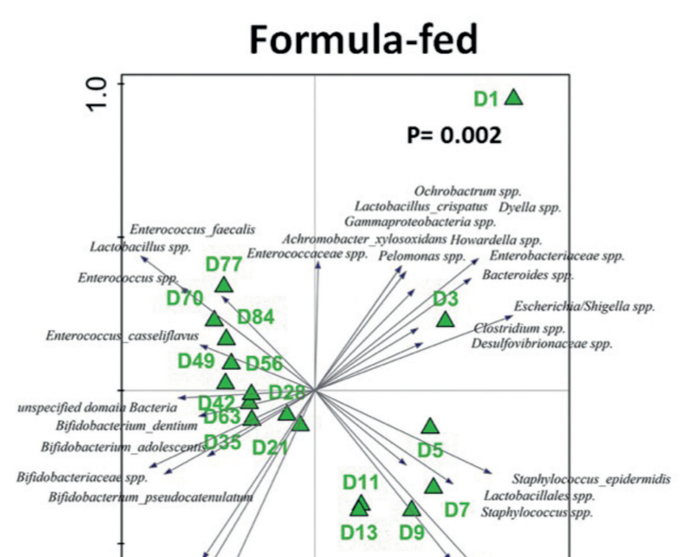
Figure 4A, B, C. Triplot of partial RDA (redundancy analyses) based on the relative abundance of detected species of the variable time (1 day until 3 months of age) after removing the effects of individual and type of feeding.

Constrained explanatory variables are indicated by triangles: day1 (D1) until day 84 (D84). Impact of time on microbiota composition was assessed in A) all breast and formula-fed infants, B) breast-fed infants only and C) formula-fed infants only. The arrows indicate the 30 species which had the highest amount of variability in their values explained by the canonical axes. Upper right shows p-value of Monte Carlo Permutation test.

4B



4C



Discussion

Infant's birth marks entry into a world densely populated by bacteria, resulting in a dynamic and complex microbial community assembly of the up to then almost sterile infant gut. Already very early aberrations of this colonization process, e.g. through antibiotics, have shown to cause long-term consequences for the developing microbiota[29,30] and ultimately deviating patterns have been associated with increased risk of developing metabolic or immunological disease.[31-33] To better harness and protect this fragile process of successional stages of bacterial communities, e.g. through nutritional approaches, a more detailed insight into the early life microbial community patterns is warranted.

In this study the dynamics of the fecal microbiota during the first 3 months of life of vaginally delivered, healthy breast-fed and formula-fed infants were investigated, using an integrative approach comprising of complementary molecular technologies, namely FISH-MS, 16S profiling and qPCR. The high number of fecal samples collected per infant (total 136, 17 samples per infant, over the first 3 months of life) allowed detailed analyses of the progression of bacterial colonization of the gastrointestinal tract in a time-resolved manner. Individuality, meaning the unique and personal microbiota development of each individual, and age-related maturation of the gut microbiome were the two most important factors explaining the variation in microbiota composition observed during the first 3 months of life. Although the initial process of colonization seems to be highly dynamic and complex, already the first fecal samples following the meconium defecation contained key taxa which were unique to the individual and resided in the ecosystem during the consecutive 3 months of the sampling period. Overall, individuality was the strongest predictor of microbiota composition, and interestingly, this unicity in strain composition appeared to be greater in breast-fed infants than in formula-fed infants, which we hypothesize to be due to the mother-specific and fluctuating composition of breast-milk that contains a highly diverse pallet of human milk oligosaccharides (HMO). HMO content has been reported to be quite variable between individual breast-milk samples and also fluctuates over time within a single breast-feeding mother,[34] which may play a determining role in the gut microbial composition and development.[35] Thereby, breast-milk can drive immuno-development effects via the direct or indirect impact of these HMO on the infant's immune system, by mediating changes in the infant's intestinal mucosal responses, or by selectively modulating the microbiota composition development, respectively.[34] In contrast to breast-milk, infant formula has a relatively simple oligosaccharide composition that is stable over time. Nowadays, different prebiotic oligosaccharides, such as galacto-oligosaccharides, fructo-oligosaccharide, polydextrose, and mixtures of these, are present in virtually all commercially available infant formula preparations. In this way, prebiotics bring infant formula one step closer to breast-milk and may have contributed to the similarity of the microbiota development signature congruency between breast- and formula-fed infants in this study. However, the supplemented prebiotics lack the reported structural diversity of HMO and mimic their effects on microbiota

development, but are not likely their functional equivalent, as is apparent from the reduced individuality detected among the formula-fed infants in this study.

Second, the present study with repetitive sampling in the first 3 months of life confirmed earlier observations that microbial succession dynamics is a non-random process.[19] Previously reported dynamics of the infant microbiota could be confirmed as the present study highlights facultative anaerobic bacteria such as Enterobacteriaceae species and members of the *Streptococcus* genus. These bacteria have been proposed to pave the way for strict anaerobes through consumption of the available oxygen.[36] In addition, in our study typical skin microorganisms were detected as a discriminatory signal in the faecal microbiome during the first two weeks of life. Previous studies have shown that infants are rapidly colonized by microbes from different environments they are exposed to, and that the mode of delivery is very determinative in this context.[7,37,38] A third developmental phase could be detected in our analyses, during which the microbiota was progressively dominated by Bifidobacteriaceae, a finding on which existing literature is conflicting. Several studies have reported that Bifidobacteria almost always dominate the intestinal community of breast-fed neonates in early life,[7,36,39,40] but paucity of bifidobacterial species by culture-independent investigations has also been described.[40] In our study-population, the last phase of intestinal colonization included the appearance of typical adult-like strict anaerobes like *Blautia*, *Eggerthella* and *Ruminococcaceae* members after two to 3 months and onwards, which could represent the starting point for the development towards an adult-like composition, of which the stabilization has been proposed to occur around 3 years of age.[5,37,41,42]

Remarkably, the sequential signatures of intestinal colonization were detected in both breast-fed and formula-fed infants in the present study population, suggesting that in the current cohort, diet was not the primary successional mechanism involved in gut microbiota maturation but other factors such as physiochemical and immunological development of the newborn are most likely more critical determinants. Even though the developmental signature between both diet groups was quite similar, there were differences between the signature taxa of the different developmental stages. For example, typical inhabitants of the human skin, like *Propionibacterium*, *Staphylococcus*, *Gemella* and *Corynebacterium* were found to be more dominant members with longer residence time in breast-fed infants, which might be a reflection of the more frequent and intense contact of breast-fed infants with the mother's skin.[43] In the present study, formula-fed infants harbored higher levels of specific bifidobacterial phylotypes than breast-fed infants, including a taxon phylogenetically closely related to *B. dentium*. The presence of the typical oral-cavity inhabitant *B. dentium* in the intestinal tract has not been reported extensively. Recently, Bäckhed *et al*. have identified with shotgun metagenomics, an approach allowing detection of variations down to the strain-level, *B. longum* to be a signature taxon for breast-feeding and *B. adolescentis* to be enriched in formula-fed infants.[19] The predominance of *B. dentium* (our study) and *B. adolescentis* at the cost of *B. longum*[19]

in the gastrointestinal tract of formula-fed infants, may be due to the differential carbon source availability in these two feeding regimes. Particularly, the relatively large fraction of the HMO pallet that is built on basis of Lacto-N-biose cannot be utilized by *B. dentium*, but is readily utilized by various other bifidobacterial species,[44] including the HMO-specialist *B. longum*.[45] Another late signature taxon for formula feeding was Enterococcaceae as detected by 16S profiling and the appearance of *C. difficile* as identified by qPCR. Although *C. difficile* was also detected in one breast-fed infant from 3 months onwards, this occurred after the introduction of supplementary feeding with formula. Higher prevalence and levels of *C. difficile* in formula-fed infants have been identified by others,[19,46] but thus far the potential protective factor contained in breastmilk remains unknown. Another explanation could be differences in microbiota composition providing colonisation resistance against *C. difficile*. Most interestingly, we found microbiota composition to be significantly altered when *C. difficile* could be detected by qPCR and four *Enterococcus* phylotypes were found to be enriched in samples with *C. difficile* carriage (Supplementary Figure 3). Intriguingly, *Enterococcus* spp. were previously also identified by others as co-occuring organisms in *C. difficile* carrying humans and infected mice.[47] Although the nature of this co-occurrence remains unknown this *de novo* acquisition of *C. difficile* and its apparent prevention through diet-microbial interactions might potentially provide novel insights aiding in the combat against this opportunistic organism.

In this study the large number of fecal samples collected per infant (17 samples per infant, during the first 3 months of life) allowed the detailed analysis of the progression of bacterial colonization of the intestinal tract in individual infants and enabled the detection of developmental microbial signatures. Importantly, the time-resolved microbial signature development is congruently detected in all infants, overrules the individuality-dependent differences in microbiota composition, and appears irrespective of the feeding regime. Nevertheless, subtle differences depending on the feeding regime could be detected and the determination of the importance of these differences and their impact on the interplay of the microbiota with the developing mucosal immune system remains to be determined. Such further knowledge of the colonization intestinal drivers and their importance for the development of appropriate mucosal immune function development, in particular tolerance development, is paramount to reduce the risk of immune-system associated diseases in later stages of life.

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Supplementary figures

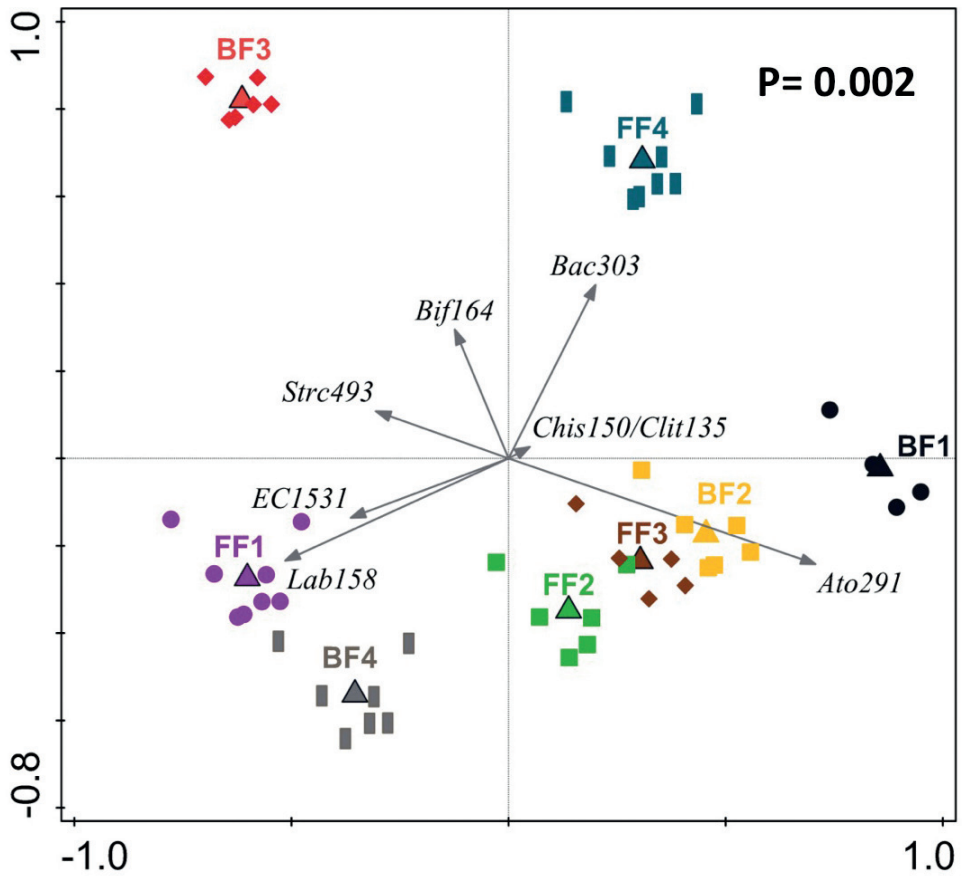

Figure S1. Triplot of partial RDA (redundancy analyses) based on the relative abundance of 8 FISH probes of the variable individual after removing the effects of time and type of feeding.

Constrained explanatory variables are indicated by triangles: BF1-4 represents infants being breast-fed and FF1-4 represents infants being formula-fed. The arrows indicate the 7 probes applied to target the different phylogenetic groups typically for the early life microbiome. Upper right shows p-value of Monte Carlo Permutation test.

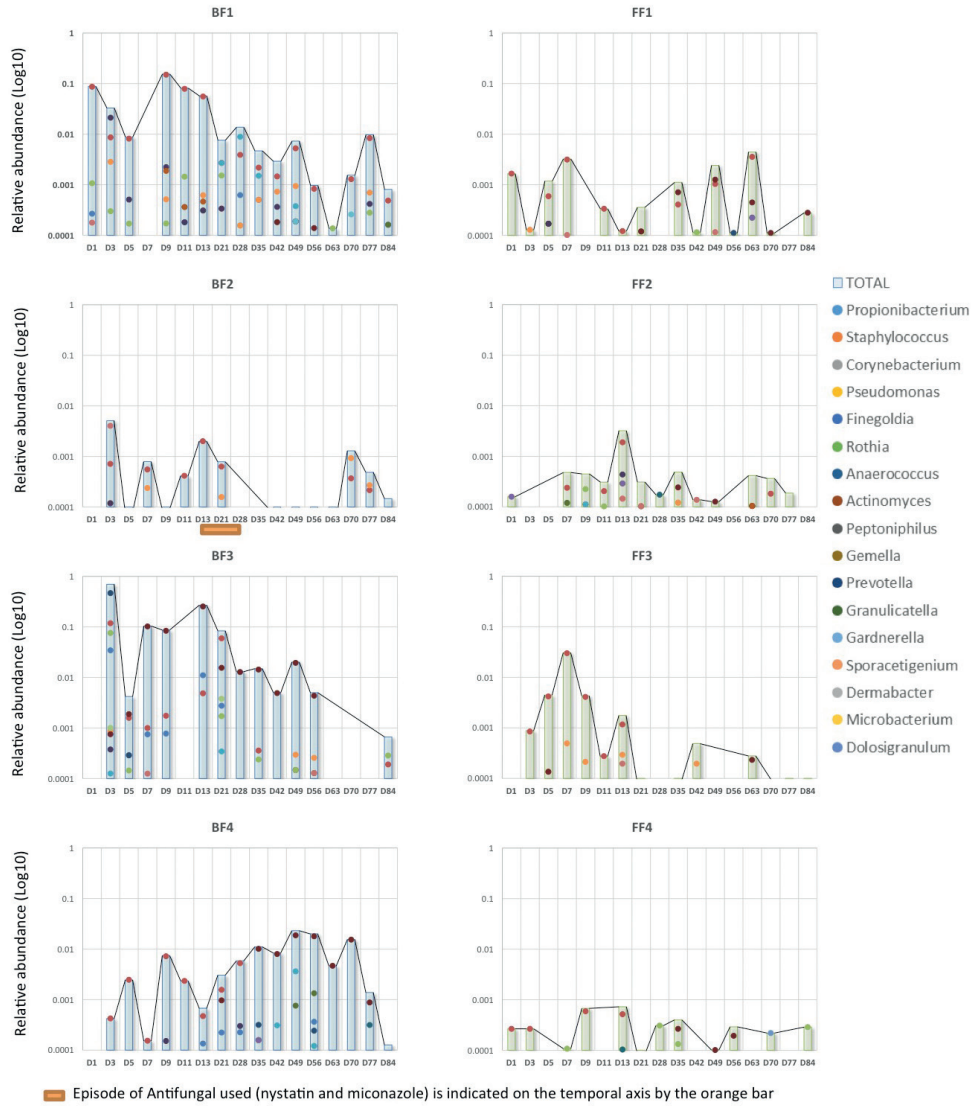

Figure S2A. Relative abundance of typical skin taxa in breast-fed and formula-fed infants from day 1 till 3 months of age onward.

Episode of antifungal used (nystatin and miconazole) is indicated on the temporal axis by the orange bar.

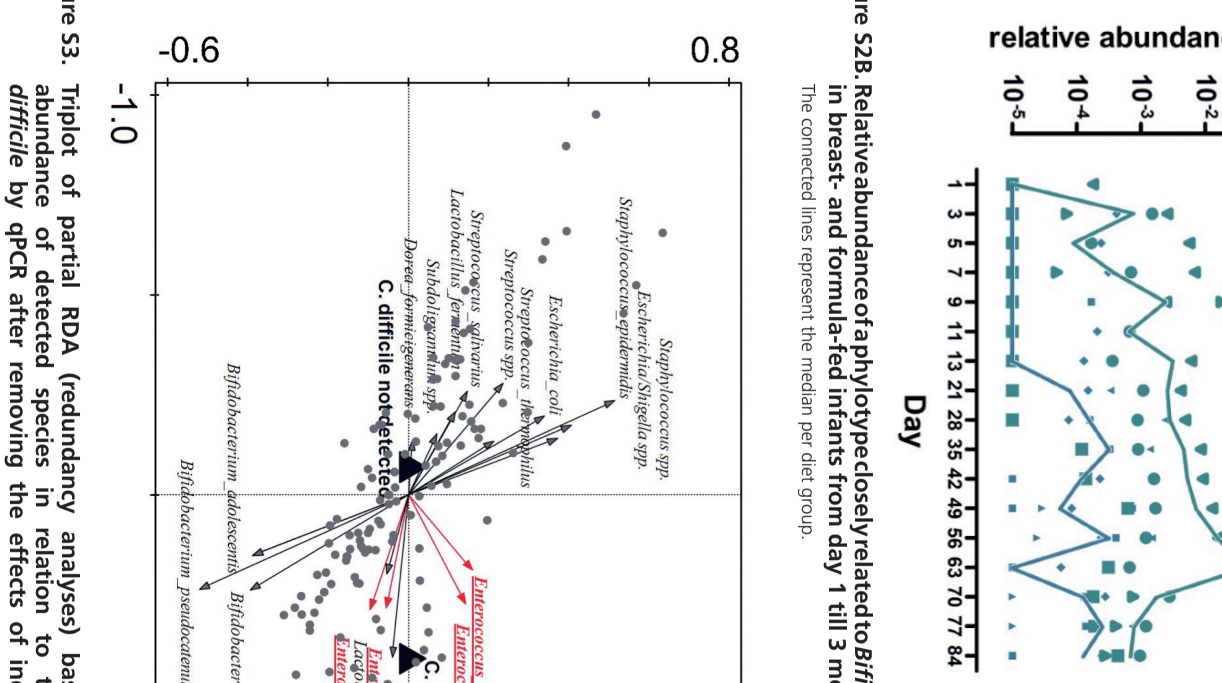

Figure S2B. Relative abundance of a phylotype closely related to *Bifidobacterium dentium* in breast- and formula-fed infants from day 1 till 3 months of age.

The connected lines represent the median per diet group.

Figure S3. Triplot of partial RDA (redundancy analyses) based on the relative abundance of detected species in relation to the detection of *C. difficile* by qPCR after removing the effects of individual.

Constrained explanatory variables are indicated by triangles: *C. difficile* detection Yes/No. The arrows indicate species which had their variation explained by the first canonical axis. Upper right shows p-value of Monte Carlo Permutation test.

Table S1. Bacterial taxa targeted by qPCR in this study

Target	Target gene (E. coli position for 16S)	Technology	Positive controls	Negative controls	References
Bacteria	16S rRNA gene	SYBR Green	*L. lactis. B. longum. B. infantis*	Fungi	(Suzuki et al 2000)
C. perfringens	16S rRNA gene (176-276)	FAM-TAMRA	*C. perfringens (DSM756)*	*C. difficile* (DSM1296)	(Wise and Siragusa 2005)
C. difficile	16S rRNA gene (57-227)	FAM-TAMRA	*C. difficile (DSM1296)*	*C. perfringens*	(Magdesian and Leutenegger 2011)
K. pneumoniae	*Phoe (outer membrane phosphate porin)*	TaqMan FAM-BHQ	*K. pneumoniae*	*E. coli.*	(Shannon et al 2007) (kit) – virulence factor
S. pneumoniae	Alpha-fucosidase	TaqMan FAM-BHQ	*S. pneumoniae*	*E. coli.*	Commercial assay– virulence factor
H. parainfluenzae	16S-23S rRNA spacer	SYBR Green	*H. parainfluenzae* (DSM8978)	*E. coli. K. pneumoniae*	(Giannino et al 2001)

Table S2. Oligonucleotide probes and hybridization conditions used in FISH analysis of fecal bacteria

Probe	Target bacterial group	Sequence (5'–3')	Hybridization conditions	References
EUB338	Total bacterial count	GCTGCCTCCCGTAGGAGT	50	(Amann et al 1990)
Ato291	*Atopobium* sp	GGTCGGTCTCTCAACCC	50	(Harmsen et al 2000a)
Bif164	*Bifidobacterium* sp.	CATCCGGCATTACCACCC	50	(Langendijk et al 1995)
Bac303	*Bacteroides/Prevotella* sp	CCAATGTGGGGGACCTT	45	(Manz et al 1996)
CLis135	*Clostridium lituseburense*	GTTATCCGTGTGTACAGGG	50	(Franks et al 1998)
CHis150	*Clostridium histolyticum*	TTATGCGGTATTAATCT(C/T)CCTTT	50	(Franks et al 1998)
Lac158	*Lactobacillus/Enterococcus* sp	GGTATTAGCA(C/T)CTGTTTCCA	50	(Harmsen et al 2000b)
Strc493	*Streptococcus/Lactococcus* sp	GTTAGCCGTCCCTTTCTGG	50	(Franks et al 1998)
Ecol1513	*Escherichia coli*	CACCGTAGTGCCTCGTCATCA	37	(Poulsen et al 1994)

Table S3. Clinical characteristics of the infants included in this study

	Breast-fed infants (n=4)*	Formula-fed infants (n=4)*
Birthweight (g) (mean ± standard deviation)	3736 ± 396	3327 ± 602
Weight at 3 months (g) (mean ± standard deviation)	6331 ± 649	5909 ± 1036
Gender (% male)	75%	50%
Duration of gestation (weeks + days)	39 weeks + 6 days	40 weeks + 2 days
Number of siblings (mean)	0.5	0.5
Use of medicines		
- **Antibiotics**	No	No
- **Antifungals**	Yes (BF2, week 2-3, treatment of sprue)	No
Illness reported by parents	BF1 : Diarrhea >2 days BF3: common cold >7 days	None

*No significant differences between the groups at the 0.05 level.

3

INTESTINAL MICROBIOTA COMPOSITION AFTER ANTIBIOTIC TREATMENT IN EARLY LIFE: THE INCA STUDY

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Abstract

Background The acquisition and development of infant gut microbiota can be influenced by numerous factors, of which early antibiotic treatment is an important one. However, studies on the effects of antibiotic treatment in early life on clinical outcomes and establishment and development of the gut microbiota of term infants are limited. Disturbed microbiota composition is hypothesized to be an underlying mechanism of an aberrant development of the immune system. This study aims to investigate the potential clinical and microbial consequences of empiric antibiotic use in early life.

Methods/Design 450 term born infants, of whom 150 are exposed to antibiotic treatment in early life and 300 are not (control group), are included in this observational cohort study with a one-year follow-up. Clinical outcomes, including coughing, wheezing, fever >38°C, runny nose, glue ear, rash, diarrhea and >3 crying hours a day, are recorded daily by parents and examined by previously defined doctor's diagnosis. A blood sample is taken at closure to investigate the infant's vaccination response and sensitization for food and inhalant allergens. Fecal samples are obtained at eight time points during the first year of life. Potential differences in microbial profiles of infants treated with antibiotics versus healthy controls will be determined by use of 16S-23S rRNA gene analysis (IS-pro). Microbiota composition will be described by means of abundance, diversity and (dis)similarity. Diversity is calculated using the Shannon index. Dissimilarities between samples are calculated as the cosine distance between each pair of samples and analyzed with principal coordinate analysis. Clinical variables and possible associations are assessed by appropriate statistics.

Discussion Both clinical quantitative and qualitative microbial effects of antibiotic treatment in early life may be demonstrated. These findings can be important, since there is evidence that manipulation of the infant microbiota by using pre- or probiotics can restore the ecological balance of the microbiota and may mitigate potential negative effects on the developing immune system, when use of antibiotics cannot be avoided.

Trial Registration ClinicalTrials.gov NCT02536560.

Background

The fetal intestine is (virtually) sterile, however, from birth onwards, the infant intestine becomes colonized with a wide variety of microorganisms.[1] The interaction between the host and its microbiota contributes fundamentally to overall health.[2,3] For example, the microbial ecosystem provides the host with valuable metabolic features, such as metabolism of otherwise indigestible carbohydrates, xenobiotic metabolism, and production of essential metabolites such as vitamin K.[4,5] Furthermore, the colonizing microorganisms play a key role in driving post-natal maturation of the infant gut and development of the mucosal immune system.[6–10] Disturbance of the microbial colonization patterns early in life can lead to long-lasting host effects and eventually disease. Aberrancies in microbial colonization patterns or distortion of the microbial ecology early in life might predispose the infant to the development of immune-mediated diseases. The gut microbiota has been associated with T-helper 2 (Th2) type diseases like allergy, wheezing and asthma, and also with T-helper 1 (Th1) type diseases, like inflammatory bowel disease, diabetes and obesity.[11–24] Also in non-immune mediated diseases, like infantile colic or irritable bowel syndrome, the fecal microbiota composition was found to be different from healthy controls.[25,26] A range of factors can influence the composition of the intestinal microbiota and its establishment, like mode of delivery, feeding mode, contact with parents, siblings, and nursing and/or hospital staff when appropriate.[27–29] Antibiotic treatment during the early postnatal period, that has become common in modern obstetric and neonatal practice,[15] is one of the important factors that can influence maturation of the infant gut microbiota and thus increase the risk of disease.[11,24] A systematic review in 2011 of longitudinal studies on the effects of infant antibiotic use showed a higher risk for subsequent development of wheezing and/or asthma.[30] Other studies have shown an association between infant antibiotic exposure and growth rate and development of adiposity.[31–33] Relatively few studies have determined the direct effects of antibiotics on the composition of gut microbiota and/or addressed the mechanisms underlying this association.[13,29,34] Initial studies were based on culture-dependent techniques, but altered intestinal microbiota in antibiotic treated infants could already be identified by these relatively limited techniques.[35,36] In the last few years, the development of culture-independent (molecular) approaches for studying the intestinal microbiota composition has changed and advanced our original perspective and insight into the impact of the composition of the microbiome.[37,38] By using molecular fingerprinting and determination of 16S rRNA genes (by quantitative polymerase chain reaction, qPCR), overgrowth of *Enterococci* and arrested growth of *Bifidobacterium* in infants exposed to antibiotics in the first week of life has been described.[34] Overall, evidence is growing that an aberrant microbiota composition as a result of antibiotic treatment can have clinical effects, but more robust studies are needed with a higher number of patients to elucidate the exact effects of antibiotics on the developing microbiota and its association with health in the first year of life. This study therefore aims to investigate the potential clinical and microbial consequences of empiric antibiotic use in early life by following a large cohort of 150 treated and 300 untreated infants and compare them with respect to their health status as well as their developing gut microbiota.

Methods/Design

Study design and population

A prospective, observational cohort study with a one-year follow-up is currently conducted to determine the clinical effects and impact on the gut microbiota composition of empiric antibiotic use in early life. Infants are recruited from the maternity wards and neonatal wards of four teaching hospitals in the Netherlands: the St. Antonius Hospital in Nieuwegein, the Tergooi Hospital in Blaricum, the Gelre Hospitals in Apeldoorn and the Meander Medical Center in Amersfoort. This study was approved by the joined Medical Ethics Committee (VCMO) of the St Antonius Hospital and Meander Medical Center (nowadays MEC-U: Medical Research Ethics Committees United (Nieuwegein)). In The Netherlands, since 2013 studies only need to be approved by one Ethics Committee. Subsequently, for other participating centers, proof of local feasibility needs to be given. The approved protocol was consequently checked for local feasibility and expertise at Tergooi Hospital and Gelre Hospitals and permission for study implementation was obtained by the respective boards of directors. The study is performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All parents of term infants (≥ 36 weeks of gestational age) who stay in the hospital for at least 24 hours, are approached for participation in the study.

In total 150 infants, treated with antibiotics because of (a high suspicion of) a perinatal infection during the first week of life, will be recruited. The control group comprises 300 healthy newborns, born in the hospital and needing clinical observation for 24-48 hours for several reasons like maternal comorbidity, low probability of neonatal infection, blood sugar monitoring, meconium containing amniotic fluid, or delivery by caesarean section. For a balanced composition of the control group, as much as possible infants that are suspected to have a neonatal infection but for whom watchful waiting is allowed (and no antibiotic treatment has to be started) will be included.

Inclusion and exclusion criteria

Inclusion criteria

1. Term-born babies (≥ 36 weeks gestational age)
2. (Short) stay on maternal ward or admission to neonatal ward because of antibiotic treatment
3. Signed informed consent by the parents

Exclusion criteria

1. Congenital illness or malformations
2. Severe perinatal infections for which transfer to the neonatal intensive care unit is needed
3. Maternal probiotic use ≤ six weeks before delivery
4. Insufficient knowledge of the Dutch language.

Infants suspected of neonatal infection will be treated with antibiotics according to the local hospital protocols. All hospitals use gentamycin in combination with either penicillin, amoxicillin or amoxicillin/clavulanic acid. During the study period, type of feeding is closely monitored, as parents monthly report the type of feeding on the calendar list (breast- or formula feeding). Parents are free in their choice of feeding regime, and after six months infants will be on solid food, but not all the infants in the study will have the exact same diet as this is neither feasible nor ethical. Mother's intake of antibiotics or drugs during pregnancy, delivery and during breastfeeding (if applicable) will be recorded. Delivery/hospital data will be extracted from patient records. All parents have to give informed consent prior to inclusion in the study. Follow up continues during the first year of life, in which parents collect and store eight fecal samples of their infant. Parents are also asked to collect a fecal sample of their child around the 2nd birthday and they give permission for a possible approach for a follow-up in about 5-6 years.

Aims

Healthy newborns born in the hospital, observed for low probability of neonatal infection will be compared to newborns exposed to antibiotic therapy in early life (first 1-2 weeks) by investigating potential differences in fecal microbiota composition. For this purpose fecal bacterial composition and diversity is determined at eight time points during the first year of life, from birth on: day one (T1), day two (T2), one week (T3), two weeks (T4), one month (T5), three months (T6), six months (T7), one year (T8). An overview of the sample time points and outcomes is shown in Figure 1. Potential differences in proportions (abundance and diversity) of intestinal bacterial groups (phyla) and species in antibiotic treated infants versus healthy controls will be determined by use of 16S-23S rRNA gene analysis. Secondly, we investigate differences in clinical outcomes between infants treated with antibiotics and controls. We evaluate incidences of atopic dermatitis (eczema), food allergy, upper respiratory tract infections (URTI), lower respiratory tract infections (LRTI), gastrointestinal infections (GITI) and excessive crying, prospectively assessed by parental reports and retrospectively assessed by doctor's diagnoses. The clinical endpoints will be linked to the developing intestinal microbiota during the first year of life. Body mass index and standard deviation (SD)-scores are calculated from individual weight and height curves.

Parents of all included infants will report the use of antibiotics (type of antibiotics and duration of treatment) during the complete study period (i.e. the first year of life) on the calendar lists. Moreover, (the type of) prescribed antibiotics will be investigated at the end of the initial study period by approaching the infant's pharmacy on medications used in the first year of life. Parents are also asked to report the moment of stool collection (date) on the collection vial, so the investigators can calculate the time between antibiotic treatment and stool sample collection. We hypothesize that in early life antibiotic treated infants develop more eczema during their first year than non-antibiotic treated infants (healthy controls). We also expect an increase in incidence of (parental reported as well as doctor's diagnosed) food allergy, respiratory tract infections (RTI's) and gastrointestinal tract infections (GITI's) in infants treated with antibiotics compared to controls.

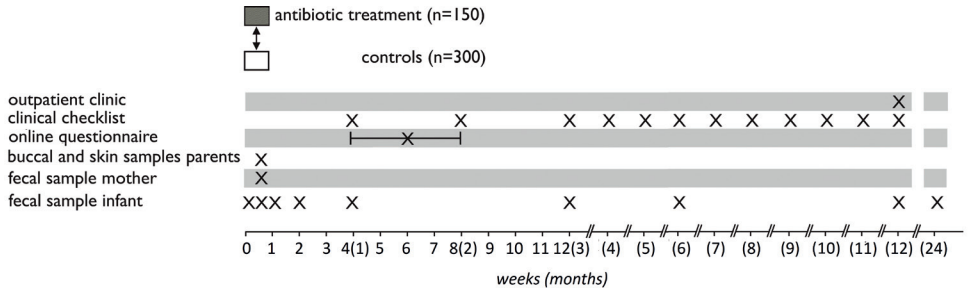

Figure 1. Overview of the sample time points and outcomes

Outcomes

Symptoms via calendar checklist

Parents are instructed to record a (for this study) designed daily checklist of clinical symptoms, which include coughing, wheezing, fever >38°C, runny nose, glue ear, rash, diarrhea, >3 hours of crying within one day (24 hours). Table 1 shows the various symptoms, which should be recorded by the parents. Descriptions standardize the way of recording as much as possible. The checklists are designed as a calendar and parents are requested to return a list (at the end of) every month. As a motivation for returning the list, we send back a contemporary Dutch magazine for young parents, monthly, after receiving their list.

At baseline, parents have to fill out an online questionnaire containing questions on demographics, comorbidity and use of medication. This questionnaire includes: gestational age and birthweight of the child, maternal comorbidity (medication during pregnancy and delivery), environmental factors (habitat, housing), parental smoking habits, siblings, pets, chronic diseases and hereditary diseases, ethnicity, education.

Regarding the daily checklists, RTI's and GITI's are defined as follows: an episode of respiratory tract infection includes at least two consecutive days of coughing and/or wheezing, runny nose and/ or earache (with or without secretion). An episode ends when the child is symptom-free for at least two consecutive days. Diarrhea is defined as increase in stool frequency to twice the usual number per day, during at least two consecutive days. Parents also report visits to the general practitioner because of respiratory or gastrointestinal symptoms of their child (since particularly respiratory infection may have a confounding effect, as antibiotic use and respiratory infections are so common and closely related in early life).[39] Parents are extensively instructed by one of the investigators, at baseline of the study, how to fill in the daily checklists and how to interpret their child's symptoms.

Symptoms via doctor's diagnosis

Primary care visits and physicians-diagnoses during the first year of life are recorded according to the International Classification system of Primary Care (ICPC)[40] and are traced through report of the computerized medical files recorded by the general practitioner.

Visits to the physician are defined as the occurrence of a "respiratory ICPC", "gastrointestinal ICPC" or "dermatological ICPC". For respiratory ICPC's, these include dyspnea (R02), wheezing (R03), cough (R05), acute upper tract infection (R74), acute bronchi(oli)tis (R78), pneumonia (R81), asthma like symptoms (R96), or other less prevalent respiratory ICPC's (breath problems [R04], sneeze [R07], other symptoms of the nose [R08], symptoms of the throat [R21], abnormal sputum [R25], concern about respiratory illness [R27], acute laryngitis [R77], influenza [R88], other infections of the airways [R83], and other respiratory diseases [R99]). For gastrointestinal ICPC's these include infectious diarrhea (D70), vomiting (D10) and susceptible gastro-intestinal infection (D73). Dermatological ICPC's include other symptoms/complaints of the skin (S21), dry skin/ flaking (S21.01). A child is considered to have eczema when the doctors diagnose was established and (1st or 2nd class) corticosteroids have been prescribed. Prescription will be checked via pharmacist's medication records. The incidence of colic is recorded as ICPC infantile colic (A14).

Table 1. Description of symptoms

Coughing	Your infant coughs several times a day and/or has coughing. Regularly there are signs of cold. Don't record cough because of choking.
Wheezing	During expiration you notice a whistling, wheezy sound coming from the lower airways. During expiration your child is trying to squeeze the air outwards. Don't record wheezing coming from or through the nose.
Fever >38°C	Clear from itself, whereby it is important you use a rectal thermometer, measure twice and the temperature is >38°C on both occasions.
Runny nose	Signs of cold with white/yellow/green mucus running from the nose.;
Glue ear	Your child seems to have earache and/or grasps its ear (the ear frequently is high-colored or bends) and/or glue egresses from the ear.
Rash	More than one day existing skin-redness (spots, rash, pimples) on the face, arms or legs, trunk. Disease-symptoms are not necessarily present. The rash can be eczema; eczema mostly is red, moistly, scaly and may itch. Infants cheeks are affected mostly. When children grow up, elbow and knees are preferred sites.
Diarrhea	Increase in stool frequency to twice the usual number per day, that continues more than one day. The content may be watery or mucous.
>3 crying hours a day	Clear from itself, whereby the total crying episodes add up to more than three hours a day (24 hours) in total.

Outpatient clinic visits

Shortly after the child's first birthday, all children visit the outpatient clinic of the recruiting hospital and parents are asked to bring the collected, frozen fecal samples (on ice). Disease episodes (as to the respiratory tract and gastrointestinal tract) during the first year of life are discussed and documented. Any prescribed (systemic) antibiotic treatments during the first year of life as well as well as use of probiotics are recorded. At the end of the visit, a venapuncture is performed to collect a 1 ml blood sample. Serum is stored at -20 °C. At the end of the study, IgG antibodies against Tetanus toxoid, Diphtheria toxoid, *Haemophilus influenza* type B, and the

capsular polysaccharides of the pneumococcal 10-valent conjugate vaccine will be determined. From the same blood sample, specific IgE to food and inhalant allergens is determined for objective evaluation of allergic sensitization.

Collection of fecal samples

Parents collect a total of 8 fecal samples during the first year of life, and one sample at or around the 2nd birthday of the child. Directly after birth, nurses of the maternity and neonatal wards collect the first fecal samples, which are frozen at -20°C. After discharge the parents start collecting samples at home. Collection of stool samples is standardized (oral instruction by the investigators at time of inclusion and there is a written instruction on the daily checklist of symptoms, that parents receive).

Parents are instructed to sample directly from the diaper by means of stool collection vials (with integrated spoons), and immediately freeze them in their home freezers at a temperature of -18 to -20°C. Moreover, in order to be able to study transmission of microbiota from the parents to the child, mother collects a stool sample of her own within the first week after the delivery and a buccal mucous membrane swab and a skin swab from the mother and the father are taken at inclusion.

16S-23S IS profiling of the microbiota

The stool samples are subjected to microbiota composition profiling by means of a high-throughput bacterial profiling technique (IS-pro, IS-Diagnostics, Amsterdam, The Netherlands). This technique combines bacterial species differentiation by the length of 16S-23S rRNA interspace region with instant taxonomic classification by phylum-specific fluorescent labeling of PCR-primers. For amplification of IS regions, phylum-specific, fluorescently labeled primers are designed, corresponding to conserved regions within the 16S rRNA and 23S rRNA genes.[41] The procedure consists of two multiplex PCRs, a combination of which provides very broad coverage for *Firmicutes*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* (FAFV), *Bacteroidetes* and *Proteobacteria*. Amplifications are carried out on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Buccal mucous membrane and skin swabs are analyzed by using the same IS-pro technique.

Data analysis

After pre-processing (IS-pro software suite, IS-Diagnostics), each sample is represented by a microbial profile, consisting of color-labelled peaks. Each peak is characterized by a specific IS fragment (measured as nucleotide length) and a color related to a specific phylum group. The intensity of peaks reflects the relative quantity of PCR product (measured in relative fluorescent units). We further consider each peak as an operational taxonomic unit (OTU) and its corresponding intensity as relative abundance. Intensity values are log2 transformed when appropriate in order to compact the range of variation in peak heights, to reduce the dominance of abundant peaks and include less abundant species of the microbiota in downstream analyses

and more related to the *in vivo* situation. This transformation results in improved consistency of the estimated correlation coefficient, lower impact of inter-run variation, and improved detection of less prominent species. This conversion is used in all downstream analyses, such as calculating within-sample and between-sample microbial diversity.[41]

Statistical analysis

We will evaluate the potential relationships between antibiotic use in early life and the various (clinical) endpoints. Variables are verified for normal distribution and *t*-tests will be used for testing the null hypothesis. Either logistic regression analysis or cox regression analysis will be applied to the data (appearance of the primary endpoint(s) will be recorded as specifically as possible), so that odds ratios or hazard ratios and their 95% confidence interval can be presented for description of the relationships. Multivariate models will be used to assess confounders and confounding effects. We will perform post hoc analyses to look for potential effects of the different antibiotic regimes. Outcomes may, possibly, be classified into subgroups. According to antibiotic treatment after the first two weeks, children will be grouped based on the number of antibiotic courses during the first year of life (0 / 1 / 2 or more courses). Within the 'treatment groups' a distinction can be made between the class or spectrum of the prescribed antibiotics.
Microbiota composition will be described by means of abundance, diversity and (dis)similarity. Diversity is calculated using the Shannon index[42] and differences in this index will be tested with Mann-Whitney U test. Dissimilarities between samples, or between-sample diversity, are calculated as the cosine distance between each pair of samples and analyzed with principal coordinate analysis (PCoA). All statistical analysis will be conducted using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and the vegan software package in R (Foundation for Statistical Computing, Vienna, Austria).

Power calculation

The power of this observational cohort study and the number of participants is based on one of the clinical relevant primary endpoints: incidence of eczema after antibiotic exposure in early life. Eczema is the most common inflammatory skin disease of childhood, affecting 5 to 20% of children at any time. The cumulative prevalence of eczema varies from 20% in Northern Europe and the USA to 5% in the south-eastern Mediterranean.[43] In high-risk populations in Northern Europe (e.g. infants with a positive family history of atopy), the incidence of eczema is increased and estimates 60%.[44] Based on these data, in this study the incidence of eczema in the control group (healthy, non-antibiotic-treated infants) is estimated 20%, whereas in the antibiotic-treated group (infants we assume at increased risk for developing eczema) the incidence may rise to 35%. This percentage, to our opinion, reflects a slightly increased risk for developing eczema after antibiotic treatment in early life in a general population of infants. Assuming a dropout rate of 15%, a total of 450 children (150 antibiotics, 300 controls) have to be included to demonstrate this difference (20% ↔ 35%) with a power of 80% and an *alpha* of 5%.
The prevalence of other disease outcomes is variable. For example, prevalence rates of

infantile colic vary between 5 and 40%,[45] and literature shows that almost half of the children experience wheezing during the first years of life.[46] So far, the effect of antibiotics (prospectively investigated) on these frequencies is unknown. Defining the size of potential differences to capture is therefore limited.

Discussion

To the best of our knowledge, this is the first prospective observational cohort study, with a follow-up of one year, addressing clinical outcomes after empiric antibiotic treatment in early life which at the same time investigates the development of intestinal microbiota composition after this antibiotic exposure. Subsequently, extensive clinical outcomes can be linked to microbiota composition over time. This study includes a total of 450 infants, of whom 150 are exposed to antibiotic treatment shortly after birth and 300 controls. Previous studies addressing consequences of antibiotic treatment in early life were hampered by a retrospective approach,[11,33,39,47] divergent study populations,[48] smaller sample sizes[17] or a limited period of follow-up.[34,49]

By assessing an extended number of clinical parameters in a fairly large group, and subsequently strictly selected homogeneous subgroups of infants, bias due to confounding factors such as mode of delivery and type of feeding is expected to be reduced. Results from this study may improve our understanding of the evolving microbiota in term infants. Also, by collecting fecal samples at eight time points during the first year of life and around the second birthday, long-term microbiota development can be investigated.

The fact that children who are sick and receive antibiotic treatment may differ from healthy children, who do not need antibiotic treatment at birth, is one of the most important limitations of this study. However, to our opinion, there are no opportunities to bypass this problem, as ethical concerns prevent us from performing a double blind randomized controlled trial. Another limitation of this study may be the fact that bacterial composition may change as a consequence of freezing fecal samples. For example, levels of *Bacteroidetes* have been shown to be reduced in frozen samples.[3,50,51] As all samples will be obtained by the parents and stored in their home freezers, no information on potential differences in storage conditions between samples can be obtained. We try to minimize this variation by instructing (by one of the investigators) all parents on how to collect the faecal samples, which makes the collection procedure as standardized as possible. We do realize that studying the microbiota composition of fecal samples may not be the best method to assess the general composition and the effect of antibiotics in the gastrointestinal tract. Stool samples therefore are used as a proxy for the study of the gut microbiota as these samples are easier to collect than biopsy samples and avoid invasive procedures (and associated ethical issues). A limitation of sampling the gut microbiota

is that once the sample is taken, alterations in the relative proportions of various bacterial species can occur so that the sample might no longer accurately reflect the composition of the microbiota in vivo by the time it is processed.[3] This is a well-known problem with respect to molecular approach of microbiota composition and to date it is impossible to collect biopsy samples from infants (and healthy volunteers generally) on a large scale due to the practical and ethical challenges indicated above.[3]

IS-pro will be used to characterize the microbiota, because this technique has specifically been designed for application in a clinical setting. It has been validated for clinical diagnostics, which makes it fully reproducible and the high-throughput nature of IS-pro makes analysis of a large number of samples feasible. IS-pro comprises two separate phylum-specific PCR reactions though, which hampers direct comparisons of relative abundances addressing all three phyla together.[41] Next to that, IS-pro does not generate sequence data in the conventional sense. This means IS-pro cannot achieve the same level of detail as next-generation/whole genome sequencing techniques (e.g. Illumina), although sequence data confirmed specificity of IS-pro in (currently) numerous peaks, underlining the validity of the technique.[41]

There is now sufficient evidence to conclude that antibiotic treatment in early life has a detrimental effect on the gastrointestinal microbiota composition. However, it is still unclear as to what extent the composition is disturbed and for how long the gastrointestinal dysbiosis remains, if and how microbiota composition will 'normalize' and what the effects are on the developing immune system. These findings can be important, since there is evidence that the manipulation of infant microbiota by using pre-or probiotics can restore the ecological balance of the microbiota.[34,52,53] If our hypothesis is proven with this study, future studies should demonstrate if targeted intervention, by using dietary supplements, pre- or probiotics or otherwise, may have beneficial effects by limiting the gastrointestinal imbalance due to early antibiotic exposure.

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EARLY LIFE ANTIBIOTIC TREATMENT DISTURBS

DEVELOPMENT OF THE INTESTINAL MICROBIOTA

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Abstract

Objective The development of the infant gut microbiota is influenced by numerous factors, including antibiotic treatment. However, data on the effects of antibiotics on gut microbiota development in term infants are limited. The aim of this study was to determine the impact of antibiotic use in the first week of life on subsequent microbial colonization and development.

Study design Fecal samples of 45 vaginally term-born and exclusively breastfed infants, of which 21 received antibiotics (AbT) and 24 were healthy controls (CtR), were obtained at one week, one month and 3 months of age. The IS-pro technique was used for bacterial profiling. Differences in bacterial abundance and diversity were assessed by conventional statistics. Longitudinal differences in stability of the population were calculated using cosine distances.

Results Antibiotic treatment in the first week of life resulted in a lower abundance, diversity and a delay in acquisition of species of the *Bacteroidetes* phylum. *Escherichia coli*, a common cause of neonatal sepsis, was more prevalent and more stable over time in CtR compared to AbT. Latter group had a less stable microbial composition over time. Regardless of antibiotic treatment, one-week-old infants could be clustered into *Bacteroidetes*-dominant or *Firmicutes-Actinobacteria-Fusobacteria-Verrucomicrobia* (FAFV) dominant microbiota.

Conclusion Early life antibiotic treatment disturbs the microbiota development in infants, mostly evident in *Bacteroidetes* species. As the long-term health implications of these effects are yet unknown, follow-up studies are warranted.

Introduction

The infant develops an initial microbiome from birth onwards, ultimately becoming colonized with a wide variety of microorganisms.[1–3] The development of the gut microbiota continues during the first years of life, a critical period for development and maturation of the immune system in which the foundation for future health is laid.[4] A range of factors can influence the intestinal microbiota and its establishment, including mode of delivery, dietary patterns, genetic factors and possibly administration of pro-, pre- and antibiotics.[5–12]

The use of peripartum broad-spectrum antibiotics has become common in modern obstetric and neonatal practice. At the same time evidence is increasing that exposure to antibiotics in early life is associated with profound effects on the gut microbiome and various disorders later in life, such as atopy, inflammatory bowel disease, diabetes and obesity.[13–19] Perturbations in microbiota composition during early life may have critical long-term effects as this period includes a crucial development phase.[13,20–24] Recent epidemiological and mechanistic data on the association between early antibiotic use, dysbiosis (imbalances in gut microbiota) and disease, support the significant concern on long-lasting effects of early antibiotics exposure on host and microbiome development.[19,25]

Earlier studies on the development of gut-microbiota using culture-dependent techniques already revealed an altered intestinal microbiota in antibiotic treated infants.[23,26] In the last few years, the development of culture-independent (molecular) approaches has dramatically changed and refined the original perspective on composition and dynamic changes of gut-microbiota.[27,28] By using molecular fingerprinting and quantitative analysis of 16S rRNA genes (quantitative polymerase chain reaction (qPCR)), overgrowth of *Enterococci* and arrested growth of *Bifidobacterium* (deemed beneficial species for promoting and maintaining host health) in infants exposed to antibiotics in the first week of life have been described.[7,9,24] Also, in preterm infants receiving antibiotics in the first week of life, increased relative abundance of *Enterobacter,* as well as lower total bacterial diversity in the second and third weeks of life was found compared with preterm neonates that did not receive antibiotics in this period of life.[21] Another study using the same techniques showed that parenteral antibiotics administered peripartum reduced, but not completely eliminated, these bacterial populations.[20] However, the interpretation of the outcome of these studies is hampered by heterogeneity in study cohorts.

The aim of this study was to investigate potential differences in the development of intestinal microbiota during the first 3 months of life in a homogeneous group of term infants. These findings may become of clinical importance, as antibiotic exposure in early life has been associated with disease later in life and strategies to restore ecological balance of the disturbed microbiota are therefore needed.[13,29,30]

Patients and Methods

Study design

Study subjects were recruited from the maternity wards of four teaching hospitals in the Netherlands: the St. Antonius Hospital in Nieuwegein, the Tergooi Hospital in Blaricum, the Gelre Hospitals in Apeldoorn and the Meander Medical Centre in Amersfoort. Patients were enrolled between January 2012 and February 2014. Parents of term-born infants (≥ 36 weeks of gestational age) who stayed in the hospital > 24 hours were approached for participation in the study. Exclusion criteria were: 1. Congenital illness or malformations; 2. Severe perinatal infections for which transfer to the neonatal intensive care unit was needed; 3. Maternal probiotic use within six weeks before delivery; and 4. Insufficient knowledge of the Dutch language. Patients were eligible for this study when they were born vaginally and exclusively breastfed. We enrolled all consecutive infants who received seven days of antibiotic treatment (AbT) according to the local hospital protocols, starting in the first week of life. We also enrolled a control group of consecutive infants who did not receive any antibiotic treatment but were observed for other reasons (such as maternal comorbidity, blood sugar monitoring or premature rupture of membranes) (CtR). Informed consent was obtained from parents at enrolment. This study was approved by the Medical Ethics Committees of all participating hospitals and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. This study was part of a larger prospective cohort study on the influence of antibiotics on microbiota and disease (the INCA study).[31]

Stool sample collection

Stool samples were collected from the infants at day seven and at the end of the first and third month. Stool samples were collected from diapers, transferred to stool collection vials and immediately frozen in the hospital or home freezers (-20 °C). Frozen samples collected at home were kept frozen during transportation on ice to the hospital and immediately stored upon arrival at -20 °C until further analysis.

Isolation of bacterial DNA

As a starting point for DNA isolation from feces, a pea-sized faecal sample (100–400 mg) was placed in an Eppendorf container. Then, a 500 µl suspension was made in nucliSENS lysis buffer, as provided for easyMAG DNA isolation, an automated system for total nucleic acid extraction (bioMérieux Clinical Diagnostics, Marcy l'Etoile, France). This suspension was vortexed for 1 minute, shaken for 5 minutes and subsequently centrifuged at 16.2 *g* for 2 minutes at room temperature. Supernatant (100 µl) was transferred to an 8-welled easyMAG container, and another 2 ml nucliSENS lysis buffer was added. After incubation at room temperature for 10 min, 70 µl of magnetic silica beads was added. Afterwards, the mixture was placed in the easyMAG machine and the "specific A" protocol was chosen, selecting the off-board workflow and finally eluting DNA in 110 µl of elution buffer. Isolated faecal DNA was diluted 10-fold in DNA/RNA free water (H_2O) before use in PCR. All DNA samples were stored at -20°C.

16S-23S IS profiling of gut microbiota

Amplification of interspace regions (IS-regions) was performed with the IS-pro technique (IS-diagnostics, Amsterdam, The Netherlands). This technique combines bacterial species differentiation by the length of 16S-23S rRNA interspace (IS) region with instant taxonomic classification by phylum-specific fluorescent labelling of PCR-primers. As described earlier, for amplification of IS regions phylum-specific, fluorescent labelled primers were designed, corresponding to conserved regions within the 16S rRNA and 23S rRNA genes.[32] The procedure consists of two multiplex PCRs, a combination which provides very broad coverage for *Firmicutes*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* (FAFV), *Bacteroidetes* and *Proteobacteria*. For detailed information on the design of the primers used we refer to a previous publication.[32] Amplifications were carried out on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Conditions for PCR were 10 cycles of 94°C for 30 s, 67°C to 57°C ('touch down') for 45s, 72°C for 1 min; 25 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 1 min; 72°C for 11 min and a final extension at 4°C. Each PCR mixture, with a final volume of 25µl, contained 10 µl of buffered DNA, 1x superTaq buffer (Applied Biosystems), 200 µM deoxynucleoside triphosphates, 0.04% BSA, 1 U of superTaq (Applied Biosystems), and 0.13 µM of each of the primers.

Data analysis

After pre-processing (IS-pro software suite, IS-diagnostics, Amsterdam, The Netherlands), each sample was represented by a microbial profile, consisting of color-labelled peaks. Each peak represented a specific IS fragment (measured as nucleotide length) and a color related to a specific phylum group. The intensity of peaks reflected the relative quantity of PCR product (measured in relative fluorescence units (RFU)). Each peak was designated as an operational taxonomic unit (OTU) and its corresponding intensity as abundance. Intensity values were log2 transformed in order to compact the range of variation in peak heights, to reduce the impact of dominant peaks and include less abundant species of the microbiota in downstream analyses and more related to the *in vivo* situation. The cut-off level for noise was set to <6 logRFU.[32] A clustered heat map was made by generating a correlation matrix based on cosine correlations of all profile data followed by clustering with the unweight pair group method with arithmetic mean (UPGMA).[33] Diversity was calculated using the Shannon index,[34] and differences in this index were tested with Mann-Whitney *U* test. Dissimilarities between samples, or between-sample diversity, were calculated using the cosine distance metric between each pair of samples' profiles. Given two vectors of attributes (two profiles in our case), A and B, the cosine dissimilarity is represented using a dot product and magnitude as:

$$\text{dissimilarity} = 1 - \cos(\theta) = 1 - \frac{\sum^{n}_{i=1} A_i \times B_i}{\sqrt{\sum^{n}_{i=1}(A_i)^2} \times \sqrt{\sum^{n}_{i=1}(B_i)^2}}$$

A lower cosine value indicates that the two profiles are more similar to each other. Alpha diversity analysis was performed using the vegan software package in R (Foundation for Statistical Computing, Vienna, Austria) and SPSS (SPSS for Mac release 22.0; SPSS Inc. Chicago, IL, USA). Data are shown as median (interquartile range) for continuous variables as appropriate. Differences between the antibiotic and control group and potentially confounding variables were tested for independence by chi-square test. Differences were considered to be significant for $p < 0.05$.

Selection of species

To validate the antibiotic effect at the species level, we selected three species that vary in their response to the antibiotic treatment. *Escherichia coli* and *Enterococcus faecalis* were selected to represent common colonizers of the newborn's intestinal tract. While *E. coli* can cause neonatal sepsis that has to be treated with antibiotics, *E. faecalis* is a common commensal bacterium that is resistant to penicillin and frequently also to gentamycin. We further selected *Streptococcus mitis*, a bacterium commonly found in the neonatal oral cavity,[35] which can also inhabit the gut, as colonization with the *Streptococcus* group has been demonstrated during early life.[36] *S. mitis* is generally sensitive to penicillin and gentamycin. We also explored bifidobacteria abundance in both groups, since microbes from the *Bifidobacterium* genus (part of the *Actinobacteria* phylum) are among the first anaerobes colonizing the gut of vaginally born, breast-fed infants.[37]

Results

Characteristics of the study population

Fecal samples of 21 infants that received antibiotic treatment after birth (AbT) and 24 otherwise healthy (CtR), term infants were included. The infant cohort characteristics are shown in Table 1. No differences in baseline characteristics between groups were identified. All participating infants in the AbT group received intravenous antibiotics for a period of seven days, because of a high suspicion of neonatal sepsis due to overt clinical signs of infection and/or an increased serum level of C-reactive protein. Blood cultures were negative in all AbT children after 48 hours.

The majority of AbT infants received a standardized combination scheme of seven days penicillin combined with gentamycin for the first two days (n=11, 52%) or amoxicillin (n=8, 38%) instead of penicillin. The other two prescribed schemes were amoxicillin with ceftazidime (a third-generation cephalosporin) and Augmentin (amoxicillin + clavulanic acid) with gentamycin. Since only a few infants received an antibiotic scheme different from the predominant schemes, we were unable to test for direct effects of different antibiotic treatment regimens. Therefore, all AbT infants were analyzed as one group.

Table 1. Study cohort characteristics

	AbT group (n=21)	***CtR group (n=24)***	***p-value***
Male (n,%)	10 (48%)	9 (37,5%)	0.49
Mean gestational age (weeks + days, SD)	40 wks, 2 days ± 1 day	39 wks, 1 day ± 1 day	0.14
Mean birth weight (gram, SD)	3773 ± 543	3427 ± 501#	0.34
Intrapartum antibiotics (mother) (n, %)	4 (20%)$	4 (22%)$	0.87
Maternal GBS carriage (n, %)	7 (50%)*	4 (31%)*	0.31
Indication for antibiotics (n, %)			
Clinical signs (respiratory distress, tachypnea, moaning, paleness, petechiae)	11 (52%)	N/A	-
Maternal fever during delivery and/or fever in the infant postpartum	7 (33%)		
Amniotic fluid containing meconium and/or low APGAR score	2 (10%)		
Pre-existing risk factors	1 (5%)		

GBS = group B streptococcus

data available for n=23 subjects

$ data available for n=20 (AbT) and n=18 (CtR)

* data available for n=14 (AbT) and n=13 (CtR)

Gut microbiota profiling; clustering of samples at one week of age

At one week, clustering revealed two distinct sub-populations, regardless of the treatment group the infants belonged to (p= 0.80). Figure 1 shows the distinct clustering of infants at one week into two groups, one with a *Bacteroidetes*-dominant microbiota and one with an FAFV dominant microbiota. Because these clusters suggested two distinct groups of early microbial settlers, we coined the groups settler type B (*Bacteroidetes* dominant) and settler type F (FAFV dominant). The group of settler type B consisted of 21 infants, of which 9 AbT infants and 12 CtR infants. The group of settler type F consisted of 20 infants, of which 8 AbT infants and 12 CtR infants at one week. Clustering was neither associated with intrapartum antibiotics of the mother (p= 1.0) nor with maternal GBS carriage (p= 1.0) or hospital of origin (p= 1.0). The one week sample of four children was missing and for that reason those were clustered according to their microbiota composition at 1 month. All these 4 had a clearly *Bacteroidetes*-dominant microbiota so they were classified as settler type B. The groups of settler types B and F did not differ in abundance or diversity of *Proteobacteria* at any time point.

Effect of antibiotic treatment on the Bacteroidetes phylum

Comparison of the antibiotic versus control groups at the phylum level showed that the antibiotic treatment mostly affected the *Bacteroidetes* phylum, in both settler types.

First, in settler type B, *Bacteroidetes* abundance was significantly lower at all time points in AbT infants as compared to CtR infants (p=0.03, p=0.003 and p<0.001 for 1 week, 1 month and 3 months, respectively; figure 2). 3 months after cessation of antibiotics, the impact on *Bacteroidetes* was still evident. In AbT infants that belonged to settler type F, *Bacteroidetes* growth was inhibited, and we observed a delayed, though not significant, acquisition rate of *Bacteroidetes* compared to CtR (3 months, p=0.4).

A borderline significant decrease over time in *Firmicutes* abundance (from 1 week to 3 months, p=0.06) was observed in CtR infants of settler type F, which was not observed in AbT infants in this group (figure 2).Second, we observed that AbT infants in settler type B had significantly lower diversity (by means of Shannon index) of species belonging to the phylum *Bacteroidetes* at all time points compared to CtR infants (p=0.009, 0.004, 0.004 for 1 week, 1 month and 3 months, respectively) (figure 3).

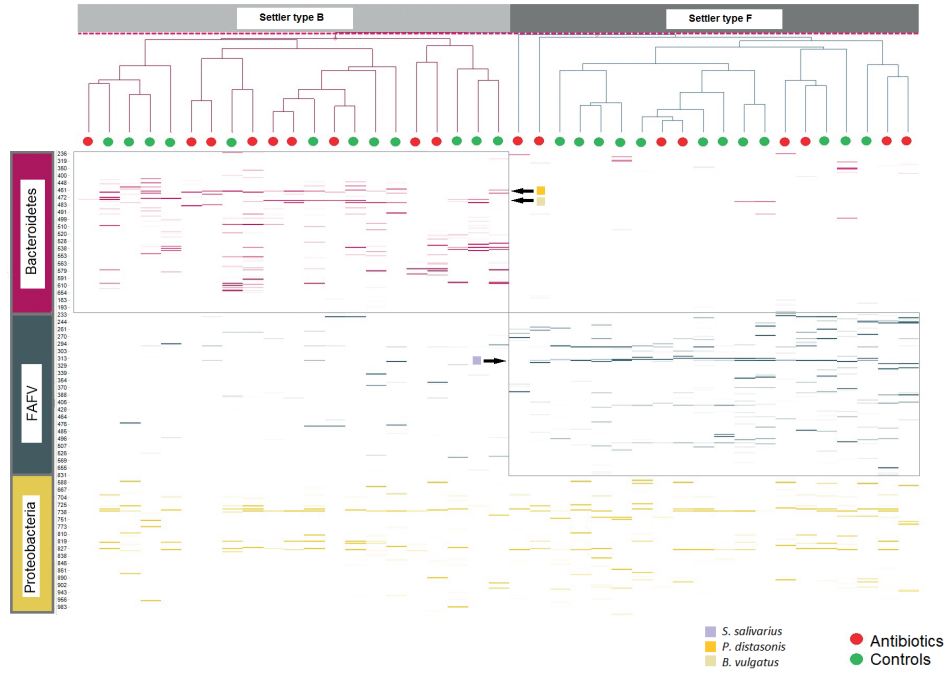

Figure 1. Heat map of all profiles at T1 (one week), sorted and colored by phylum.
Abundance of OTUs in each sample at T1 (one week). Columns correspond to samples (antibiotic treatment: red; controls: green);rows correspond to OTUs. Phyla color shades represent the abundance of each OTU in a sample (*Bacteroidetes*: pink; *FAFV*: blue; *Proteobacteria*: yellow). Cosine correlations and hierarchical clustering were calculated on the raw data, but for a better interpretation we only present the called peaks in this visualization. Samples on the left cluster (pink) belong to settler type B and share Bacteroidetes-dominant microbiota, in which *P. distasonis* and *B. vulgatus* are determinatives. Samples on the right cluster (blue) belong to settler type F and share FAFV-dominant microbiota, in which *S. salivarius* is a determinative species.
FAFV = *Firmicutes, Actinobacteria, Fusobacteria, Verrucomicrobia*
OTU = operational taxonomic units.

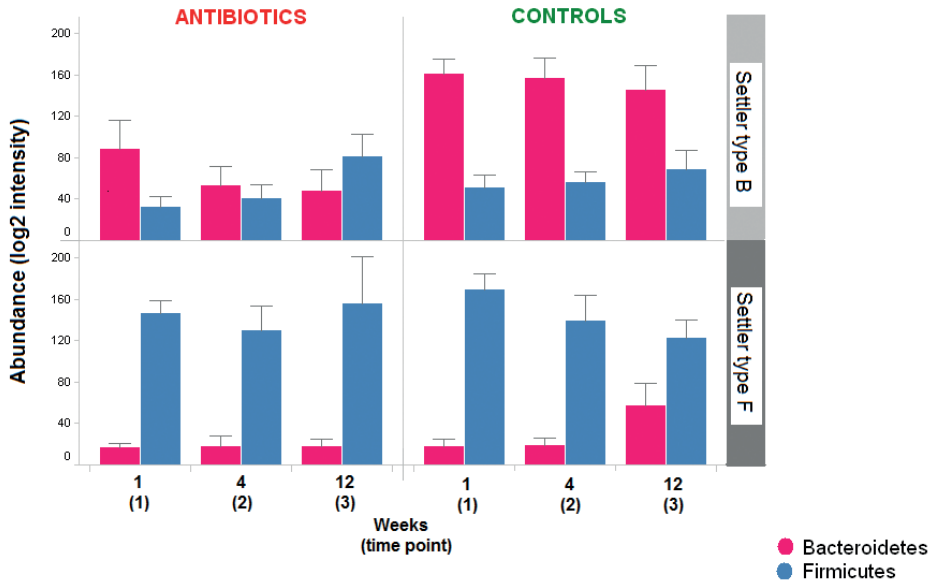

Figure 2. Abundance of *Bacteroidetes* and *Firmicutes* by settler type in infants treated with antibiotics compared to control infants.

Average abundance of *Bacteroidetes* (pink) and *Firmicutes* (blue) over time by settler type B (top) and F (bottom) in antibiotic treated infants (left) and controls (right). Values are log2 transformed intensities (measured as log2 RFU). Error bars denote the standard error. Time point 1: one week of age; Time point 2: one month of age; Time point 3: 3 months of age. RFU = relative fluorescent units.

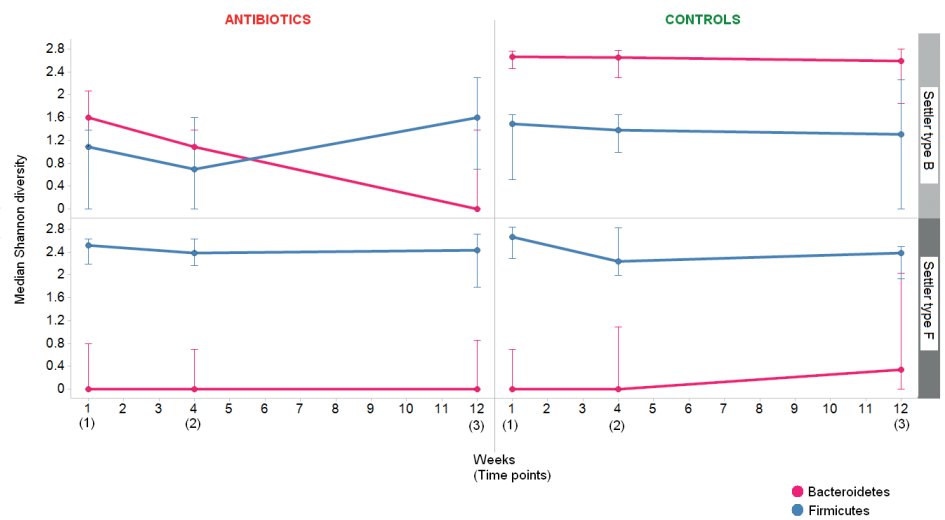

Figure 3. Shannon diversity index of the gut microbiota by settler type in infants treated with antibiotics compared to control infants.

Median Shannon diversity index of *Bacteroidetes* (pink) and *Firmicutes* (blue) over time by settler type B (top) and F (bottom) in antibiotic treated infants (left) and controls (right). Error bars denote the upper and lower IQR. Time point 1: one week of age; Time point 2: one month of age; Time point 3: 3 months of age. IQR = inter quartile range.

Differential development of the microbiota

To examine the effect of antibiotics on the development of the microbiota in time, the cosine distance between two profiles of each infant for all possible combinations of time points was calculated. Infants were stratified according to settler types, and antibiotic treated infants were compared to controls. In the control group of settler type B, profiles were the most stable over time as can be seen by the lowest overall cosine distances (figure 4) ($p < 0.0001$ for the displayed interval between 1 week and 3 months).

Infants of settler type B who received antibiotics had the most unstable microbiota reflected by the highest cosine distances. Antibiotic treatment did not affect stability over time in infants of settler type F, as cosine distances over time did not differ between the antibiotics and control group.

To determine whether the different settler types B and F would converge towards a common microbiota composition over time, we calculated cosine distances between individuals of different settler types within each group (AbT or CtR). Figure 5 shows that the settler types became more similar to each other with time. The antibiotic group showed a delayed convergence of the clusters compared to controls ($p<0.0001$ for all time points).

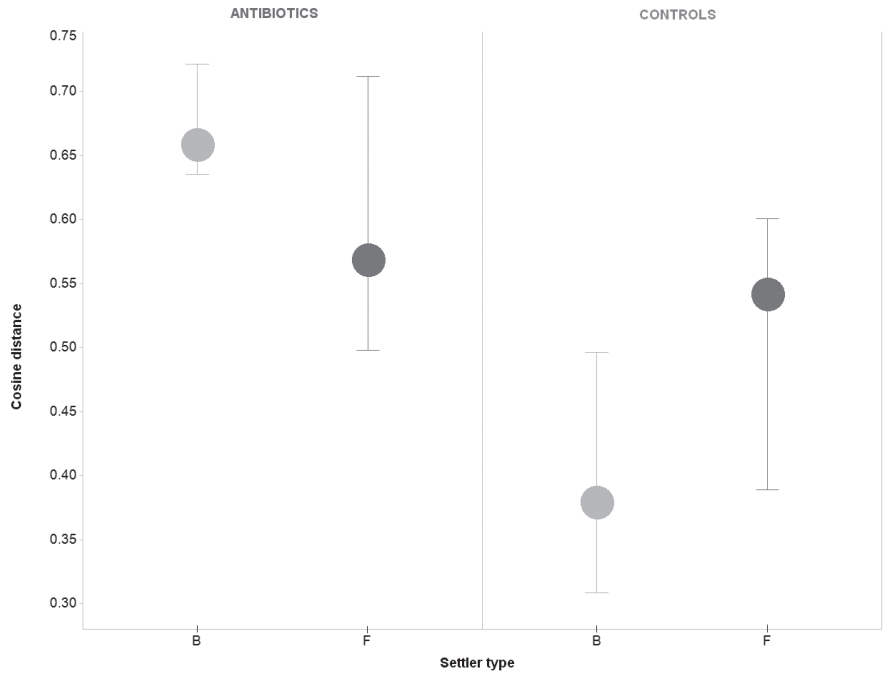

Figure 4. Cosine distances as a measure of community dissimilarity within individuals over time by settler types B and F in antibiotic treated infants and controls.

Cosine distances displayed by settler type B (light grey) and F (dark grey) in antibiotic treated infants (left) and controls (right). Distances were calculated between one week of age (time point 1) and 3 months of age (time point 3) within each individual. Dots indicate median cosine distances (during the 3-month interval) of all individuals of that settler type grouped together; bars indicate inter quartile range (IQR).

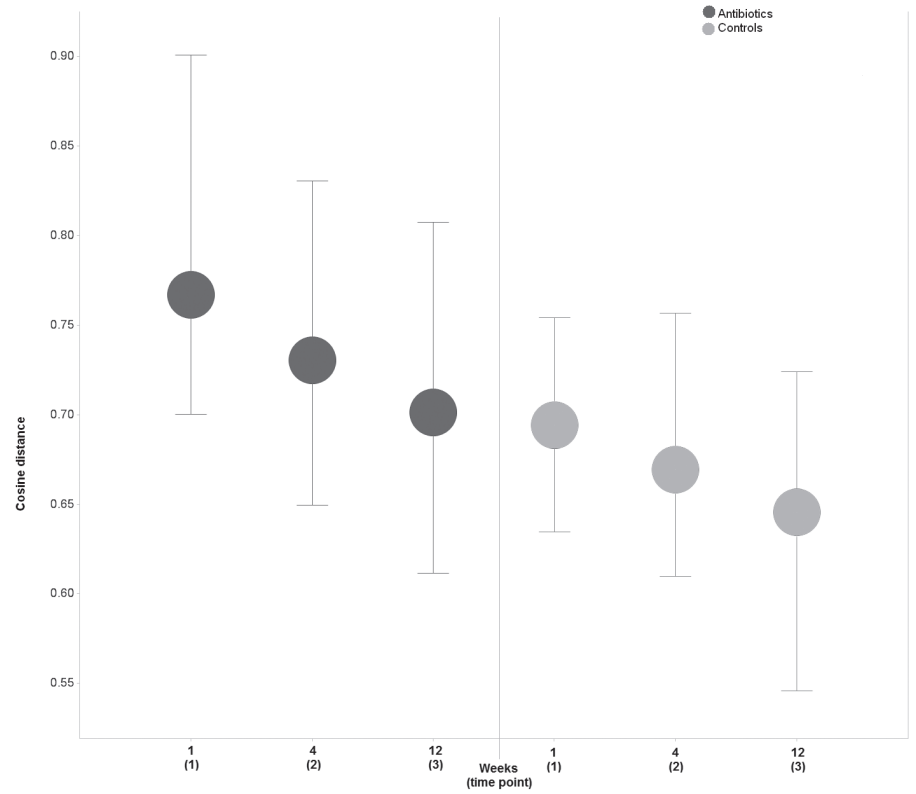

Figure 5: Cosine distances as a measure of community dissimilarity between settler types (B and F) within antibiotics and control infants.

Cosine distances displayed over time by treatment group (antibiotic treatment: dark grey; controls: light grey). Dots indicate median cosine distances, bars indicate inter quartile range (IQR). Time point 1: one week of age; Time point 2: one month of age; Time point 3: 3 months of age.

Antibiotic effect on specific bacterial species

Abundances of *E. coli, E. faecalis* and *S. mitis* are shown in Figure 6. In general, during this 3-month period, the abundance of the common gut colonizers – *E. coli* and *E. faecalis* - increased over time, while *S. mitis* abundance decreased. In AbT infants, levels of *E. coli* and *S. mitis* were lower compared to controls, while *E. faecalis* levels were more abundant in the AbT group. At the age of 3 months, control children were colonized with a higher abundance of *E. coli* compared to AbT (p=0.07). Moreover, in AbT infants, *E. coli* was less prevalent and we observed a shorter and a more fluctuating colonization compared to CtR infants; only four infants continuously carried *E. coli* at all three time points in the AbT group, compared to 12 infants in the CtR group (p=0.06, chi square test).

Effects on key bacterial species over time

In order to gain a deeper view of the neonatal microbial composition in the 2 separate settler types, we determined the 'driver species' of the two settler types (B and F). These were defined as species being predominant (>50% prevalence) in that settler type. Settler type B was mainly dominated by *Bacteroides vulgatus* and *Parabacteroides distasonis*, species that are generally common and abundant in the human gastrointestinal tract.[38,39] In settler type F, the 'driver species' were *Staphylococcus epidermidis* and *Streptococcus salivarius*.

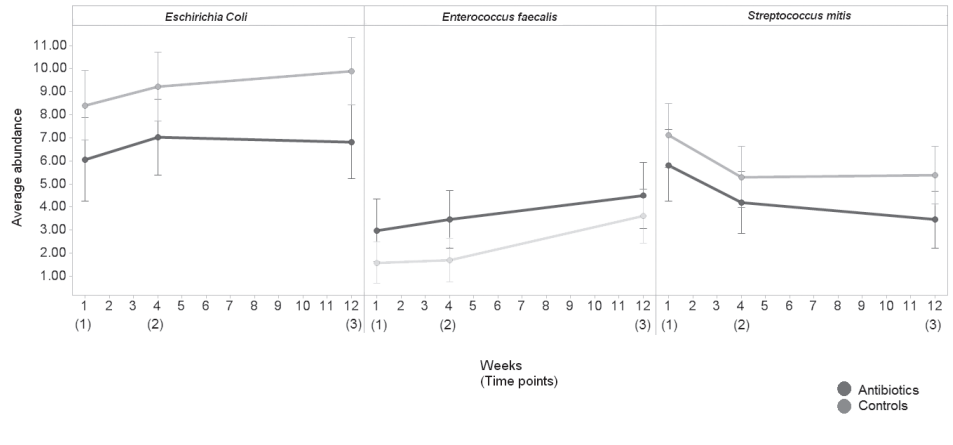

Figure 6. Antibiotics effect on selected species by treatment group

Average abundance (log2 transformed) intensities by treatment group (antibiotics: dark grey; controls: light grey) of three selected species that vary in their response to the antibiotic treatment: *E. coli* and *S. mitis* are sensitive to the antibiotic treatment, whilst *E. faecalis* is resistant. Error bars indicate standard error. Time point 1: one week of age; Time point 2: one month of age; Time point 3: 3 months of age.

Within the first two weeks of life beneficial *Bifidobacterium* species start dominating the microbiota and reach high levels compared to the species of other phyla.[37,40] The IS-pro technique allows for positive identification of four particularly abundant *Bifidobacterial* species in breastfed children: *B. longum*, *B. breve*, *B. bifidum* and *B. lactis.*[5,41] We observed a trend, although not statistically significant, towards lower abundance of these four *Bifidobacterium* species in the antibiotic group compared with controls, during the first 3 months of life (data not shown).

Discussion

In this study the gut microbiota of exclusively breastfed and vaginally born term infants, receiving antibiotic treatment in the first week of life, was characterized, to assess the effect of antibiotics on the development of the gut microbiota. We showed that administration of antibiotics results in a lower diversity of bacterial species of the *Bacteroidetes* phylum and/or a delay in *Bacteroidetes* colonization, and that these effects persist for at least 3 months. It is known that infants delivered by caesarean section also have a lower abundance and diversity of the phylum *Bacteroidetes*[42] and that this aberrant microbiota composition is associated with an increased risk of developing type 1 diabetes, asthma and allergic diseases.[43–45] Whether the pronounced effects of antibiotics on the *Bacteroidetes* composition, as seen in this population, are also associated with an increased risk of developing auto-immune or allergic diseases is unknown at this moment. In addition, a trend towards lower abundance of four *Bifidobacterium* species in the antibiotic group was observed, compared with controls. Beneficial properties are attributed to *Bifidobacterium* species with respect to their positive influence on development and maturation of the immune system. Deviations in abundance or diversity of bifidobacteria may predispose the infant to the development of immune-mediated diseases later in life.[18,46]

In both antibiotic treated infants and controls, a positive correlation was observed between the time span of the interval and measured cosine distances between profiles. This can be explained by the continuous general increase in richness during infancy. However, AbT infants' profiles of both settler types B and F (although not significant for type F) diverged more over 3 months, which suggests a differential development of the microbiota and a more aberrant colonization pattern in this group. It has been described that microbial communities in adults return to their initial state after antibiotic treatment, but this return is often incomplete.[25,47] Since the microbiota in infants has not yet reached a stable state,[48] the effects of antibiotics cannot be expressed as a measure of return to initial state. Antibiotics may cause a permanent shift to an alternative state of the microbiota, as was described in adults,[47] or towards a different developmental trajectory.[13] The neonatal period is a crucial development phase in life, during which perturbations in microbiota composition may have critical long-term health effects.

An important finding of this study was the clustering of IS-profiles of all infants, into two distinct subgroups regardless of treatment. This clustering was most distinct at one week of age: a *Bacteroidetes*-predominant subgroup (settler type B) and a FAFV (*Firmicutes*, *Actinobacteria*, *Fusobacteria*, *Verrucomicrobia*)-predominant subgroup (settler type F) could be distinguished. To the best of our knowledge, the presence of two clearly demarcated subgroups in vaginally delivered infants has been described before in only one, relatively small study. A group of seven term, vaginally delivered and exclusively breast-fed neonates could be divided into two groups according to their levels of the genus *Bacteroides* over the neonatal period.[40] In one group detectable levels of *Bacteroides* were observed for the first time towards the fourth week of

life, while the other group immediately showed levels comparable to those in maternal feces that remained relatively high and stable throughout the neonatal period. In accordance with the driver species identified in our study, the authors demonstrated that the *Bacteroidetes* phylum mainly comprised the genera *Bacteroides* and *Parabacteroides* and the genera *Streptococcus* and *Staphylococcus* reached the highest relative abundances within the *Firmicutes* phylum.

It has been suggested that the human gut microbiome can be categorized into three distinct types or 'enterotypes'.[49,50] In a large cohort of 330 infants, it was shown that enterotype establishment occurs between 9 and 36 months of age with cessation of breast-feeding as a major driver in shifts in composition. Since we examined faecal microbiota only in the first 3 months of life and allocation to a settler type was based on the one-week-of-age stool sample, it would be premature to term our settler types as 'proxy enterotypes'.[41] Moreover, the described settler types may possibly be determined by, and reflect, physiological fluctuations in community composition in early life. Gut microbiota development is a highly variable process in which host, genetic and environmental factors play a role.[47,51] Because our data show that individuals may move from one settler type to the other over time, the clustering could also reflect a snapshot of the real situation. One hypothesis however could be that the foundations for microbiota development are already laid within the first week of life. Differentiation into described settler types is not induced by antibiotic exposure. Birth mode however could be an influencing factor, since it has already been shown that vaginally delivered infants acquire bacterial communities resembling their own mother's vaginal microbiota.[52] Moreover, the proximity of the birth canal to the anus suggests that the maternal faecal microbiota may also play an important role in the development of the different settler types. Maternal transmission and other potential mechanisms need to be further elucidated.

A major strength of this study is that it controlled for two of the most important confounding factors in the development of the gut microbiota, that have hampered previous studies: type of feeding and delivery mode. This study also has a few limitations. First, all samples were obtained by the parents and stored in their home freezers, so no information on potential differences in storage time between samples could be obtained. Parents, however, were clearly instructed on how to collect and store the samples. Secondly, the IS-pro technique comprises two separate phylum-specific PCR reactions, which currently precludes direct quantification of *Proteobacteria* relative abundance against the other phyla. Furthermore, we found a relative paucity of *Bifidobacterial* species, which may have been caused by the techniques employed, such as DNA isolation protocols and PCR primers.[37,53]

Conclusion

Antibiotic treatment in the first week of life resulted in a lower diversity of the *Bacteroidetes* phylum and/or delay in acquisition. Regardless of antibiotic treatment, one-week-old neonates could be clustered into two settler types, with either a *Bacteroidetes*- or a FAFV dominant microbiota. Since short-term changes of the establishing infant gut microbiota may have long-term health consequences in the form of allergies, asthma, diabetes and obesity later in life,[25,54–57] follow-up studies are warranted to further elucidate this potential causality. In cases when antibiotic treatment cannot be avoided, these findings can be clinical important as the use of pre- or probiotics may mitigate these potential negative health effects by influencing the ecological balance of the microbiota.

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5

NEONATAL MICROBIOTA CAN BE CLASSIFIED INTO TWO DISTINCT SETTLER TYPES WITH DIFFERENT DEVELOPMENT OVER TIME

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Abstract

Background Intestinal microbiota development of newborns is very much dependent on delivery mode and feeding type. We have recently shown that neonatal microbial profiles can be separated into two distinct compositional clusters, coined settler types. These two settler types are characterized by a high abundance of either *Bacteroidetes* (settler type B) or *Firmicutes* (settler type F). In the present study, we investigate the relation of settler types with delivery mode and maternal microbiota composition. We further investigate the development of these settler types in relation to feeding (breast-fed versus formula-fed).

Methods Fecal samples were collected from neonates at three time points: one, four and twelve weeks after birth. A fecal sample of all mothers was taken during the first week after delivery. 32 children were exclusively breast-fed and 45 were exclusively bottle fed. All analyses were done with IS-pro, a clinically validated microbiota analysis tool, based on length and sequence variation of the 16S-23S interspace (IS) region.

Results In total, 45 vaginally delivered (VD) infants and 32 infants born after cesarean section (CS) were included in this study. At week one, the *Bacteroidetes* dominant cluster was found only in VD infants (n=27), while the *Firmicutes* dominant cluster was present in 18 VD and all CS infants. Microbiota of children in settler type B showed a high similarity to gut microbiota of their mothers at all time points, while those in settler type F showed a low similarity to that of their mothers at all time points. We could show that many *Bacteroidetes species* were transferred from mother to child in settler type B, but Alistipes spp., present in all mothers, were never acquired by a child. Microbiota in settler type F showed a clear effect of feeding type (breast-fed vs formula-fed), with breast-fed children harboring more *Staphylococcus epidermidis* than formula-fed children.

Conclusion The neonatal microbiome can be separated into two distinct settler types: a *Bacteroidetes* and a Firmicutes dominant type. Vaginally delivered children can have either settler type, while children delivered by caesarean section almost exclusively have settler type F. By classifying children into settler types instead of delivery mode, group effects may become clear and associations with disease later in life might prove easier to establish.

Introduction

After birth, the neonatal human intestine rapidly obtains large numbers of facultative anaerobes or aerobes, such as *Staphylococcus*, *Enterobacteriaceae*, and *Streptococcus*. These microbes are transient and are replaced by a wide variety of obligate anaerobes, such as *Bifidobacterium*, *Bacteroides*, and *Clostridium*, in a matter of days.[1] During the first few months, the diet of the infant almost exclusively consists of milk, thereby enabling milk oligosaccharide fermenters, such as *Bifidobacterium* to thrive. Initially, the intestinal microbiota of an infant has a low diversity and complexity, but it develops and matures slowly, reaching an adult state at around 3 years of age or perhaps somewhat later in childhood.[2,3] There is high variability in the profiles of fecal microbiota among the infants, being generally dominated by *Actinobacteria* (mainly the genus *Bifidobacterium*) and *Firmicutes* (with diverse representation from numerous genera).[4] The development of intestinal microbiota in neonates is critical, since initially present bacteria can modulate the expression of genes in host epithelial cells. Furthermore, as has been reviewed extensively, the composition of the microbiota is important for the development of the immune system and immunological tolerance.[1,5–7]

Probably the most influential external factor for the development of the infant's microbiome is the mother's microbiome, due to intimate contacts during birth, nursing, and early feeding.[2,8,9] The microbes from mother's mouth and skin transfer horizontally to the newborn through several processes from the beginning of life.[2,8,10,11] During delivery, transmission of microbiota from the birth canal and anus occurs from mother to the offspring, but to what extent this happens is not clear. In vaginally delivered infants, colonization with maternal vaginal and fecal microbes, including *lactobacilli* and group B *streptococci*, strongly suggests a maternal signature and successful transmission of specific gut microbes has been demonstrated in a limited number of *bifidobacterial* species, which were obtained from the feces of both mothers and (vaginally delivered) infants.[12–14] Gut microbiota of infants delivered by caesarean section strongly resembles maternal skin microbes (*staphylococci*).[11,15,16] Moreover, caesarean born infants harbor less *Bifidobacterium, Escherichia–Shigella* and *Bacteroides* species compared to children born vaginally.[4,13] Differences in the postnatal microbial colonization may explain the higher incidence of immune mediated diseases such as allergy in children born by caesarean section as compared with those born vaginally.[1,17]

Type of feeding after birth also seems to play a role in the colonization process, although only minor differences have been noted between breast- and formula-fed infants over the past years.[8] The microbiota of breast-fed neonates is dominated by the genera *Bifidobacterium* and *Ruminococcus* and low presence of clostridia.[18] There is evidence for direct inoculation of the infant gut microbiota by bacteria present in breast milk (*lactobacilli*, *enterococci* and *staphylococci)*, while exclusively formula-fed infants have an increased richness of species compared with breast-fed neonates and harbor a diverse microbiota including *Enterobacteriaceae*, *Enterococcus*

and *Bacteroides*, as well as *Bifidobacterium* and *Atopobium*[18–20] and overrepresentation of *Clostridium difficile.*[4]

In our previous study we showed a clear clustering of vaginally born, breast-fed infants into two so-called settler types at one week of age: one group of infants was *Bacteroidetes* dominant in their feces, and the other groups microbiota consisted mainly of bacteria from the phylum *Firmicutes* [Chapter 4 of this thesis]. The origin of such a clustering, of which *Bacteroides* dominance has also been shown in another study,[21] with a small study population, remains to be explained. The aim of this study is to further investigate these settler types, by comparing gut microbiota composition in term infants, born vaginally (VD) or by caesarean section (CS) and who received breast-feeding (BF) or formula-feeding (FF) during the first 3 months of life. Moreover, a possible relation between settler type and maternal microbiota is investigated.

Patients and Methods

Study design

The women and children included in this study were part of a larger prospective cohort study investigating the potential clinical and microbial consequences of empiric antibiotic use in early life.[22] Study subjects were recruited from the maternity wards of four teaching hospitals in the Netherlands. Patients were enrolled between January 2012 and March 2014. Parents of term-born infants (≥ 36 weeks of gestational age) that stayed in the hospital for at least 24 hours were approached for participation in the study. Exclusion criteria were 1. Congenital illness or malformations; 2. Severe perinatal infections for which transfer to the neonatal intensive care unit was needed; 3. Maternal probiotic use ≤ six weeks before delivery and 4. Insufficient knowledge of the Dutch language.

The selected study group comprised 77 healthy women and their infants, divided into four groups:
group A] 24 infants, born by vaginal delivery, breast-fed,
group B] 21 infants, born by vaginal delivery, formula-fed,
group C] 24 infants, born by caesarean section, formula-fed, and
group D] 8 infants born by caesarean section, breast-fed.
Informed consent was obtained from parents at enrollment. This study was approved by the joined Medical Ethics Committee (VCMO, nowadays MEC-U: Medical Research Ethics Committees United (Nieuwegein)) and proof of local feasibility was given for all participating centers. The study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Stool sample collection

One stool sample from mother (M), collected within the first week after the delivery, was obtained as well as stool samples from the infants at day seven (T1), at the end of the first month (T2) and third month (T3). Mother's stool sample was caught in a tray and infant stool samples were collected from diapers, transferred to stool collection vials and immediately frozen in the hospital or home freezers (-20 °C). Frozen samples collected at home were transported on ice to the hospital and immediately stored upon arrival at -20 °C until further analysis.

Isolation and identification of bacteria

As starting point for DNA isolation from feces, a pea-sized fecal sample (100–400 mg) was placed in an Eppendorf container. Then, a 500 µl suspension was made in nucliSENS lysis buffer, as provided with the easyMAG, an automated system for total nucleic acid isolation (bioMérieux Clinical Diagnostics, Marcy l'Etoile, France). This suspension was vortexed for 1 minute, shaken for 5 minutes and subsequently centrifuged at 16,200 *g* for 2 minutes at room temperature. Supernatant (100 µl) was transferred to an 8-welled easyMAG container, and 2 ml nucliSENS lysis buffer was added. After incubation at room temperature for ≥10 min, 70 µl of magnetic silica beads was added, as provided with the easyMAG machine. Afterwards, the mixture was inserted in the easyMAG machine and the "specific A" protocol was chosen, selecting the off-board workflow and eluting DNA in 110 µl of buffer. Fecal DNA was diluted 10-fold before use in PCR. All DNA was stored at -20°C.

16S-23S IS profiling of gut microbiota

Amplification of interspace regions (IS-regions) was performed with the IS-pro technique (IS-diagnostics, Amsterdam, The Netherlands). This technique combines bacterial species differentiation by the length of 16S-23S rDNA interspace region with instant taxonomic classification by phylum-specific fluorescent labeling of PCR-primers. As described previously, for amplification of IS regions phylum-specific, fluorescent labeled primers were designed, corresponding to conserved regions within the 16S rDNA and 23S rDNA.[23] The procedure consists of two multiplex PCRs: a first PCR has two different fluorescent color labeling: one for the phyla *Firmicutes*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* (FAFV) and a second color for the phylum *Bacteroidetes*. A separate PCR is performed for the phylum *Proteobacteria*. Amplifications were carried out on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Cycling conditions for PCR were 10 cycles of 94°C for 30 s, 67°C to 57°C ('touch down') for 45 s, 72°C for 1 min; 25 cycles of 94°C for 30 s, 57°C for 45 s, and 72°C for 1 min; 72°C for 11 min and a final extension at 4°C. Each PCR mixture, with a final volume of 25µl, contained 10 µl of buffered DNA, 1x superTaq buffer (Applied Biosystems), 200 µM deoxynucleoside triphosphates, 0.04% BSA, 1 U of superTaq (Applied Biosystems), and 0.13 µM of each of the primers.

Data analysis

After pre-processing (IS-pro software suite, IS-diagnostics, Amsterdam, The Netherlands), each sample was represented by a microbial profile, consisting of color-labeled peaks. Each peak was characterized by a specific IS fragment (measured as nucleotide length) and a color related to a specific phylum group. The intensity of peaks reflected the quantity of PCR product (measured in relative fluorescence units). We further considered each peak as an operational taxonomic unit (OTU) and its corresponding intensity as abundance. Intensity values were log2 transformed in order to compact the range of variation in peak heights, to reduce the impact of dominant peaks and include less abundant species of the microbiota in downstream analyses. This transformation results in improved consistency of the estimated correlation coefficient, lower impact of inter-run variation, and improved detection of less prominent species. This conversion was used in all downstream analyses, such as calculating within-sample and between-sample microbial diversity. A clustered heat map was made by generating a correlation matrix based on cosine correlations of all log2 transformed profile data followed by clustering with the unweighted pair group method with arithmetic mean (UPGMA).[24]

Diversity analysis and discriminative features selection

Diversity was calculated using the Shannon index[25] and differences in this index were tested with Mann-Whitney *U* test. Dissimilarities between samples, or between-sample diversity, were calculated using the cosine distance measure between each pair of samples' profiles. Diversity analysis was performed using the vegan software package in R (Foundation for Statistical Computing, Vienna, Austria) and SPSS (SPSS for Mac release 22.0; SPSS Inc. Chicago, IL, USA). Data are shown as median (interquartile range) for continuous variables as appropriate. Differences were considered to be significant for $p < 0.05$.

Results

Fecal samples of 77 term infants and their mothers were obtained. 45 infants were vaginally born and 32 were born by caesarean section. 32 children were exclusively breast-fed and 45 were exclusively bottle fed. One mother in group A received antibiotics during labor (more than four hours before the child was born), but antibiotics during labor was not designated an exclusion criterion. Participant characteristics are shown in Table 1. Baseline characteristics were not statistically different between the groups (groups were compared by ANOVA).

Table 1. Study cohort characteristics

INFANTS	**Group A: VD +BF (n=23)**	**Group B: VD +FF (n=21)**	**Group C: CS +FF (n=24)**	**Group D: CS +BF (n=8)**
Male	10 (42%)	12 (57%)	12 (50%)	2 (25%)
Mean gestational age	39 wks, 1 day	39 wks, 5 days	39 wks, 6 days	39 wks, 6 days
Birth weight, in gram (SD)	3423 (463)	3381 (430)	3995 (647)	3597 (500)
Number of infants receiving antibiotics (during first 3 months of life)	1*	0	0	0
MOTHERS	**(n=24)**	**(n=18)**	**(n=21)**	
Age in years (SD)	32,6# (3,7)	32,9 (4,8)	32,3 (4,1)	No maternal samples analyzed
Number of mothers receiving antibiotics during labor	1	0	0	

data available for n=23 mothers. *antibiotic treatment shortly before 3th month stool sample; this sample was excluded from the analysis. BF: breast-fed; CS: caesarean section; FF: formula fed; VD: vaginally delivered.

At one week of age, a clear separation into two clusters of neonatal microbiota profiles could be made (Figure 1). One cluster was characterized by a high abundance of the *Bacteroidetes* phylum; the other cluster by a high abundance of species from the *Firmicutes, Actinobacteria, Fusobacteria* and *Verrucomicrobia* (FAFV) group. The clusters were coined 'settler types' B and F respectively, representing the preponderant bacterial phylum. A cutoff at a relative abundance of Bacteroidetes at 0.3 (total load of Bacteroidetes/ total bacterial load) was used to discriminate between the Bacteroidetes and Firmicutes settler type. With only a single exception, *Bacteroidetes* dominant samples came from children who were delivered vaginally. All children who were delivered by caesarean section harbored a *Firmicutes* dominant microbiota. However, a significant proportion (40%) of children delivered vaginally also had a *Firmicutes* dominated microbiota, similar to children delivered by caesarean section.

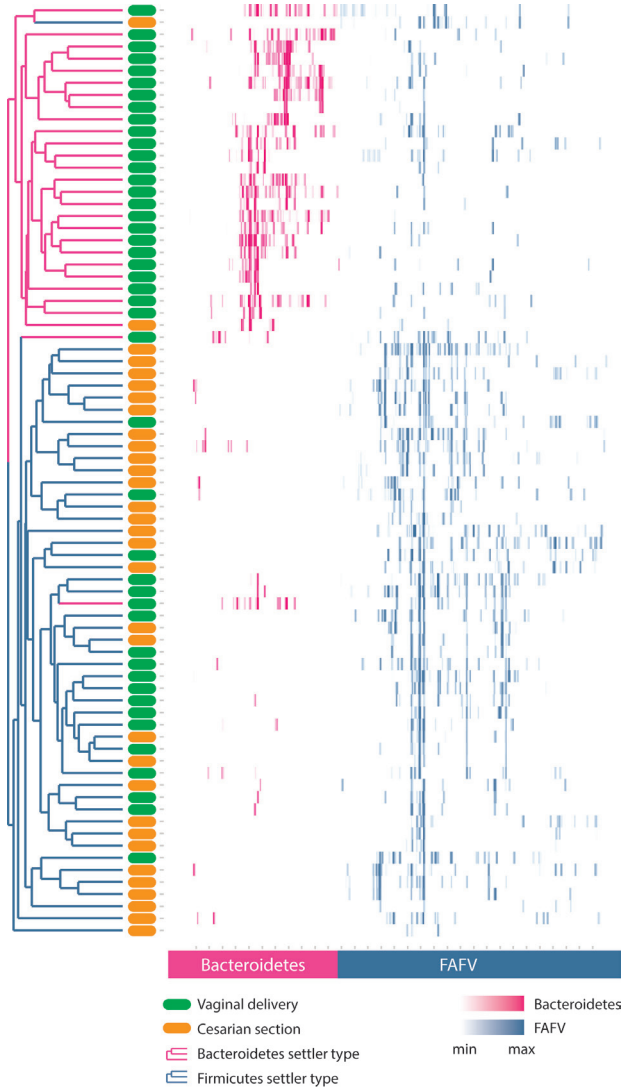

Figure 1. Heat map of samples of all children at T=1 (one week).

To investigate whether children with different settler types differed in microbiota development over time, the microbial composition at the three time points were explored per settler type (Figure 2 and 3).

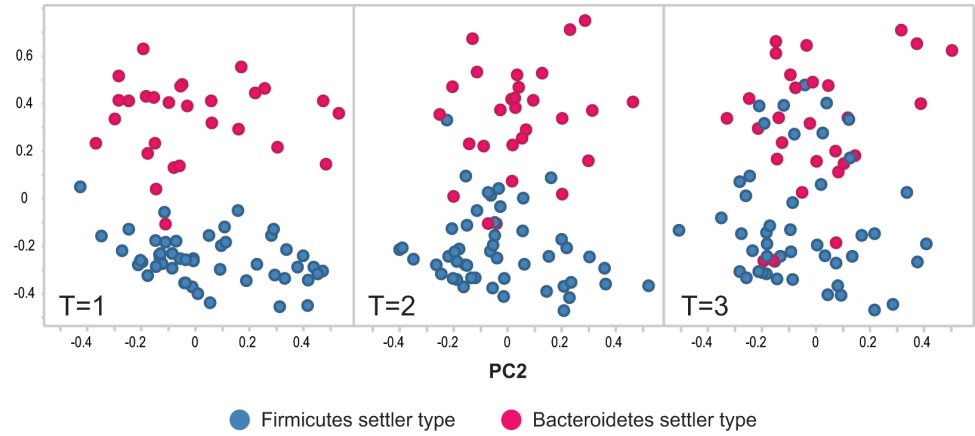

Figure 2. PCoA analysis of intestinal microbiota of all children at week 1 (T=1), month 1 (T=2) and month 3 (T=3).
Samples are colored by settler type.

In this analysis it became apparent that settler types were the main driving force of separation of microbial samples in a principal coordinate analysis (PCoA). This effect was most outspoken at week 1, but remained evident at 1 month and 3 months of age. At each time point, the abundance of *Bacteroidetes* and FAFV group differed significantly between the two settler types ($p<0,005$ at all time points), while abundance of *Proteobacteria* was the same for the two groups (figure 4). The Shannon diversity of *Bacteroidetes* and FAFV group also differed significantly between the settler types ($p<0,005$ at all time points). *Bacteroidetes* were most abundant and had highest diversity in settler type B, while FAFV group had the highest abundance and diversity in settler type F. Total diversity was significantly lower in settler type F at T=2 and T=3 ($p<0,05$). *Proteobacteria* diversity did not differ between groups. In settler type F, *Bacteroidetes* did increase in abundance and diversity over time, but they did not reach the levels of settler type B in three months of time.

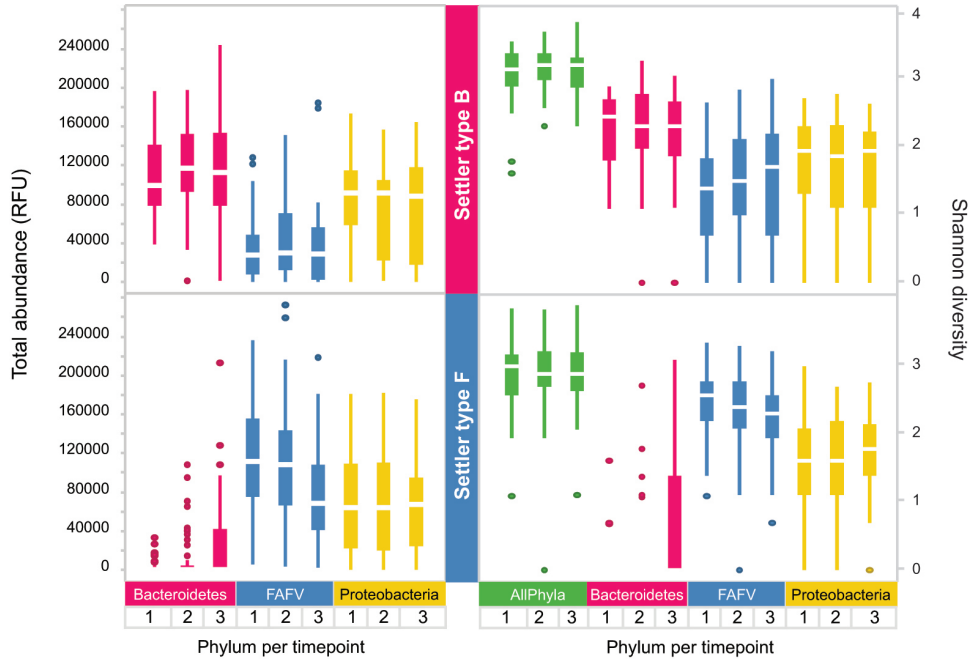

Figure 3. Abundance (left) and diversity (right) per phylum for all time points per settler type.
1 week (T=1), 1 month (T=2) and 3 months (T=3) of age.

Thus, while some microbiota profiles of children with settler type F appeared more similar to microbiota profiles of children with settler type B after three months, in the PCoA analysis abundance and diversity analysis showed that at group level microbial compositions still differed significantly. However, there also appears to be some convergence of microbiota profiles on group level: abundance and diversity of *Bacteroidetes* increase over time in settler type F, while FAFV abundance and diversity decrease. At the same time FAFV diversity increases in settler type B, towards a value similar to that of settler type F. Hence, while microbiota composition is significantly different at all time points for settler type B and F, there does seem to be some convergence towards a similar microbiota over time.

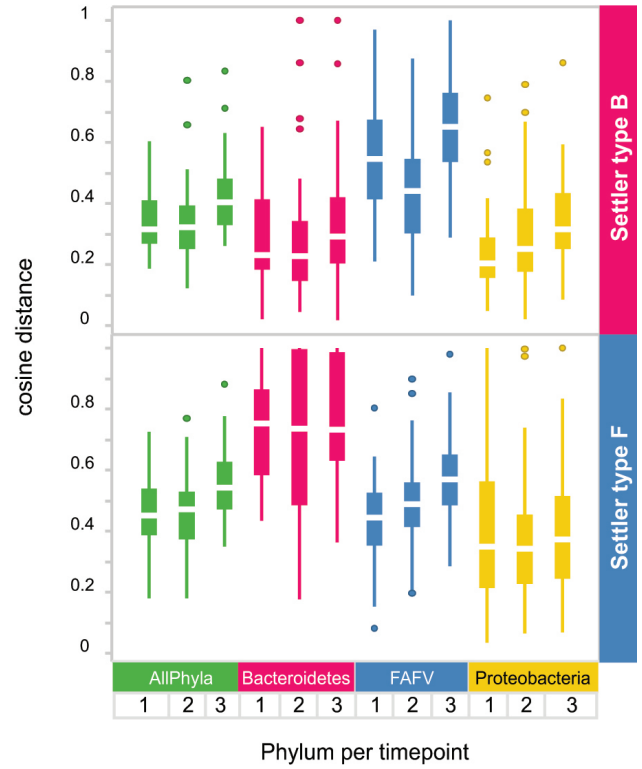

Figure 4. Stability analysis of neonatal gut microbiota per settler type.
1 week (T=1), 1 month (T=2) and 3 months (T=3) of age.

Stability of the population, expressed as cosine distance, also differed per settler type (figure 4). Lower values in this respect mean higher stability of the population over time. Cosine values of all phyla combined, as well as cosine values of *Bacteroidetes*, were lower at all time points in settler type B compared to settler type F. This implies that settler type B microbiota is more stable in the first 3 months of life than settler type F at the level of *Bacteroidetes* as well as for the total microbial population.

Finally, maternal stool samples were analyzed to investigate the extent of vertical transmission of microbiota from mother to child and to explore whether the maternal gut microbiota has impact on the neonatal settler type. By performing a principal coordinate analysis (PCoA) of all maternal and infant samples, it became apparent that the most important drivers separating samples were origin (maternal vs infant) and settler type (Figure 5). Maternal samples all clustered together, regardless of the settler type of the child and infant samples clustered together per settler type. Clustering of infant samples per settler type remained apparent at all time points.

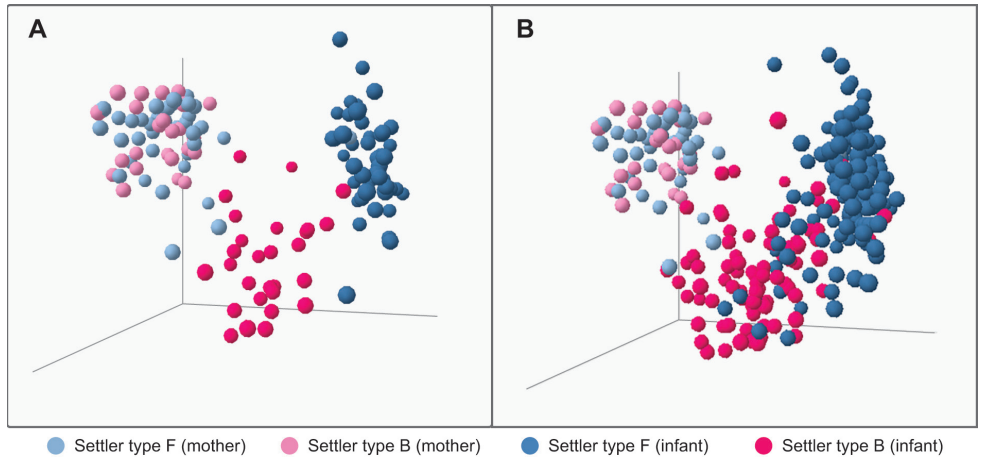

Figure 5. Principal Coordinate Analysis (PCoA) of samples from mothers and children colored by neonatal settler type.
Main drivers of separation are origin of the samples (maternal or infant) and infant settler type. Maternal samples cluster together regardless of the child's settler type. Settler types cluster together at T=1 (panel A), but also when all time points are compared (panel B).

By performing analyses per settler type, group effects become clear. Children in settler type B showed a higher similarity to gut microbiota of their mothers at all time points compared to children in settler type F, in particular for the phylum *Bacteroidetes*. However, similarity to other mothers than their own was correspondingly high for children in settler type B. Two reasons turned out to be responsible for this phenomenon: firstly, not all species from maternal microbiota are transferred successfully to infants. This is especially apparent for Alistipes spp, that are present in all mothers, but in none of the infants at one week of age. Secondly, some species are present in most mothers and are particularly likely to be transferred from mother to child, such as *Bacteroides vulgatus* and *Bacteroides fragilis* (Figure 6). It can also be seen that children harbor some species that apparently are undetectable in the maternal microbiota. These may be species with low abundance in mothers that became successful settlers in the infant's microbiota, or these species may derive from a different environmental source. Taken together, these effects render mothers and children distinct groups, with similarity between members of the two groups comparable for all individuals. However, when studying individual cases as shown in figure 6, it can be seen that children in settler type B often seem to inherit selected bacterial species from their mothers gut microbiota.

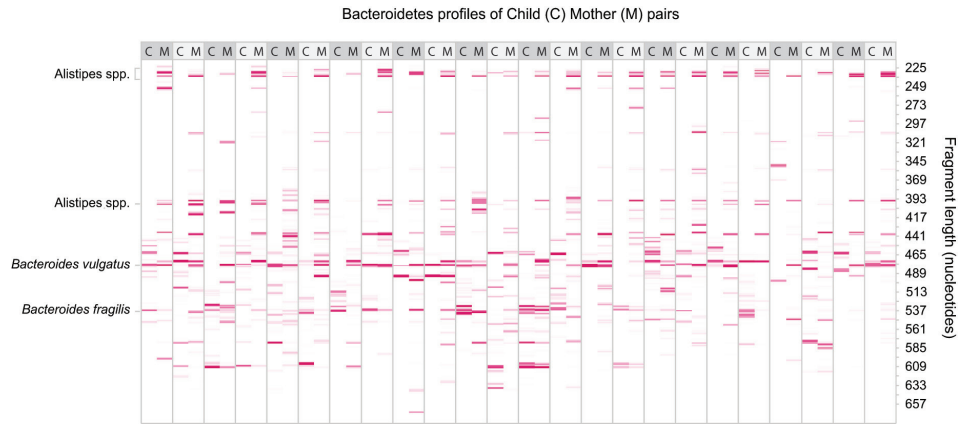

Figure 6. Comparison of Bacteroidetes profiles of mother-child pairs from settler type B at 1 week of age (T=1).
Increasing intensity of the pink bars represent higher abundance of the species indicated.

However, differences can be seen in the composition of the FAFV cluster in both settler types, when investigating dominant species. Settler type F is, at one week of age, dominated by *Staphylococcus epidermidis*, *Streptococcus mitis* and *Streptococcus salivarius*, common members of oropharyngeal and skin microbiota. With time, the predominance of these species decreases, and those are at the same time replaced by a diverse panel of bacterial species.

The type of feeding, breast- or formula-feeding did not result in significant differences at the phylum level (vaginally delivered, breast- versus formula feeding (A-B) and caesarean section, formula versus breast-feeding (C-D)). This may have been due to the small group sizes and/or subtle differences that were not detected by the technique applied. Further analysis revealed that settler types were not determined by feeding type; the numbers of infants breast- and formula fed were distributed evenly between settler type B and F. However, in settler type F, an effect of feeding type was shown, with breast-fed children harboring more *Staphylococcus epidermidis* compared to formula fed children. Formula-fed children acquired higher numbers of *Enterococcus faecalis* over time.

Discussion

In this study gut microbiota composition during the first 3 months of life of healthy term infants, receiving breast-feeding or formula-feeding who were born vaginally or by caesarean section, was characterized. At one week of age a clear separation into two clusters of neonatal microbiota profiles, as shown in our previous study [Chapter 4 of this thesis], could be confirmed. The emergence and development of the so-called settler types, in which *Bacteroidetes* (settler type B) or *Firmicutes, Actinobacteria, Fusobacteria* and *Verrucomicrobia* (FAFV) (settler type F) are highly abundant, was explored. Importantly, we found that only vaginally delivered children had settler type B. Children born by cesarean section all had settler type F. However, 40% of vaginally delivered children also had settler type F.

Moreover, the potential relation between settler type and maternal microbiota was investigated. It was shown before that infants are rapidly colonized by microbes from different environments they are exposed to shortly after birth, and that the mode of delivery is very determinative in this context.[21,26,27] In vaginal delivery, the child being born has an extensive opportunity to acquire its mother's vaginal and fecal microbiota.[12,14,18] Although there have been suggestions that the ingestion of amniotic fluid allows some colonization of the infant's gut in utero, infants born by caesarean section have no access to the mother's (vaginal and fecal) microbiota.[11,14,29] Gut microbiota of caesarean delivered infants strongly resembles skin microbes, suggested to be derived from their mother and caregivers/hospital staff,[11,15,16] but also from microbes coming from operating rooms: it was recently shown that bacterial content of operating room dust corresponded to human skin bacteria.[30] Vertical transmission of microbes from mother to infant thus especially takes place during vaginal delivery. However, the data presented in this study indicate that a substantial number (40%) of vaginally delivered children have an initial microbiota very similar to that of children delivered by caesarean section. They share the underrepresentation of *Bacteroides* species. Literature shows that microbiota of infants delivered by caesarean section remains more heterogeneous over time compared to the vaginally born infants, and *Bacteroides* spp in particular are less prevalent or missing in the caesarean section-delivered newborns.[14] This study demonstrated that settler type was the main driver of separation of microbiota composition at one week of age, expressed as high versus low abundance and diversity of *Bacteroidetes* phylum members. Interestingly, settler type B (*Bacteroidetes* species dominant) consists exclusively of vaginally delivered children, while settler type F (mainly species of the *Firmicutes* phylum dominant) consists of children delivered vaginally and by caesarean section. Based on the present data, we hypothesize that not delivery mode per se has the greatest impact on initial development of the microbiota, but that exposition to maternal fecal microbiota during delivery is a prerequisite for acquisition of *Bacteroidetes* dominant microbiota. The importance of the mother's intestine as source for the for the transmission of specific Bifidobacterium strains was shown before.[12] Group effects became clear by classifying children according to the assigned settler types, and subsequent studies on

development of disease later in life might turn out to be easier to establish by this classification. Next to exposure to maternal microbiota, other characteristics of and around the delivery process may play a role regarding settler type determination and/or the degree of mother-to-infant transmission of microbes. Because epidemiological evidence suggests that acquiring bacteria from the *Bacteroidetes* phylum (both abundance and diversity of these species) is associated with a lower risk of asthma and allergic diseases as well as type 1 diabetes,[31-34] the specific conditions during labor and delivery may be important: duration of ruptured membranes, total duration of delivery, maternal bowel movements during labor, and environment (home versus hospital (delivery room, operating room), to name a few. At present we don't have sufficient detailed data on those conditions for this group of vaginally delivered children, but are currently investigating those conditions. They could be part of an updated version of the original "hygiene hypothesis", which associated increased proportion of people affected by allergic diseases with differences in diet or in childhood exposure to microbes and allergens.[35] Accordingly, it was shown that increased exposure to microbial compounds has to occur early in life to affect maturation of the immune system and thereby reduces risk for development of allergic diseases.[36] The impact of the present study for the hygiene hypothesis would be that not vaginal versus caesarean delivery as such, but exposure to vaginal, and particularly fecal microbiota during vaginal delivery would have far-reaching long-term consequences. Future investigations should elucidate this possibility.

Over a course of three months the separation into clusters remained, though some children with settler type F moved towards settler type B children. This merging was caused by the acquisition of *Bacteroidetes* by settler type F children; *Bacteroidetes* did increase in abundance and diversity over time, but they did not reach the levels of settler type B in three months of time. The difference between an infantile versus adult-like microbial composition in general was confirmed in this study, as maternal samples cluster together regardless of the child's microbiota (that clustered together based on settler type). The development of the gut microbiota continues during the first years of life and its composition is considered to resemble the adult gastrointestinal tract by the age of 3 or somewhat later in childhood.[3,26]

When comparing microbiota composition of mothers and infants, transmission of *Bacteroidetes* from fecal microbiota from mother to child seems to determine infant settler type: when transmission takes place, children develop settler type B, but when transmission does not take place, children develop settler type F. Children in settler type B show a higher similarity to gut microbiota (*Bacteroidetes*) of their mothers at all time points, while those in settler type F show a lower similarity to that of their mothers at all time points. Interestingly, microbiota similarity was not significantly higher for mother-child pairs than for children compared to unrelated mothers. This effect was largely caused by the fact that only a limited number of *Bacteroidetes* species were transferred from mother to child; some species that were present in all mothers were never transferred to the child, such as *Alistipes* species, while other species that were

commonly present in high abundance in mothers, such as *B. vulgatus* and *B. fragilis*, seemed to be transferred preferentially to children. Furthermore, infants also harbor species that were not detected in the maternal microbiota. These may be species with low abundance in mothers that became dominant in the infant's microbiota, or they may possibly originate from other body sites or the environment. This was shown before and a decline over time may reflect reduced fitness to persist in the human gut.[14]

Results presented here confirm data from literature that demonstrated only minor differences in microbiota composition in breast- and formula-fed infants.[8,37,38] Feeding type thus does not seem to be one of the dominating factors involved in gut microbiota maturation, at least of less importance than settler type. An effect of feeding type that could be shown in infants with settler type F was breast-fed children harboring more *Staphylococcus epidermidis* compared to formula fed children, who acquire higher amounts of *Enterococcus faecalis* over time. Our results are in accordance with literature showing typical inhabitants of the human skin to be dominant members with longer residence time in breast-fed infants,[39] and with our own data describing *Streptococcus* and *Enterococcus* species being discriminatory signature taxa associated with formula feeding (chapter 2 of this thesis). Possibly the relatively less robust microbiota of infants belonging to settler type F, compared to those of settler type B, was more susceptible to subtle changes caused by feeding type.

The fairly high number of healthy, term infants included in this study is a strength of the study as it allowed thorough analyses of the progression of bacterial colonization in the gastrointestinal tract during the first 3 months of life. Although the initial process of colonization is known to be dynamic and complex, clustering into settler types at one week of age could be clearly demonstrated. This study also has a few limitations. First, IS-pro consists of two separate PCRs. One for amplification of *Bacteroidetes* and FAFV and a second for *Proteobacteria*. This precludes direct comparison of abundances of *Proteobacteria* to *Bacteroidetes* and *FAFV*. Furthermore, IS-pro does not generate 16S sequence data but instead amplifies the 16S-23S IS region for bacterial identification. While IS-pro can generally differentiate bacterial taxa to species level, direct comparison to results of other studies performed with 16S sequencing may be less straightforward. However, the IS-pro technique has been extensively validated for reproducibility on fecal samples and has been shown to correlate well to sequence data.[23,40] Secondly, there may have been differences in storage conditions between samples, because all samples were obtained by parents and stored in their home freezers. Parents, however, were clearly instructed on how to collect and store the samples, so we assume no major influence on fecal compositions.

Conclusion

The neonatal microbiome can be separated into two distinct settler types: a *Bacteroidetes* (settler type B) and a *Firmicutes/ Actinobacteria/ Fusobacteria/ Verrucomicrobia* (settler type F) dominant type. Vaginally delivered children can have either settler type, while children delivered by caesarean section almost exclusively have settler type F. By classifying children into settler types instead of delivery mode, group effects may become more clear and associations with disease later in life might prove easier to establish. Future studies should focus on establishing the presence of these settler types and factors associated with settler type development as long-term consequences remain unknown up to now.

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PART II - EFFECTS OF USING PROBIOTICS IN EARLY CHILDHOOD

6

PREVENTIVE EFFECTS OF SELECTED PROBIOTIC STRAINS ON THE DEVELOPMENT OF ASTHMA AND ALLERGIC RHINITIS IN CHILDHOOD. THE PANDA STUDY.

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Abstract

Background Administration of probiotic bacteria may be a potential approach to prevent allergic disease, by modification of the intestinal microbiota and establishing and/or restoring the physiological balance between Th1 and Th2 cells. In this study, we hypothesize a possible beneficial long-term effect of perinatal administration of selected probiotic strains on the prevalence of asthma and allergic rhinitis in high risk children at age 6 years. We here describe the long term follow-up of children included in our previous described clinical trial, showing a preventive effect on the prevalence of eczema at 2 years of age. The primary objective of this study was to determine the potential preventive effect on the prevalence of asthma and allergic rhinitis and eczema.

Methods In a double-blind, randomized, placebo-controlled follow-up trial, a mixture of probiotic bacteria, consisting of *B. bifidum* W23, *B. lactis* W52 and *Lc. Lactis* W58, selected by *in vitro* experiments, was perinatally administered to pregnant women and their offspring. In the current single-blind follow-up trial, 84 high-risk children aged 6 years completed the follow-up with a questionnaire, physical examination, lung function testing and specific IgE measurements.

Results Asthma was diagnosed in 5/39 (12.8%) of participants in the probiotic group and in 8/44 (18.2%) of the placebo group (p=0.50). Allergic rhinitis was diagnosed in 5/39 (12.8%) of participants in the probiotic group and in 1/44 (2.3%) of the placebo group (p=0.09). The severity of allergic rhinitis and the prevalence of eczema were comparable between both groups. No differences were found in lung function parameters nor in sensitization between both groups.

Conclusion Perinatal supplementation with Ecologic® Panda had no preventive effect on the development of asthma and allergic rhinitis in children at high-risk for allergy, at age 6 years. The preventive effect on the prevalence of eczema during the first 2 years was no longer present at age 6. Therefore, Ecologic® Panda cannot be generally recommended for long term primary prevention of asthma and allergic rhinitis. Prolonged gut microbiota management beyond 1 year of age may be required to achieve a long-lasting impact.

Introduction

During the past half century an overwhelming rise in allergic airway disease was observed, and more recently, a convincing rise in the prevalence of food allergy. Evidence is mounting that progressive westernization and allergic disease are associated with disturbance of the intestinal microbial balance. This includes altered early colonization patterns and reduced bacterial diversity[1,2,3] Therefore, it seems logical to attempt to restore a more optimal pattern of microbiota.[4] Recently, Olszak *et al*.[5] showed that colonization of a conventional microbiota in neonatal—but not adult—germfree mice protected the animals from mucosal T-cell accumulation and related pathology. These results indicate that age-sensitive contact with commensal microbes may be critical for establishing mucosal natural killer T-cell tolerance to later environmental exposures.

Besides this evidence for a crucial role of early microbial colonization in animal models, a number of human randomized controlled trials assessing the potential benefit of probiotics, prebiotics or synbiotics have been performed with mixed outcome. A meta-analysis[6] concluded that there is some evidence that specific probiotics may reduce the incidence of eczema in infants at high risk of disease, but there is no evidence for any preventive effect on other allergic conditions or sensitization. Several clinical trials found no effect on the prevalence of asthma or asthma-like symptoms.[7-10] In most of these trials, *Lactobacillus* species were used. Because probiotic effects are strain-specific, other probiotic strains like bifidobacteria might be more efficacious, since specific prebiotics stimulating growth of bifidobacteria seem to lead to a significant reduction in the incidence of recurrent wheezing in two year old children.[11] Both the timing and duration of exposure, as well as targeted selection of microbiota might explain the differences between the promising results in animal models and the inconclusive results in humans. Additionally, the long-term effects of probiotics have scarcely been studied in placebo-controlled trials.

In 2009 we reported results of early and long-term administration of selected probiotics to high-risk infants. In these children, administration of a probiotic mixture consisting of *B. bifidum*, *B. lactis* and *Lc. lactis* (Ecologic®Panda) during pregnancy and during the first year of life resulted in a preventive effect on the incidence of eczema, but not atopic eczema (eczema and sensitization).[12] This preventive effect seemed to be established within the first 3 months of life, together with significant changes in the intestinal microbiota and decreased IL-5 production. No differences were observed in respiratory symptoms indicative for asthma or allergic rhinitis at the age of 2 years.

In this study, we hypothesize a possible beneficial effect of long-term perinatal administration of selected probiotic strains on the prevalence of asthma, allergic rhinitis and eczema in these high risk children at age 6, and we describe the long term follow-up of the children included in our previous clinical trial.

Materials and methods

Participants and treatment

For detailed information on the design of the initial randomized double-blind placebo-controlled trial, we refer to our previous publication.[12] All participating children had a positive family history of allergic disease, such as atopic eczema, food allergy, asthma or allergic rhinitis in either the mother, or the father plus an older sibling. The selected probiotic mixture, consisting of *B. bifidum* W23, *B. lactis* W52 and *Lc. Lactis* W58 (Ecologic® Panda, Winclove Probiotics B.V., Amsterdam, The Netherlands) or placebo was administered prenatally to pregnant women for the last 6 weeks of pregnancy and postnatally to their offspring during the whole first year of life. We approached all previous participants of the PandA study still willing to participate. They were 108 in total, of which all fully completed the initial prenatal intervention and 98 fully completed the initial postnatal intervention.

Study design

The original study was carried out during the first 2 years of life. After the child's second birthday, the parents were informed on the nature of the treatment their child was exposed to. Subsequently, follow-up of the participants was continued prospectively in a single-blinded (investigator blinded) design. At the child's age of 6 years, parents were asked to complete a slightly modified ISAAC questionnaire,[13] evaluating symptoms in their child indicative of food allergy, eczema, asthma or allergic rhinitis. The treating family doctor was asked for doctors diagnosed allergic diseases. Complete physical examination, blood sampling and lung function testing were performed. The study was approved by the Medical Ethics Committee of the University Medical Centre Utrecht, the Netherlands. Written informed consent was obtained.

Clinical outcome definitions

Current asthma at age 6 years was defined as at least one of the following four criteria: doctors diagnosed asthma active in the past 12 months, parental reported wheezing in the past twelve months, use of asthma medication in the past twelve months and/or an at least 9 percent reversibility in the forced expiratory volume in half a second (FEV0.5) or in one second (FEV1).[14] We defined current allergic rhinitis as doctor diagnosed allergic rhinitis active in the past twelve months or periodic or perennial sneezing and/or runny or blocked nose, when not having a cold or flu active in the past twelve months combined with allergic sensitization to inhalant allergens.[15] Food allergy was defined as doctor diagnosed food allergy, combined with sensitization to food allergens (cow's milk, egg white, soybean, wheat, peanut, hazelnut). Eczema was defined according to the Williams UK Working Party's criteria, i.e. an itchy skin plus 3 or more of the following: a family history of atopy, dry skin in previous 12 months, history of eczema, or visible eczema at typical sites. Sensitization was studied using measurement of specific IgE levels (IMMULITE 2000, Siemens Healthcare). Sensitization was defined as sIgE levels above 0.35 IU/ml. For inhalant allergens we tested house dust mite (*Dermatophagoides pteronyssinus*), grass

panel, tree panel, weed panel, mold panel, cat and dog. For food allergens we tested egg white, cow's milk, wheat, peanut, soybean and hazelnut.

Results

From the initial 123 participants evaluated in the former PandA-study, only 102 consented to be contacted for follow-up. Of these, a total of 84 participants were willing to participate in this follow-up study at the age of 6 years. Reasons for not willing to participate primarily were lack of time or priority. Baseline characteristics did not differ between the initial cohort and the patients who participated at follow-up. No differences were observed in basic descriptors between the placebo and intervention group (Table 1). Parental smoking habits also were comparable. Mean age at follow-up was 5,9 years, 40% were boys.

The prevalence of current asthma was 15.7%, with no statistically significant difference between the intervention group and the placebo group (Table 2). The prevalence of allergic rhinitis was 7.2%, with a trend to more allergic rhinitis in the intervention group (12.8% versus 2.3%, p=0.094). There was no statistically significant difference in the severity of allergic rhinitis between the group receiving probiotics and the group receiving placebo (data not shown). Also, the prevalence of food allergy and eczema did not differ significantly between the intervention group and the placebo group (Table 2).

Table 1. Baseline characteristics of the initial cohort and follow up cohort

	INITIAL COHORT		FOLLOW-UP COHORT	
Family characteristics	**Probiotic group (n=50)**	**Placebo group (n=52)**	**Probiotic group (n=40)**	**Placebo group (n=44)**
Maternal atopy	41 (84%)	44 (85%)	32 (82%)	39 (88%)
Age of mother at birth (years)	32.3	31.4	32.8	31.8
Older siblings				
- None	17 (34%)	18 (35%)	13 (32%)	15 (34%)
- One or more	33 (66%)	34 (65%)	27 (68%)	29 (66%)
Pets at home	11 (22%)	23 (44%)	14 (35%)	18 (41%)
- Cat(s)	8 (16%)	11 (21%)	8 (20%)	8 (18%)
- Dog(s)	3 (6%)	8 (15%)	4 (10%)	5 (11%)
- Bird(s)	0 (0%)	2 (4%)	1 (3%)	2 (5%)
- Other	2 (4%)	9 (17%)	5 (10%)	5 (11%)
Birth characteristics				
Male gender	18 (36%)	23 (44%)	16 (40%)	18 (41%)
Mode of delivery				
- Vaginal	46 (92%)	46 (89%)	36 (90%)	36 (86%)
- Caesarean section	4 (8%)	6 (11%)	4 (10%)	6 (14%)
Birth weight (grams)	3558	3658	3587	3688
Gestational age (weeks)	39.7	39.7	39.7	39.7
Breast feeding during first year of life	42 (84%)	41 (79%)	33 (87%)	35 (83%)
- Mean duration of breast-feeding (months)	7.0	7.2	6.8	7.1
Duration intake study products mother (weeks)	6.0	6.0	5.9	5.9

Table 2. Outcomes of allergic disease (% of children) at 6 years of age

	Probiotics (n=39)	**Placebo (n=44)**	**Relative Risk (confidence interval)**	**p-value**
Asthma	5 (12,8%)	8 (18,2%)	0.69 (0.24-1.93)	0,502
Allergic rhinitis	5 (12,8%)	1 (2,3%)	5.64 (0.68-46.2)	0,094
Food allergy	4 (12,1%)	2 (5,9%)	2.26 (0.44-11.6)	0,427
Eczema	13 (33,3%)	14 (31,8%)	1.05 (0.56-1.95)	0,883

Furthermore, all spirometry measures were essentially equal in both groups. We also could not identify any statistically significant differences between exhaled bronchial NO nor nasal NO between both groups (Table 3).

For sensitization rates, table 4 shows that overall sensitization did not differ between the probiotics group and placebo group. A trend was observed towards more sensitization in the probiotics group versus the placebo group. This seems to be related to a higher rate of sensitization to food allergens before the age of 5 years. This difference however, was not statistically significant. Persistent sensitization is only observed in a minority of children, with no relation to intervention. Also, the total IgE levels did not differ between both groups (probiotic group: mean 139 kU/l, (95% CI 32-245 kU/l, placebo group: mean 144 kU/l (95% CI 72-215 kU/l) , p = 0,942)).

Table 3. Outcomes of lung function at 6 years of age (mean values (SD))

	Probiotic group (n=37)	**Placebo group (n=40)**	**p-value**
FVC (% of predicted)	97 (14)	96 (13)	0,64
FVC reversibility (%)	2,7 (4,8)	1,1 (6,0)	0,22
FEV1 (% of predicted)	102 (12)	103 (11)	0,79
FEV1 reversibility(%)	5,6 (5,8)	3,5 (6,3)	0,13
FEV0,5 (% of predicted)	126 (21)	121 (18)	0,30
FEV0,5 reversibility (%)	8,7 (9,2)	8,0 (7,3)	0,72
MEF25-75 (% of predicted)	92 (20)	94 (26)	0,83
MEF25-75 reversibility (%)	0,25 (0,23)	0,20 (0,19)	0,40
FeNO	13,8 (14,3)	9,0 (3,5)	0,13
Nasal NO	510,9 (240,7)	437,3 (187,7)	0,30

Table 4. Development of sensitization at 6 years of age (%)

	Probiotic group (n=33)	Placebo group (n=34)
Any sensitization		
Never	13 (39%)	16 (47%)
Ever before age 6	8 (24%)	3 (9%)
At age 6 but not before	4 (12%)	6 (18%)
Persistent	8 (24%)	9 (26%)
Inhalant sensitization		
Never	25 (76%)	24 (71%)
Ever before age 6	0 (0%)	0 (0%)
At age 6 but not before	7 (21%)	9 (26%)
Persistent	1 (3%)	1 (3%)
Food sensitization		
Never	16 (49%)	23 (68%)
Ever before age 6	7 (21%)	2 (6%)
At age 6 but not before	6 (18%)	4 (12%)
Persistent	4 (12%)	5 (15%)

Column proportions do not differ significantly from each other at the 0.05 level.

Discussion

During 6 years follow-up of a randomized placebo-controlled trial we could not demonstrate any benefit on the development of allergic diseases at the age of 6 years from prenatal and one-year long postnatal use of a probiotic mixture consisting of *B. bifidum*, *B. lactis* and *Lc. Lactis* (Ecologic®Panda). Neither a significant effect was seen on asthma nor allergic rhinitis or food allergy. Interestingly, our results show a trend towards a higher prevalence of allergic rhinitis in the intervention group. Besides this, the positive effect on the prevalence of eczema, seen in the former study at the age of 2 years, was not present anymore at the age of 6 years. No differences in number of sensitizations nor grade of sensitization was observed between both groups.

Since the prevalence of allergic diseases, evaluated in our study, do correspond with the prevalence in high risk populations, we think our results do apply to this category. Our study results, showing no preventive effect of intervention with probiotics on allergic airway disease, are in line with other reports in literature.[16] To date, only two studies have investigated the effect of probiotics on allergic airway diseases such as asthma and allergic rhinitis at the age above 4 years, which we think to be the earliest age to make a firm diagnosis of asthma.

Kalliomäki *et al.* performed follow-up studies[7,8] of their initial study[17] cohort when the children were 4 and 7 years old. They found that asthma and allergic rhinitis tended to be more common in the probiotic group. We found a trend towards a higher prevalence of allergic rhinitis, but not asthma. Furthermore, Kalliomäki *et al.* found no difference in sensitization rates in both groups, as we did. We think our results for asthma are however more robust than theirs, since our diagnosis of asthma is based partially on objective measurements (lung function testing), whereas Kalliomäki based the diagnosis of asthma merely on (reimbursement of) prescription of asthma medication. For allergic rhinitis, we found an identical effect to what they found with the same definition of allergic rhinitis. Kuitunen *et al*. showed no effect of probiotics on asthma and allergic rhinitis at the age of 5 years in an elegant study with definitions of allergic disease comparable to ours in combining objective measurements with outcomes of questionnaires.[9] They found an identical prevalence of allergic rhinitis in both groups at the same age as our population. As we did, they demonstrated a temporary effect of probiotics on the prevalence of eczema. Abrahamson *et al*., who used *L. reuteri*, found no reduction of asthma (RR 1.16 (0.33-4.10),[18] neither did Wickens *et al*. for *L. rhamnosus* HN001 (RR 0.95 (0.62-145))[19] nor West *et al*. for *L. paracasei* spp *paracasei* F19 (RR 1.05 (0.39-2.81).[20] Overall, our present and all former studies fail to show a long term clinical benefit of early administration of probiotics on the development of allergic airway disease.

A relative strength of our study is the selection method of the applied probiotics. The selection of probiotics used in the other studies might be crucial to resort any clinical result. In contrast

to the studies of Kalliomäki *et al.* and of Kuitunen *et al.* our study is the first in which probiotic bacteria were selected on the basis of *in vitro* modulation of cytokine production for use in an intervention study. *Bifidobacterium bifidum*, *B. lactis* and *Lc. lactis* were selected because of their good IL-10-inducing capacity in vitro as well as efficient inhibition of IL-5 and IL-13.[21,22]

The duration and timing of treatment with probiotics might also play a crucial role in the long term effects. The intervention period with Ecologic® Panda in our study was one year, which is relatively long compared to other studies on this issue. There is only one other study[23] with an intervention period of one year or longer, while all other studies[8,9,11,24-28] had a much shorter intervention period (of three or six months postnatally). Although there are no studies that investigated the optimal length for an intervention period, we believe that the relative longer duration of our probiotic intervention further supports the reliability of our results. Besides this, starting supplementation of the probiotic intervention during pregnancy is another strength of our study, since prenatal start of the probiotics might be crucial to colonize mothers so that they transfer them to their offspring during vaginal delivery.

Also some weaknesses of our study need to be observed. One is the single-blind design of our randomized controlled trial, because parents were already aware of which intervention their child got during the initial study. This might have resulted in parental bias. We believe this will not have strongly influenced our outcome since both objective and subjective (parental) parameters failed to show any effect of the intervention. Furthermore, we had a loss to follow-up of 18% compared to the initially evaluated children, which might have induced some selection bias. However, the baseline characteristics of the initial group were identical to the baseline characteristics at the start of this follow up study, indicating that the loss to follow-up will not have significantly influenced our outcomes.

We conclude that a one-year long-term pre- and postnatal administration of a selected mix of probiotics (Ecologic® Panda) did demonstrate a beneficial effect on the development of eczema at the age of two years. In accordance with other studies published thus far, to beneficial effect does not extend to the age of 6 years and does not lead to primary prevention of asthma. Given the fact that development of the gut microbiota composition may continue for at least the first 3 years of life,[29,30] prolonged gut microbiota management may be required to achieve a long-lasting impact.

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7

LONG TERM DEVELOPMENT OF GUT MICROBIOTA COMPOSITION IN ATOPIC CHILDREN: IMPACT OF PROBIOTICS

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Abstract

Introduction Imbalance of the human gut microbiota in early childhood is suggested as a risk factor for immune-mediated disorders such as allergies. With the objective to modulate the intestinal microbiota, probiotic supplementation during infancy has been used for prevention of allergic diseases in infants, with variable success. However, not much is known about the long-term consequences of neonatal use of probiotics on the microbiota composition. The aim of this study was to assess the composition and microbial diversity in stool samples of infants at high-risk for atopic disease, from birth onwards to six years of age, who were treated with probiotics or placebo during the first year of life.

Methods In a double-blind, randomized, placebo-controlled trial, a probiotic mixture consisting of *B. bifidum* W23, *B. lactis* W52 and *Lc. Lactis* W58 (Ecologic® Panda) was administered to pregnant women during the last 6 weeks of pregnancy and to their offspring during the first year of life. During follow-up, faecal samples were collected from 99 children over a 6-year period with the following time points: first week, second week, first month, three months, first year, eighteen months, two years and six years. Bacterial profiling was performed by IS-pro. Differences in bacterial abundance and diversity were assessed by conventional statistics.

Results The presence of the supplemented probiotic strains in faecal samples was confirmed, and the probiotic strains had a higher abundance and prevalence in the probiotic group during supplementation. Only minor and short term differences in composition of microbiota were found between the probiotic and placebo group and between children with or without atopy. The diversity of Bacteroidetes was significantly higher after two weeks in the placebo group, and at the age of two years atopic children had a significantly higher Proteobacteria diversity ($p < 0.05$). Gut microbiota development continued between two and six years, whereby microbiota composition at phylum level evolved more and more towards an adult-like configuration.

Conclusion Perinatal supplementation with Ecologic® Panda, to children at high-risk for atopic disease, had minor effects on gut microbiota composition during the supplementation period. No long lasting differences were identified. Regardless of intervention or atopic disease status, children had a shared microbiota development over time determined by age that continued to develop between two and six years.

Introduction

The gastrointestinal tract is home to a diverse microbiota of about 10^{14} bacteria, representing up to 1500 bacterial species. The interaction between host and its microbiota contributes fundamentally to overall health.[1] Imbalance and disruptions of the human gut microbiota in infancy and early childhood have been suggested as a risk factor for a number of lifestyle-related and immune-mediated disorders such as atopic diseases, diabetes, obesity, necrotizing enterocolitis and inflammatory bowel disease.[2–6] The neonatal period is critical in terms of mucosal defense and immunologic priming and presence of aberrant gut bacteria during this time could therefore have profound effects on immune maturation.[2,3]

A reduction in overall diversity, a reduced abundance of commensal bacteria and an increased abundance of potentially pathogenic bacteria in the gut microbiota have been associated with the development of immune-mediated disorders later in life.[2,3,7] Data from observational studies are however conflicting, exemplified by a recent study showing more diverse microbiota in children with eczema[5] and with no clear allergy-promoting or allergy-protective taxa, as opposed to another study revealing that high diversity of total microbiota and high abundance of butyrate-producing bacteria are inversely associated with severity of atopic eczema.[8] Moreover, there is ongoing debate whether in allergy development an altered diversity of the gut microbiota is more important than the altered prevalence of particular bacterial species.[9]

Representative species and strains of lactobacilli and bifidobacteria have been used as probiotics, with the aim to colonize the infant's intestine and modulate the host's immune response.[10] Several studies have indeed shown benefits from treatment with probiotics in atopic children and thereby modulation of the infant's gut microbiota,[10–14] even by solely supplying mothers during late pregnancy.[15] Meta-analyses provide evidence in support of a moderate role of probiotics in the prevention of atopic dermatitis and IgE-associated atopic dermatitis in infants,[16] but more heterogeneous results are found for probiotics supplementation in the treatment of eczema.[17] Strain-specificity and the role for timing of administering probiotics are of particular importance, since some studies did not show any beneficial effect on the prevention of eczema after supplementation with probiotics.[3,5,18–21] Therefore, it seems crucial to administer probiotic supplementation during pregnancy and at least in the first months of life to be able to reasonably evaluate the effects of this intervention on microbial colonization of the gut and possible immunological effects.

The growing recognition of the role of gut microbiota in lifelong health and disease emphasizes the need of understanding the dynamics that lead to acquisition and colonization of intestinal microbiota. Moreover, it is important to study long-term effects of probiotic supplementation, both clinically and microbiologically. The aim of this study therefore was to assess the long-term effects of added probiotics on the composition and diversity of gut microbiota over time in infants at risk for atopic disease. Moreover, we aimed to investigate the differences in microbiota between children who did and did not develop atopic disease.

Methods

Participants and treatment

Subjects were part of a randomized, double-blind, placebo-controlled trial (the PandA-study, registered at ClinicalTrials.gov, Identifier NCT00200954) addressing the effect of pre- and postnatal administration of selected probiotic bacteria in primary prevention of allergic disease. More detailed information about the participants, received treatments and (long-term) clinical results were described in our previous publications.[11,22] In short, all participating children had a positive family history of allergic disease (i.e. atopic eczema, food allergy, asthma or allergic rhinitis in either the mother plus an older sibling, or the father plus an older sibling with a history of allergic disease) and either received a probiotic mixture consisting of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52 and *Lactococcus lactis* W58 (Ecologic® Panda, Winclove Probiotics B.V., Amsterdam, the Netherlands) or placebo (the carrier of the probiotic mixture, containing rice starch and maltodextran) during their first year of life. Moreover, the probiotic mixture or placebo was prenatally administered to the mothers. The intervention group received once daily $3 \cdot 10^9$ colony forming units (CFU) ($1 \cdot 10^9$ CFU of each strain) of freeze dried powder of the probiotic mixture. The control group received placebo consisting of the carrier of the probiotic product, i.e. rice starch and maltodextran. Both supplements were dispensed as a stable powder in identical individually packed sachets containing 3 g of material.

After the child's second birthday, the parents were informed about the nature of the treatment their child was exposed to. Subsequently, follow-up of the participants was continued in a single-blinded (investigator-blinded) design. When the child was approximately six years old, parents were asked to complete a follow-up visit during which clinical parameters were investigated. Parents were also asked to complete questionnaires evaluating symptoms in their child indicative of allergy. The definition of allergy in the initial study[11] and at the age of six years[22] was made and led to the classification of 'atopic' versus 'non-atopic' in this follow-up study. Atopic children should have had at least one of the following between age 0 and 6:

A positive doctor's diagnosis for eczema, asthma or allergic rhinitis Persistent sensitization to inhalant allergens and/or food allergens (>0.35 IU/ml allergen specific IgE antibodies)

Ethics Statement

The study was approved by the Medical Ethics Committee of the University Medical CenTER Utrecht, the Netherlands and has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Written informed consent from the parents was obtained.

Stool sample collection

Parents collected stool samples of their child at different time points between birth and the child's sixth birthday. Samples of 8 time points in early life were used: one week (T1), two weeks (T2), one month (T3), three months (T4), one year (one week after the intervention was stopped) (T5), one and a half year (T6), two years (T7) and six years of age (T8). Stool samples were collected from diapers or caught on a sheet, placed in stool collection vials and immediately frozen by the parents in their home freezers (-20 °C). Frozen samples were transported on ice to the hospital at time of follow-up visits. Upon arrival, samples were immediately stored at -20 °C until further analysis.

Isolation and identification of bacteria

To isolate DNA from faeces, a pea-sized fecal sample (100–400 mg) was placed in an Eppendorf container. Then, a 200 µl suspension was made in nucliSENS lysis buffer, as provided with the easyMAG, an automated system for total nucleic acid isolation (bioMérieux Clinical Diagnostics, Marcy l'Etoile, France). This suspension was vortexed for 1 minute, shaken for 5 minutes ≥ 1400 *rpm* and subsequently centrifuged at 14,000 *rpm* for 2 minutes. Supernatant (100 µl) was transferred to an 8-welled easyMAG container, as provided by the manufacturer, and 2 ml nucliSENS lysis buffer was added. After incubation at room temperature for ≥10 min, 70 µl of magnetic silica beads was added, as provided with the easyMAG machine. Afterwards, the mixture was inserted in the easyMAG machine and the "specific A" protocol was chosen, selecting the off-board workflow and eluting DNA in 110 µl of buffer. Faecal DNA was diluted 10-fold before use in PCR. All DNA was stored at 4°C.

16S-23S IS profiling of gut microbiota

The technique for determination of bacterial species in faecal samples and monitoring of microbiota development over time, deviates of the description in the initial study protocol. PCR and denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal DNA genes together with Fluorescent *in situ* hybridization (FISH) combined with flow cytometric analysis were performed at that time. In recent years however, the rapid development of sequencing techniques including the application of high-throughput methodology enables the analysis of hundreds of samples from different origins to conduct genetic audits of faecal material to a much greater depth than previously possible. Based on this, amplification of the interspace regions (IS-regions) was performed with the high-throughput bacterial profiling technique IS-pro (IS-diagnostics, Amsterdam, The Netherlands). This technique combines bacterial species differentiation by the length of the 16S-23S rRNA interspace region with instant taxonomic classification by phylum-

specific fluorescent labeling of PCR-primers. The 16S-23S rRNA IS region is variable in size and sequence, making it well suitable for analysis of complex communities. The procedure consists of two multiplex PCRs, a combination of which provides very broad coverage for *Actinobacteria, Firmicutes, Fusobacteria and Verrucomicrobia* (AFFV), *Bacteroidetes* and *Proteobacteria*. For detailed information on the design of the used primers we refer to our previous publication.[23] Amplifications were carried out on a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Cycling conditions for PCR were 94°C for 4 min; 94°C for 30 s; 35 cycles of 56°C for 45 s; 72°C for 1 min, 72°C for 11 min and a final extension at 4°C. Each PCR mixture, with a final volume of 25 µl, contained 10 µl of buffered DNA, 1x superTaq buffer (Applied Biosystems), 200 µM deoxynucleoside triphosphate, 0.04% BSA, 1 U of superTaq (Applied Biosystems), and 0.13 µM of each of the 5 primers.

Data analysis

After pre-processing, which consisted of baseline correction and noise-filtering of data using a standardized 'rolling ball-algorithm' (IS-pro software suite, IS-diagnostics, Amsterdam, he Netherlands), each sample was represented by a microbial profile, consisting of color-labeled peaks. Each peak was characterized by a specific IS fragment (measured in number of nucleotides) and a color related to a specific phylum group. The intensity of peaks reflected the quantity of PCR product (measured in relative fluorescence units (RFU)). Each peak was designated as an operational taxonomic unit (OTU) and its corresponding intensity as abundance. Intensity values were log2 transformed in order to compact the range of variation in peak heights, to reduce the dominance of abundant peaks and to include less abundant species of the microbiota in downstream analyses. The cutoff level was set to <6 logRFU.[23] This transformation results in improved consistency of the estimated correlation coefficient, lower impact of inter-run variation, and improved detection of less prominent species. This conversion was used in all downstream analyses, such as calculating within-sample and between-sample microbial diversity. A clustered heat map was constructed by generating a correlation matrix based on cosine correlations of all log2 transformed profile data followed by clustering with the unweighted pair group method with arithmetic mean (UPGMA).[24]

Diversity analysis and discriminative features selection

Diversity was calculated using the Shannon index,[25] representing diversity per time point, and differences in this index were tested with Mann-Whitney *U* test. Dissimilarities between samples, or between-sample diversity, were calculated using the cosine distance measure between each pair of samples' profiles. Given two vectors of attributes (two profiles in our case), A and B, the cosine dissimilarity is represented using a dot product and magnitude as:

$$\text{dissimilarity} = 1 - \cos(\theta) = 1 - \frac{\Sigma^{n}_{i=1} A_i \times B_i}{\sqrt{\Sigma^{n}_{i=1}(A_i)^2} \times \sqrt{\Sigma^{n}_{i=1}(B_i)^2}}$$

Principal coordinate analysis (PCoA), to explore similarities between groups, was calculated based on the cosine distance matrix. Discriminative OTUs between the groups were detected by LEfSe[26] with α parameter for pairwise tests set to 0.05 and the threshold on the logarithmic score of linear discriminant analysis (LDA) set to 2. Diversity analysis was performed using the vegan software package in R (Foundation for Statistical Computing, Vienna, Austria) and SPSS (SPSS for Mac release 22.0; SPSS Inc. Chicago, IL, USA). Differences were considered to be significant for $p < 0.05$.

Results

From the 123 participants evaluated in the initial PandA-study, 108 fully completed the initial prenatal intervention and 98 fully completed the initial postnatal intervention (until two years of age). 102 consented to be contacted for follow-up and a total of 83 participants were willing to participate in the follow-up study at the age of six years. Reasons for not willing to participate primarily were lack of time or priority. Clinical data of this follow-up study have been published previously.[22] In short, administration of the selected combination of probiotics did demonstrate a beneficial effect on the development of eczema up to the age of two years. This preventive effect was established within the first 3 months of life (12% parental reported eczema in the probiotics group vs 29% in the placebo group).[11] No differences were observed in respiratory symptoms indicative for asthma or allergic rhinitis at the age of 2 years. The beneficial effect on development of eczema did not extend to the age of 6 years and did not lead to primary prevention of asthma.[22] Comparison of baseline characteristics of the initial group to those of this follow-up group did not indicate selection bias due to selective drop-out. In total, of 99 children a stool sample at one or more different time points during the complete study period could be collected (until six years of age). A flow diagram of the initial clinical trial and follow-up studies is represented in Figure 1. It also shows how this follow-up study relates to the previous studies.

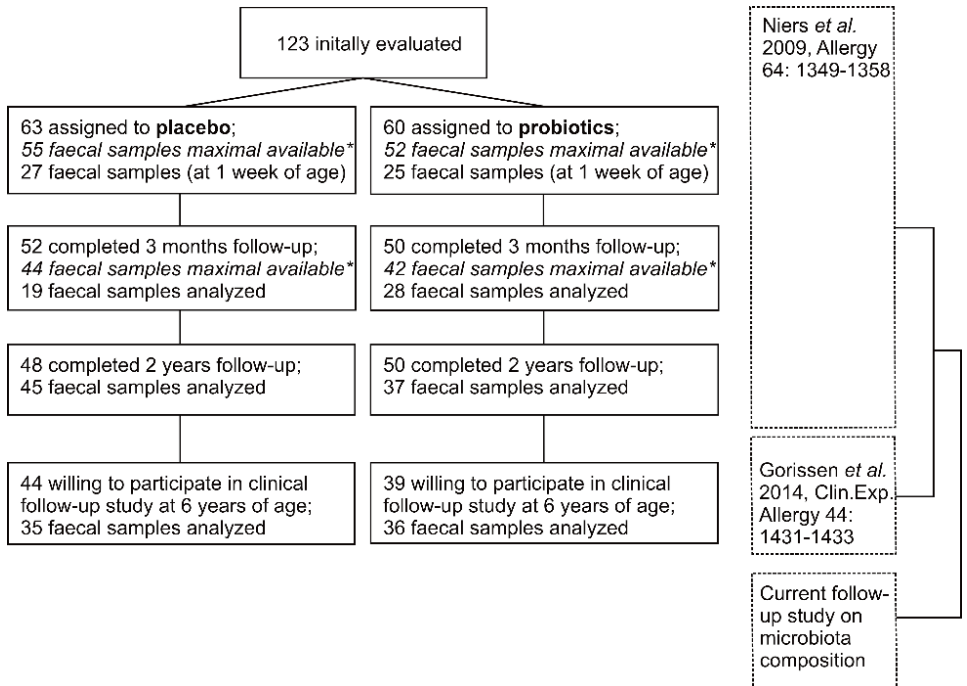

Figure 1. Overview of the inclusion of subjects in the initial PandA study and follow-up phases, including the number of faecal samples per time point.

***stool samples of eight randomly chosen participants from the placebo and intervention group at seven different time points, including those of one week and three months of age, were analyzed in the initial PandA study.

Relatively low percentages of subjects with faecal samples at early time points are due to the hectic postpartum period (one week of age) and previous analyses (in the original PandA study) that decreased the number of samples maximal available for the first five time points. The distribution of number of samples per time point, subdivided in probiotic/placebo and atopic/non-atopic groups, are shown in table 1.

Gut microbiota migration over time

First, we looked for general features of the development of gut microbiota composition between 0 and 6 years of age. The microbiota development was highly associated with age, as shown in Figure 2. Samples are most heterogeneous at early age and from there follow a more or less conserved vector towards a fairly homogeneous cluster at the age of six. Stability of the microbial community on phylum level was investigated by calculating average cosine distances for each phylum between two subsequent time points. Figure 3 gives a depiction of community stability over time and underlines that the largest variations in microbiota composition occur very early in life. Moreover, it can be seen that *Bacteroidetes* populations stabilize between one and two years of age, and variations clearly decrease from two years of age.

Table 1. Number of samples per time point per group (n, %).

	Placebo		Probiotic	
Time point	**Atopic**	**Non-atopic**	**Atopic**	**Non-atopic**
(T1) One week, *n = 52*	16 (30.8%)	11 (21.2%)	15 (28.8%)	10 (19.2%)
(T2) Two weeks, *n = 39*	9 (23.2%)	10 (25.6%)	10 (25.6%)	10 (25.6%)
(T3) One month, *n = 42*	9 (21.4%)	8 (19.1%)	14 (33.3%)	11 (26.2%)
(T4) Three months, *n = 47*	11 (23.4%)	8 (17.0%)	19 (40.5%)	9 (19.1%)
(T5) One year, *n = 63*	17 (27.0%)	13 (20.6%)	21 (33.4%)	12 (19.0%)
(T6) One and a half year, *n = 69*	23 (33.3%)	13 (18.9%)	22 (31.9%)	11 (15.9%)
(T7) Two years, *n = 82*	30 (36.6%)	15 (18.3%)	26 (31.7%)	11 (13.4%)
(T8) Six years, *n = 71*	24 (33.8%)	11 (15.5%)	24 (33.8%)	12 (16.9%)

T = time point

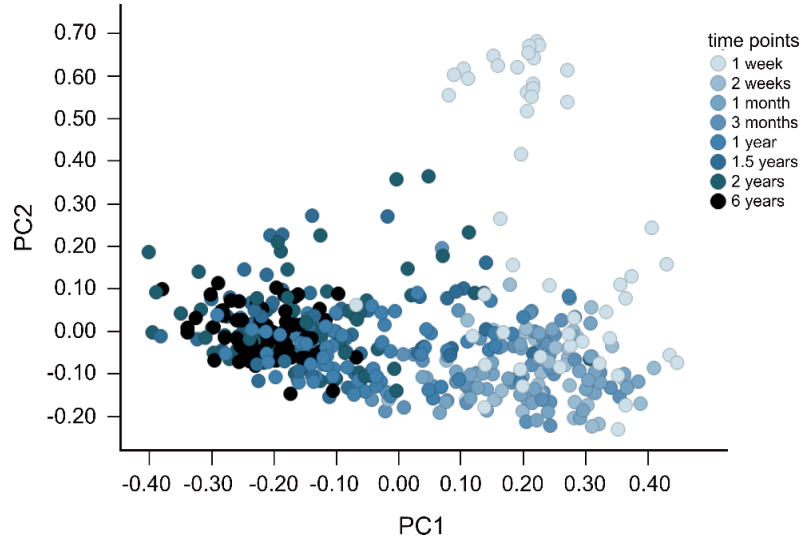

Figure 2. Principle coordinate analysis (PCoA) scatterplot to express gut microbiota development over time (T1-T8), all phyla combined, in the study population.
Smaller inter-individual distances indicate more similar microbiota composition. Color intensity (light to dark) reflects the age of a child at which the sample was taken.

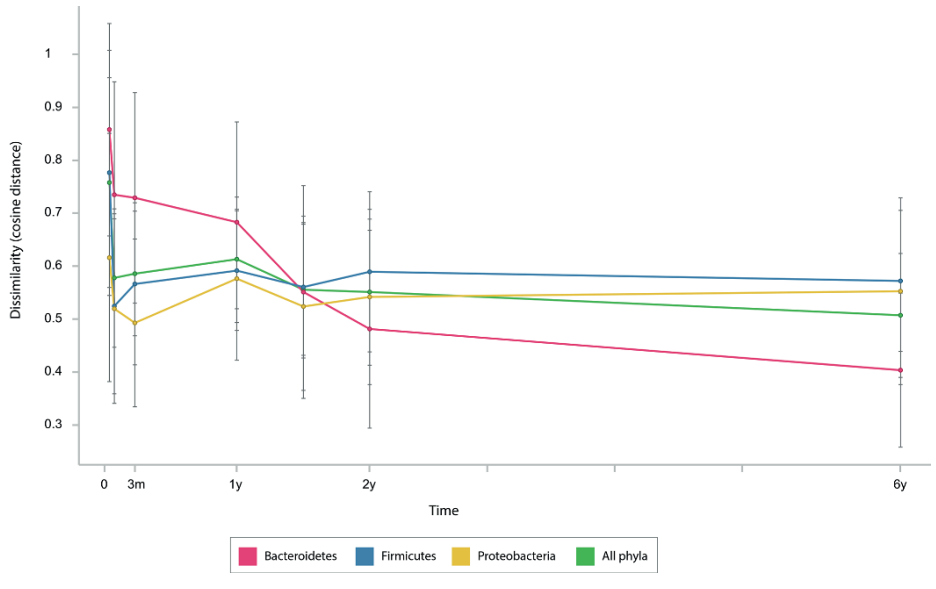

Figure 3. Microbiota stability over time.
Depicted are average cosine distances (y-axis) between two subsequent time points (x-axis) for each phylum and all phyla combined.

Effect of probiotic supplementation on gut microbiota composition

Probiotic versus placebo group

Secondly, we investigated whether the probiotic product, containing *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, and *Lactococcus lactis* W58 could be detected in the faecal samples. Indeed, the presence of the supplied product was confirmed. IS-pro could not discriminate between the two *Bifidobacterium* strains, since both gave a partially overlapping microbial profile.

At time of supplementation (T1-T5) the probiotic species had a higher abundance and prevalence in the probiotic group, whilst after stopping (T6-T8) prevalence was comparable between the two groups. Figure 4 shows the relative abundances per time point for the *Bifidobacterium* strains and *Lactococcus* strain. Bifidobacteria were significantly higher in the probiotic group at one month of age (T3, p= 0.003) and *Lactococcus lactis* was significantly higher at two weeks of age (T2, p= 0.001), and one month of age (T3, p= 0.03). Moreover, *Lactococcus lactis* was substantially absent in the placebo group during the intervention period and had a significantly higher abundance at the age of two years in this group (T7, p= 0.01).

Subsequently, we searched for potential effects of the intervention on microbiota composition at the species or genus level. By using LEfSe,[26] a method designed to explain differences between microbial communities, no bacterial species or genus that were significantly different between the treatment groups could be identified. At phylum level, there were no major differences between the probiotic and placebo group according to microbiota diversity (of *Bacteroidetes*, *Firmicutes* and *Proteobacteria*), except at the age of two weeks where the diversity of Bacteroidetes and Proteobacteria in the placebo group was higher (T2, p< 0.05) compared to the probiotic group (Figure 5A). Relative abundances per phylum in the probiotic group and placebo group, that show the similar pattern compared with diversity, are shown in supplementary Figure 1.

Figure 4. Relative abundance (y-axis) per time point (x-axis) of the probiotic strains B. Bifidum/B. Lactis and L. Lactis (dots) and error bars.
Marked bar on the x-axis indicates intervention period. *significant different time points.

Atopic versus non-atopic group

As described above, no major differences between the probiotic and placebo group could be identified according to microbiota diversity. The design of the study also allowed us to study potential differences in gut microbiota development in children who did and did not develop atopy in early life. Figure 5B shows diversity per phylum in the atopic and non-atopic groups. Diversity of all three phyla increased over time and no significant differences were found between the atopic and non-atopic group, except at the age of two years when atopic children had a significantly higher *Proteobacteria* diversity (T7, p< 0.001, Mann-Whitney U-test).

The relative abundance of the phyla *Firmicutes* and *Bacteroidetes* generally increased over time and followed a similar trend at all time points in the atopic and non-atopic group; no statistical differences were found (supplementary Figure 1). As *Proteobacteria* are amplified in a separate PCR reaction, their relative abundance could not be quantified together with the other two phyla. But in accordance with *Bacteroidetes* and *Firmicutes*, relative abundance of *Proteobacteria* increased over time.

At species or genus level, differences between the microbial community in the atopic and non-atopic group was tested using LEfSe,[26] but no discriminative bacterial species that were significantly different between the disease groups, could be identified.

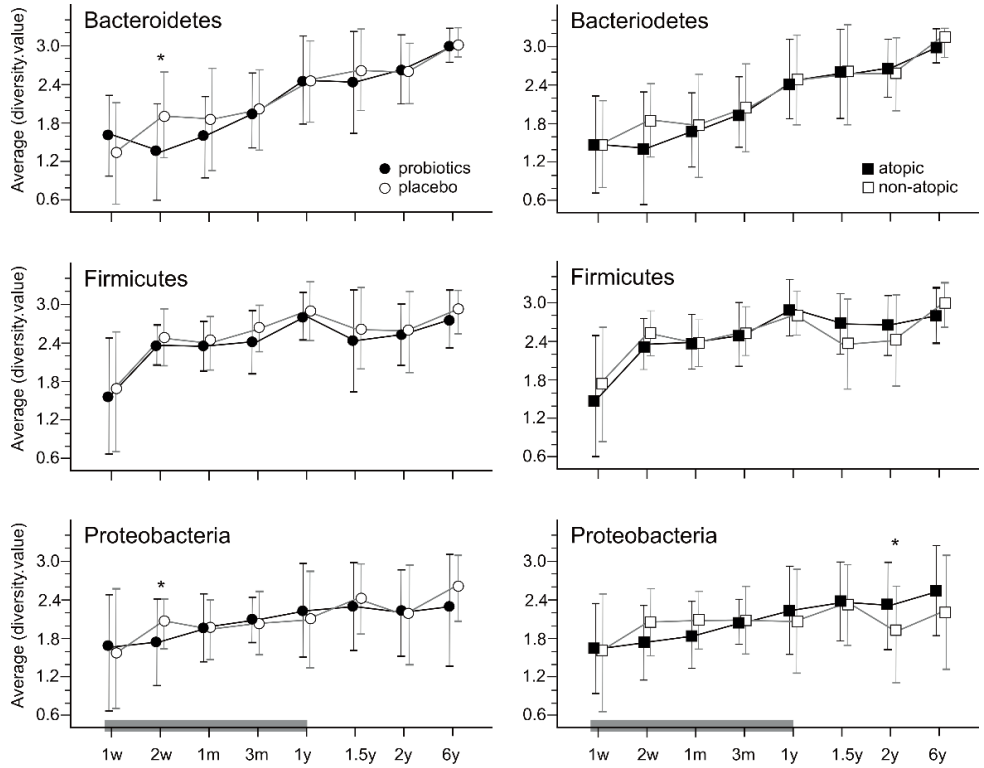

Figure 5. Diversity index (Shannon, y-axis) of the phyla Bacteroidetes, Firmicutes and Proteobacteria per time point (x-axis) for probiotics versus placebo group (A) and atopic versus non-atopic group (B) (dots, with error bars).
Marked bar on the x-axis indicates intervention period. *significant different time points.

Effects of probiotic intervention or disease status over age

Finally, we investigated the development of microbiota composition over age depending on the intervention or a child's atopic disease status. A similar development of a stable, robust microbiota composition over time was found in all subgroups, and therefore was not associated with later development of atopic disease or influenced by supplementation with probiotics (Figure 6).

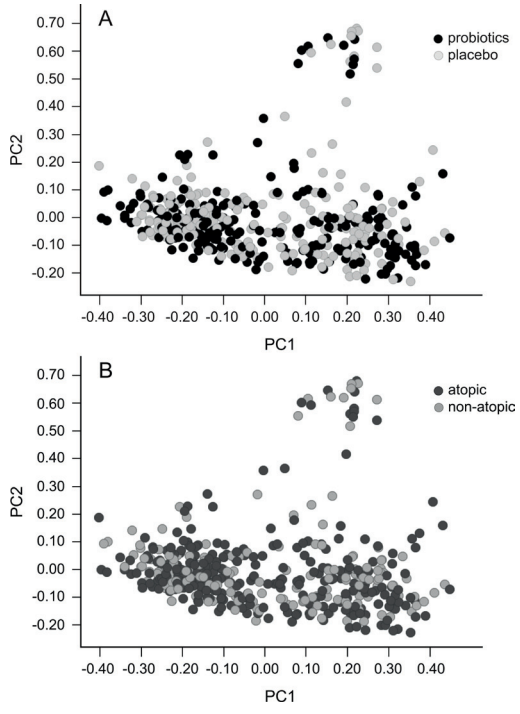

Figure 6. (Principle coordinate analysis) PCoA representing gut microbiota development.
Samples are grouped by intervention (A) and disease (B). Dots represent microbiota composition (all phyla combined) per individual per time point.

Discussion and Conclusion

In this study the long-term development of gut microbiota was investigated in infants at high-risk for atopic disease, who were supplemented with probiotics or placebo during the first year of life. Follow-up ended at the age of six years. Our data suggest that, regardless of intervention or atopic disease status, children develop a stable, converging gut microbiota during their first years of life. Age was the major driver of microbiota composition, overriding the differences based on intervention or development of atopic disease and the gut microbiome development is suggested to continue between 2 and 6 years of age.

In line with this finding, intestinal microbiota development is currently thought to stabilize by the end of the first year of life and the composition considered to resemble the adult gastrointestinal tract by the age of 2 to 2.5 years.[6,27–29] Koenig *et al*.[29] showed that the qualitative measures of diversity responded to time, but the quantitative measures (such as abundance) responded to life events (illnesses, dietary changes, antibiotic treatment). Yatsunenko *et al*.[30] described an adult-like configuration within a three-year period after birth and showed some differences in

clustering between very young children and adults. Results of Ringel-Kulka *et al*.[4] extend this difference (by describing significant differences in diversity between the group of 3–4 years old children and adults) and indicate that the microbiota has not yet reached the climax of bacterial diversity at the age of 4 years. The authors suggest that the evolvement into adult-like microbiota continues beyond 3 years of age. Our data support the last suggestion as the gut microbiome development seemed to continue between 2 and 6 years of age, though a specific time point for microbiome stabilization could not be indicated.

The presence of probiotic strains in stool samples was confirmed during supplementation. The fact that *Lactococcus lactis* was substantially absent in the placebo group during the intervention period is not surprising as the *Lactococcus* genus is not considered a commensal in infants. There was however a significantly higher presence and abundance of *Lactococcus lactis* at the age of two years (T7) in the placebo group compared to the probiotic group (9/45 versus 1/37) as we specifically analyzed the presence of this strain being part of the supplied probiotic product. This difference can be caused by the introduction of solid food and the variability of the children's diet at this age, because *Lactococcus lactis* is a well-known ingredient of buttermilk and cheese. When analyzing the prevalence of *Lc. lactis* at the age of two years in more detail, there was a tendency towards a higher prevalence in the atopic children within the placebo group (7/45) when compared to the non-atopic ones (2/45). This difference was however not significant after correction for multiple testing.

At the age of two weeks (T2) the diversity of Bacteroidetes was significantly higher in the placebo group compared to the probiotic group, a finding also described by Enomoto *et al*.[14] at the age of four months. This could suggest that probiotics suppress the acquisition of genera belonging to the phylum Bacteroidetes. Hypothetically, this could cause relevant disturbances on gut microbial patterns over time and have effects on clinical outcomes, since lower diversity of the phylum Bacteroidetes has been related to increased risk of asthma and atopic sensitization.[5,31] However, clinical results of the present study showed significantly lower eczema during the first 3 months of life in the intervention group compared with placebo, which does not point towards a predisposing role for the development of asthma and atopy in our population. Moreover, the difference demonstrated here seems to be without long-term consequences as microbiota composition of placebo and probiotic children showed no significant differences anymore during follow-up.

In this study no evident long-term consequences of probiotic supplementation on microbiota composition were found, as the abundance of probiotic strains decreased after the intervention and effects on diversity and abundance disappeared afterwards. Our results are in line with those described by Nylund *et al*.[5] who showed that *Lactobacillus rhamnosus* GG supplementation during the first 6 months of life, in children with and without eczema, had only minor long-term effect on the microbiota composition. Enomoto *et al*.[14] also demonstrated limited changes in

the composition of faecal microbiota after bifidobacteria supplementation during the first six months of life to healthy infants.

The exact in vivo mechanism of action of probiotics in shaping the immune response still needs to be determined and a number of unanswered questions remain regarding how probiotics mediate their clinical effects. The intestinal microbiome may contribute to the pathogenesis of allergic diseases due to its substantial effect on mucosal immunity. A probiotic may have a direct interaction with the ecosystem within the gut lumen by providing enzymatic activities that cause metabolic effects, it can interact with the gut lumen mucus and epithelium, and may be signaling the host to other organs beyond the gut.[32]

The design of the present study allowed us also to study potential differences in gut microbiota development in children who did and did not develop atopy. Variations in early gut colonization have been associated with the development of atopic disease. Results have, however, been highly variable across studies. Wang *et al.*[7] examined overall patterns of faecal microbial colonization in healthy and atopic infants and found that infants who developed eczema had significantly lower faecal bacterial diversity at 1 week of age. Others have confirmed that reduced gut microbial diversity in early life was associated with an increased risk of eczema[9,33] and multiple differences in specific bacterial groups result in microbiota profiles that are significantly distinct between healthy and eczematous infants.[5]

Johansson *et al.* showed that the kinetics of colonization postnatally seemed to differ generally in non-allergic children compared to allergic children, with a delayed colonization in early infancy in the allergic group. At 12 months of age however, the groups of children were similar in the frequencies of the different species investigated. Bisgaard *et al.*[2] described a population of high-risk infants comparable to the present study and showed an association between reduced bacterial diversity of the infant's intestinal microbiota with increased risk of allergic sensitization, allergic rhinitis and peripheral blood eosinophilia, but not asthma or atopic dermatitis, in the first 6 years of life.

In contrast to the above findings, in our study, abundance and diversity were the same over time in atopic and non-atopic children though small differences were seen. This is in accordance with a prospective study from 3 European birth cohorts that also found no differences in gut microbiota among infants developing or not developing atopic eczema and food allergy.[34]

Differences in findings between all these studies, including ours, may reflect differences in methods used for investigating the faecal microbiota or even differences in defining atopy. Our study does endorse the importance of the gut microbiota composition in early life, because of the beneficial clinical effect of probiotics on eczema,[11] but besides effects on gut microbiota composition during the intervention, no long lasting differences could be identified. In this

respect, recent data point towards a major role for short chain fatty acids production, especially butyrate, in regulation of development of mucosal regulatory T cells. Therefore, next to the 16S-23S rRNA gene analysis, as performed in this study, microbial metagenomics (whole genome shotgun sequencing) could further expand the understanding of gut microbiota development and composition and microbial metabolomics may reveal mechanisms by which gut microbiota interacts with the human host.[35,36]

In conclusion, supplementation with a probiotic mixture consisting of *B. bifidum* W23, *B. lactis* W52 and *Lc. Lactis* W58 to children at high-risk for atopic disease had only minor effects on gut microbiota composition. Future studies should identify the functional activities of the gut microbiota, but also further elucidate the working mechanisms of probiotics, to illustrate host and microbiota interactions and identify optimal timing and duration of probiotic supplementation as strategy for prevention of allergic diseases.

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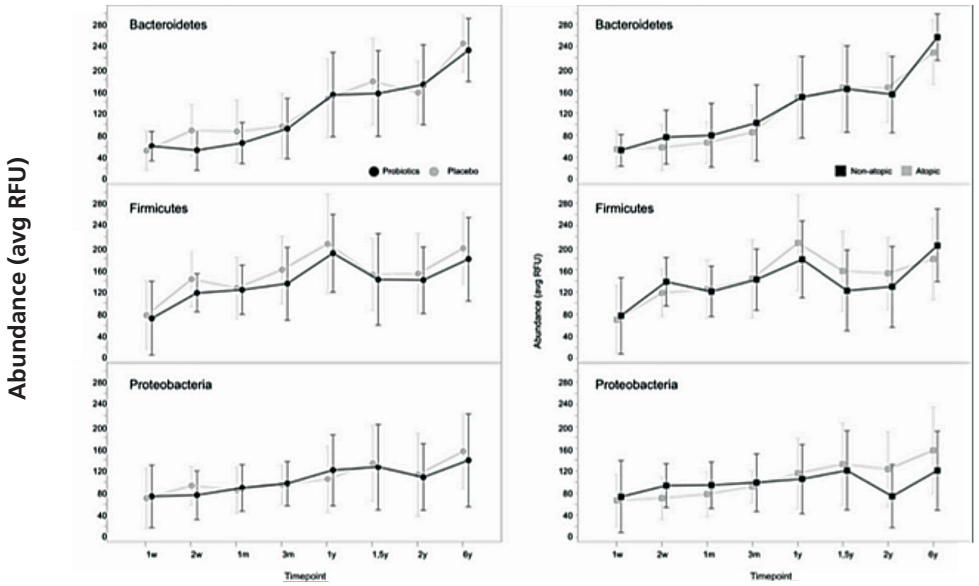
Supplementary figure



Supplementary figure 1. **Average abundances of the phyla Bacteroidetes, Firmicutes and Proteobacteria per time point (x-axis) for probiotics versus placebo group and atopic versus non-atopic group (dots/squares, with error bars)**

8

PROBIOTIC SUPPLEMENTATION INFLUENCES FAECAL SHORT CHAIN FATTY ACIDS IN INFANTS AT HIGH RISK FOR ECZEMA

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Abstract

The composition of the gut microbiota plays a role in the development of allergies. Based on the immunomodulating capacities of bacteria, various studies have investigated the potential role for probiotics in the prevention of childhood eczema. In a previous study we have shown that significantly less children developed eczema after probiotic supplementation (Bifidobacterium bifidum W23, Bifidobacterium animalis subsp. lactis W52 and Lactococcus lactis W58, Ecologic®Panda) at three months of age as compared to controls. Here, metabolites in faecal samples of these 3-month old children were measured by ^{1}H-NMR to investigate possible gut metabolic alterations. Lower amounts of short-chain fatty acids (SCFAs), succinate, phenylalanine and alanine were found in faecal samples of children later developing eczema, whereas the amounts of glucose, galactose, lactate and lactose were higher compared to the children not developing eczema. Although these differences were already present at the age of 3 months, eczema did not develop in the majority of children before the age of 1 year. Supplementation of multispecies probiotics seems to induce higher levels of lactate and SCFAs, and lower levels of lactose and succinate when compared with the placebo group. This might explain the temporary preventive effect of probiotics on the development of eczema. These results highlight the role bacterial metabolites may play in development of the immune system, even before clinical manifestations of allergic disease arise.

Introduction

The worldwide prevalence of allergic diseases such as eczema, food allergy, asthma and allergic rhinitis has increased considerably in the past few decades.[1,2] Up to 40% of the population of the USA and Europe suffer from at least one type of allergy and the prevalence of allergies in industrial societies has doubled over the past 15 years.[3] Amongst these, eczema is one of the most common inflammatory skin diseases in childhood. The etiology of allergic diseases is unknown although it has been well established that both genetic and environmental factors do play a role. There is growing evidence that the composition of gut microbiota is a crucial environmental factor in the development of allergic disease. The development of allergies has been linked to reduced microbial exposure in early childhood, reduced bacterial diversity and an altered composition of gut microbiota.[3-6] In this context, the window of opportunity for influencing the composition of the intestinal microbiota and possibly modulate the development of allergic diseases, would be the perinatal period. The composition of the gut microbiota in infants is influenced by several environmental factors, including mode of delivery, nutrition, medication such as antibiotics and by the supplementation of pre- and probiotics.[7] Probiotics are living microorganisms that, when administered in adequate amounts, confer a beneficial health effect on the host.[8,9] The most widely used probiotic bacteria belong to the *Lactobacillus* and *Bifidobacterium* genera. Certain probiotics have shown to be effective in the prevention and/or management of disorders such as necrotizing enterocolitis, acute infectious diarrhea, antibiotic-associated diarrhea, inflammatory bowel disease (pouchitis and ulcerative colitis) and lactose maldigestion.[10,11] Various studies have investigated the potential of probiotics, both for primary prevention and for treatment of eczema and other atopic diseases. Results are inconsistent; while some studies have shown a preventive effect of probiotics on the development of eczema, others showed no effects.[12,13] This inconsistency could be explained by differences in study design, amount and duration of probiotic supply, and the strain-specific effects of probiotic bacteria. Moreover, the biological mechanisms involved in successful clinical outcome of probiotic supplementation on the prevention of eczema still remain unknown.

One of the potential mechanisms by which probiotic bacteria could regulate the development of the mucosal immune system is stimulation of the differentiation of naive T-cells towards T-helper 1 cells (Th1) or regulatory T-cells, thereby shifting the balance between Th1 and T-helper 2 cells (Th2). In our clinical trial (the Panda-study),[14] a mixture of three probiotic strains was administered to infants at high-risk for atopy, from birth onwards during the first year of life. The probiotics were also given to the mothers during the last 6 weeks of pregnancy. The selection of probiotic strains was based on the ability to down-regulate Th2 cytokine production *in vitro* and to induce regulatory T-cell cytokines.[15] Clinical supplementation of the multi-species probiotic product proved to have a significant preventive effect on the development of eczema in high-risk children within the first three months of life.[14] This clinical difference was maintained up to the age of two years, although it lost significance. Recently, the long-term outcomes of this intervention were published,[16] showing that the beneficial effect was not extended to the

age of 6 years and did not lead to the primary prevention of asthma, in accordance with other studies published so far.[17] The biological pathways that mediate the clinical effects of probiotics are still unclear and it remains to be demonstrated whether immunoregulatory properties are an important mechanism *in vivo*. In order to gain insight into the biological mechanisms underlying the clinical outcome of probiotics, an analysis of bacterial metabolites was made. To that end the faecal metabolic profiles of the eczema-prone children in the Panda-study were investigated. The aim of this study was to identify potential differences in gut metabolites between children at-risk for atopic disease, that later developed eczema and those that did not develop eczema. Out of the several techniques available for metabolic profiles, ^{1}H NMR spectroscopy was used. The advantages of NMR are robustness, reproducibility and the absence of extensive sample preparation , a disadvantage is the lower sensibility compared to other metabolomics techniques like mass spectrometry.[18-21] It thus has been demonstrated that the metabolite profiles of gut microbiota can be constructed by targeted profiling of NMR spectra.[22,23]

Methods

Design of study

A double blind, randomized, placebo-controlled trial as described in detail by Niers *et al*. was performed.[14] Briefly, pregnant women and their offspring (with a positive family history of allergic disease) received either once sachet daily with 3 grams of probiotics (Ecologic®Panda, Winclove Probiotics, the Netherlands) or a placebo during the last six weeks of pregnancy (mothers) and during the first 12 months after birth (infants). The probiotic product contained the bacterial strains *Bifidobacterium bifidum* W23, *Bifidobacterium animalis* subsp. *lactis* W52 and *Lactococcus lactis* W58, a total 1x10^{9} colony forming units per strain per day) In total 123 participants were included (per protocol) in the clinical study (63 assigned to the placebo, 60 to the probiotic group). In the placebo group 52 participants and in the probiotic group 50 participants completed the 3 months clinical follow-up. Follow-up continued up to six years of age, investigating the clinical manifestation of eczema, IgE sensitization and, in time, allergic rhinitis and asthma. Blood samples and faecal samples were collected at different time points during follow-up.

Clinical outcomes

Children were clinically examined at the age of 3, 12, 24 months and 6 years of age, as described previously.[14,16] For the present study, we grouped the children according to the presence or absence of eczema till two years of age. Parental-reported eczema was defined as eczema reported by parents, in diaries that they were asked to complete weekly for complaints of eczema, infectious or atopic symptoms, feeding habits and use of medication. Doctor-diagnosed eczema was defined as clinical signs of eczema diagnosed by the family doctor or consulted physician at the time of visit to the doctor's office or outpatient clinic.

Fecal sample collection

As the clinical effects were significantly different between the probiotic and the placebo group at three months of age, faecal samples of this time point were chosen to perform metabolite analysis on. The faecal samples (n=34) were collected directly from the diapers by the parents and stored at -20 degrees Celsius until analysis. Only samples from children of whom complete information on disease development was recorded were analyzed, resulting in a different number of samples for each group (Group A, placebo eczema n=9, group B, probiotic eczema n=9, group C, probiotic no eczema n=9, group D, placebo no eczema n=7.)

Fecal sample extraction

Fecal extracts were prepared by mixing 20 mg of frozen faecal material with 1 mL of phosphate buffered saline that consists of 1.9 mM Na_2HPO_4, 8.1 mM NaH_2PO_4, 150 mM NaCl and 1 mM TSP (sodium 3-(trimethylsilyl)-propionate-d_4) in D_2O (Le Gall *et al.*, 2011). After mixing thoroughly, samples were centrifuged (17,000 g, 5 min). The supernatant was filtered through a 0.2 μm membrane filter and 600 μL of the filtrate was transferred to a 5 mm NMR tube for analysis.

NMR analysis

High resolution ^{1}H NMR spectra were recorded using a Bruker AV 600 spectrometer (Bruker, Karlsruhe, Germany). Sample temperature was controlled at 25 °C. Each spectrum consisted of 128 scans and *noesypr 1d* pre-saturation sequence was used to suppress the water signal with low power selective irradiation at the water frequency during the recycle delay (D1 = 2s) and mixing time (D8=0.15 s). A 90° pulse length of 8.2 μs was set for all samples. ^{1}H NMR spectra were Fourier transformed (LB = 0.3 Hz). After zero filling, each spectrum was manually phased and baseline corrected using TOPSPIN 3.0 software. All resonances of metabolites were confirmed by comparison with reported data[24] or by 2D NMR spectroscopy (J-resolved, COSY and HMBC).

Data analysis

The AMIX software (Bruker) was used to reduce the ^{1}H NMR spectra to an ASCII file, with total intensity scaling. Bucketing or binning was performed and the spectral data were reduced to include regions of equal width (0.04 parts per million) equivalent to the region of δ 10.00–0.40. The regions of δ 4.88–4.64 were not included in the analysis because of the remnant D_2O signal. Due to the complexity of the NMR spectra, it was necessary to apply multivariate data analysis to explore differences between the groups. In this case, we applied orthogonal partial least squares-discriminant analysis (OPLS-DA) which is a supervised multiple regression analysis similar to PLS-DA, but by orthogonolizing non-correlated variables.[25] Principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis were performed with the SIMCA-P+ software (v. 13.0, Umetrics, Umeå, Sweden). The individual short chain fatty acids were quantified and differences between the groups were tested by ANOVA as described previously.[26]

Results

NMR analysis was performed for 34 samples. One sample from the placebo eczema group showed signals corresponding to N-acetyl-*p*-aminophenol (Acetaminophen®), probably administered as an analgesic or antipyretic drug. This sample was excluded from further data analyses. There were no significant differences in baseline characteristics between groups (Table 1). A total of 33 faecal samples were classified according to the presence or absence of parental-reported eczema. In a number of cases the reported eczema was confirmed by a doctor (doctor's diagnosis) (Figure 1). Within the eczema group, consisting of 8 children who received placebo (group A) and 9 children who received probiotics (group B), the majority of children did not have eczema at the moment of stool sampling (3 months) but developed symptoms later (Figure 1). In the non-eczema group (group C (probiotics) n=9 and group D (placebo) n=7), none of the children showed clinical signs of eczema at any of the time points indicated.

Table 1. Baseline characteristics of study participants

Group		N	Gestational age in weeks (mean ± SD)[a]	Birth weight in kg (mean ± SD)[a]	Mode of delivery (caesarean section/ total)	Feeding type
A	Placebo, eczema	8	40.5 ± 1.8	3.9 ± 0.4	2/8	7 breast, 1 mixed
B	Probiotics, eczema	9	39.3 ± 1.8	3.4 ± 0.4	0/9	5 breast, 2 bottle, 2 mixed
C	Probiotics, no eczema	9	40.5 ± 1.7	3.8 ± 0.4	1/9	4 breast, 2 bottle, 3 mixed
D	Placebo, no eczema	7	40.0 ± 1.4	3.8 ± 0.8[b]	0/7	3 breast, 3 bottle, 1 mixed

[a] SD = standard deviation

[b] Data available from 6 infants. No significant differences, as measured by nonparametric repeated measures ANOVA, were detected between groups in gestational age, birth weight, mode of delivery or type of feeding.

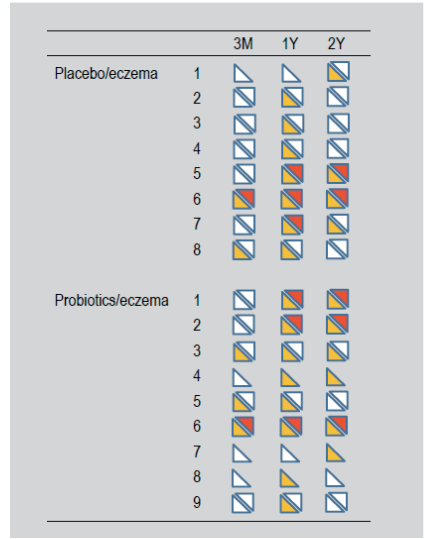

Figure 1. Overview of the eczema score of the participants.
A missing triangle means that no information was available on doctor's diagnosed eczema. Open lower triangles: no parental reported eczema; closed lower triangles: parental reported eczema; open upper triangles: no doctor's diagnosed eczema; closed upper triangle: doctor's diagnosed eczema.

Differences in faecal metabolites between the eczema and non-eczema group

^{1}H NMR spectroscopy of faecal extracts revealed the presence of various metabolites such as lactate, SCFAs (butyrate, propionate, acetate), amino acids (alanine, valine, leucine, phenylalanine), sugars (glucose, galactose, xylose) and sugar alcohol (glycerol). The major identified metabolites and its chemical shifts found in faecal samples are listed in Table 2.

Comparison of the ^{1}H NMR spectra, analyzed by OPLS-DA, showed separation of the groups based on presence or absence of parental reported eczema (R^2= 0.7, Q^2 = 0.013, Figure 2A). The R^2 value estimates the goodness of fit, whereas the Q^2 value is a measure of the quality in multivariate models. A Q^2 value > 0.5 is considered large, so our observed differences were relatively small. Lower amounts of SCFAs (butyrate, acetate), alanine, succinate, and phenylalanine were detected in the faecal samples of the eczema group, whereas the amounts of glucose, galactose, lactose and lactate were higher in the eczema group when compared to the non-eczema group (Figure 2B). As expected with the low Q-value we observed, groups could not be discriminated based on a single metabolite, as displayed by the non-significant differences between groups (table 3).

Table 2. Chemical shifts of metabolites found in faecal samples

Metabolite	Chemical shift (δ)[1]
n-butyrate	0.90 (t), 1.56 (m), 2.16 (t)
Isovalerate	0.91 (d), 2.06 (d)
Isoleucine	0.95 (t), 1.02 (d)
Leucine	0.96 (d), 0.97 (d)
valine	1.00 (d), 1.05 (d)
propionate	1.06 (t), 2.19 (q)
Lactate	1.33(d), 4.11 (q)
threonine	1.33 (d), 4.26 (m)
Isobutyrate	1.07 (d), 2.39 (m)
Alanine	1.48 (d), 3.79 (q)
Acetate	1.92 (s)
glutamate	2.11 (m), 2.36 (m), 3.76 (dd)
Lysine	1.48 (m), 1.73 (m), 1.91 (m), 3.03 (t), 3.77 (t)
aspartate	2.68 (dd), 2.82 (dd), 3.90 (dd)
succinate	2.42 (s)
trimethylamine	2.91 (s)
Taurine	3.27 (t), 3.44 (t)
α-glucose	5.24 (d), 3.53 (dd)
β-glucose	4.65 (d), 3.25 (dd)
Glycine	3.57 (s)
Fumarate	6.52 (s)
Uracil	7.54 (d), 5.80 (d)
phenylalanine	7.44 (m), 7.38 (m), 7.32 (d)
Tyrosine	7.20 (d), 6.90 (d)
4-hydroxyphenylacetate	7.19 (d), 6.86 (d), 3.44 (s)
Formate	8.45 (s)
Adenine	8.23 (s), 8.19 (s)
α-galactose	5.27 (d)
β-galactose	4.59 (d)
α-xylose	5.20 (d)
β-xylose	4.58 (d)
Glycerol	3.57 (dd), 3.67 (dd), 3.79 (m)
Lactose	5.22 (d), 4.46 (d), 4.67 (d)

1 d= doublet, dd = double doublet, q= quartet , s = singlet, t= triplet, m= multiplet.

Table 3. Concentrations short chain fatty acids (SCFAs) for the different groups.[1]

	Acetate	Propionate	n-butyrate	total SCFA
Eczema group	0.48 ± 0.42	0.08 ± 0.09	0.10 ± 0.10	0.65 ± 0.56
Non-Eczema group	0.52 ± 0.39	0.10 ± 0.08	0.10 ± 0.11	0.72 ± 0.46
	p= 0.75	**p=0.34**	**p=0.96**	**p=0.68**
Probiotic group	0.50 ± 0.27	0.11 ± 0.09	0.13 ± 0.12	0.74 ± 0.43
Placebo group	0.49 ± 0.52	0.07 ± 0.06	0.07 ± 0.08	0.63 ± 0.61
	p = 0.95	**p=0.19**	**p= 0.13**	**p=0.57**

1 The concentrations are given in arbitrary units ± standard deviation

For 21 out of 33 children (64%) of whom data on parental reported eczema were available, there was a corresponding doctor's diagnosis. A separate OPLS-DA analysis for eczema versus non-eczema based on doctor's diagnosis did not show significant differences (Q^2 value negative, data not shown).

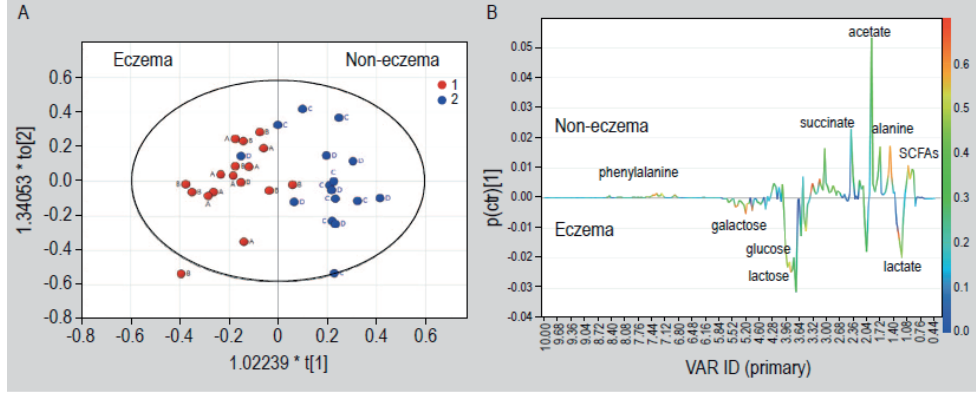

Figure 2. Orthogonal partial least squares-discriminant analysis of faecal samples between eczema group (group A+B, n= 17, red circles [1]) and non-eczema group (group C+D, n= 16, blue circles [2]), based on parental reported eczema. A) scatter plot, B) s-line plot. R^2Y= 0.691, Q^2Y= 0.013.

To determine the effect of probiotic supplementation on identification of specific gut metabolites, we analyzed the metabolic profiles of the probiotic and placebo group. In OPLS-DA, the probiotic group could be separated from the placebo group (R2= 0.7, Q2 = 0.3, Figure 3A). The probiotic group showed higher levels of lactate and SCFAs (acetate, butyrate, propionate, isobutyrate) and lower levels of lactose and succinate (Figure 3B).

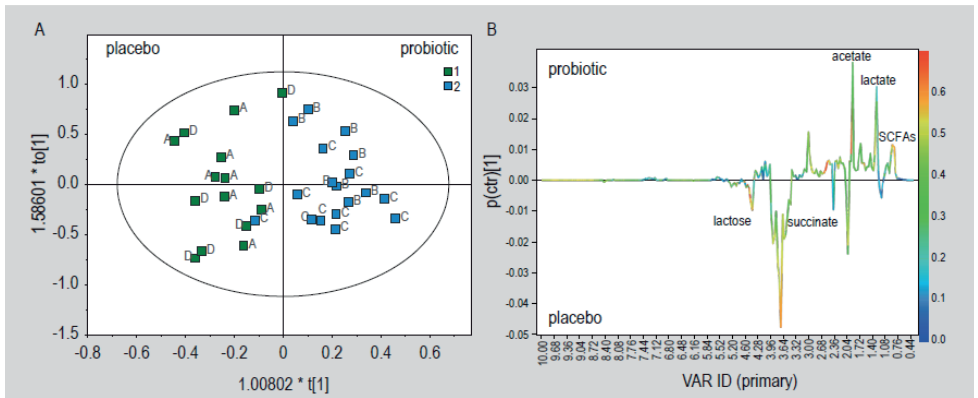

Figure 3. Orthogonal partial least squares-discriminant analysis of faecal samples between placebo group (group A+D, n= 15, green squares [1]) and probiotic group (group B+C, n= 18, blue squares [2]). A) Scatter plot, B) S-line plot. R^2Y= 0.748, Q^2Y= 0.296

Discussion

SCFAs were found to be the major discriminating metabolites between infants at-risk for developing atopy, expressed as eczema. Some SCFAs, mainly acetate, propionate and butyrate, are end-products of the breakdown of carbohydrates by gut microbial action. Among the SCFAs, butyrate is important for maintaining a normal colonocyte population.[27] Moreover, it has anti-inflammatory properties by the reduction of different pro-inflammatory cytokine expression and signaling, induction of nitric oxide synthesis and metalloproteinases and the reduction of lymphocyte proliferation and activation.[28] The potential role of SCFAs in eczema has been addressed before: Bottcher *et al.* showed lower levels of different SCFAs as compared with non-allergic controls in one-year old allergic children.[29] Another study showed the severity of eczema to be inversely correlated with the amount of butyrate producing bacteria.[30] In our study, the eczema group consisted of children that developed eczema in the first two years of life. By analyzing the 3 month-old samples, when the majority of children had not yet developed eczema, the demonstrated low levels of SCFAs seem to precede the clinical manifestation of eczema. Sandin *et al*. have shown before that children with positive skin prick tests and allergic

symptoms during the first four years of life, also revealed lower levels of SCFAs preceding the clinical manifestation of allergy.[31] Allergic diseases can be regarded as Th2 immune-mediated diseases in which regulatory T-cell function is impaired.[1] Regulatory T-cells therefore are a major target for preventive or therapeutic intervention. Recent data indicate that SCFAs, in particular butyrate and acetate, can directly promote regulatory T-cell generation in the colon of mice.[32,33] Thus, differences in faecal SCFAs levels of infants who did and did not develop eczema could provide the missing link between gut microbiota, the mucosal immune system and the development of eczema. Elucidating the role of SCFAs could also lead to a better understanding of the biological mechanism for functional effects of probiotic bacteria.

Our data indicate that early life supplementation of specifically selected probiotic strains might influence SFCA production, including butyrate. In model systems of the human gut it has been shown that certain probiotics and synbiotics can influence SCFAs levels,[34] a finding that supports the data presented here. There are also indications that probiotics can increase the amounts of SCFAs in healthy adults *in vivo*, without changing the overall faecal microbiota composition.[35,36] On the other hand, another study addressing the effects of *L. acidophilus* 74-2 and *B. animalis* subsp *lactis* DGCC 420 on faecal microbiota in healthy adults failed to show any effect on faecal SCFAs levels.[37] In accordance with that study, no increase of SCFAs could be indicated, as in a mouse model with humanized infant microbiota supplementation with *Lactobacillus paracasei* NCC2461 and *L. rhamnosus* NCC4007 resulted in a decrease in acetate and butyrate.[38] However, it is difficult to generalize results of these studies, as the effects of probiotics supplementation, including metabolic effects, can be highly strain-specific.

Naturally, when investigating gut metabolites, composition of and changes in the microbial population are of great importance. This was initially evaluated by MCPC, which is a qualitative analysis based Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis.[14] This analysis showed significantly more frequent colonization with higher numbers of *Lc. lactis* in the probiotic group compared with the placebo group during the first 3 months of life. Moreover, no differences were observed in the first 4 weeks of life in the number of children colonized by bifidobacteria but at the age of 3 months, all children in the probiotic group and 85% of the placebo group were colonized with bifidobacteria. *Lc. lactis* was shown to be present in all fecal samples from the intervention group and in significantly higher amounts, and it was present only in 2/8 samples from the placebo group. Bifidobacterium spp. were present in all of the individuals in high numbers.[14] The overall microbiota composition did not show major differences.[14,39] We hypothesize the observed difference in SCFA production most probably is indirect, as none of the probiotic strains included in the used probiotic product is a butyrate producer itself. How this effect is induced cannot be concluded directly from our data. It could be affected by upregulation of the number of butyrate-producing bacteria in the gastrointestinal tract or by increased metabolic activity in general. The main limitation of this study was the small sample size. However, even with these small numbers of participants, differences between groups could be demonstrated. Based on the data presented here, a suggested mechanism

could be that colonic regulatory T-cell development is impaired when SCFA production by gut microbiota is reduced. This mechanism then may contribute to the development of allergic disease. This specific combination of probiotics might then have the capacity to modulate the metabolic activity of resident gut microbiota for producing higher concentrations of SCFAs. The SCFAs, in particular butyrate, diffuse through gut epithelial cells, stimulating the differentiation of naive T-cells into regulatory T-cells, thereby preventing allergy.

Succinate, lactate and alanine were found to be more abundant in the non-eczema group, whereas glucose, galactose and lactose (sugars) were more prevalent in the eczema group. It can be hypothesized that the higher amounts of different sugar metabolites in the eczema group are caused by decreased absorption of these metabolites in the intestine. Children with eczema are known to have an impaired intestinal mucosal barrier, which could lead to decreased absorption.[40] A comparable finding was observed in faecal extracts of patients with ulcerative colitis compared with healthy controls, as glucose levels were higher in diseased patients.[24] The roles these metabolites play in the development of eczema remain unclear, but do warrant further investigation. In conclusion, our study suggests a role for intestinal SCFAs in the development of eczema in early life. Group enlargement in comparable studies addressing primary prevention of atopic disease, by supplementation of pre- or probiotics, can strengthen the understanding of possible working mechanisms. Moreover, it can lead to rational development of sustainable microbiota management for prevention and treatment of allergy and other immune mediated diseases, where the production of specific metabolites would be the leading criterion for selection.

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9

IN VITRO ASSESSMENT OF THE IMMUNOMODULATORY EFFECTS OF MULTISPECIES PROBIOTIC FORMULATIONS FOR MANAGEMENT OF ALLERGIC DISEASES

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Abstract

Modulation of the composition of the intestinal microbiota with probiotics could possibly offer a way of prevention or management of allergic diseases. The objective of this study was to determine the immunomodulating effects of various multispecies probiotic combinations , as preamble to application . Multispecies probiotic combinations were formulated and tested for their effects on cytokine production by human mononuclear cells and were compared to products that already have shown beneficial effects . All 4 tested combinations of probiotics showed a 40-71% decrease of Th2 cytokine production (IL-4, IL-5, and IL-13) and a variable increase of Th1 (IFN-g) and Treg cytokine (IL-10) production compared to the medium. A specific probiotic mixture that contained W25,ATCC SD 5219, ATCC SD 5220, W62, W57 and W19 was superior in its stimulating effect on IL-10 production (significant better than the other tested combinations; = 0.001). Modulation of cytokine production profiles can be used to differentiate between selected probiotic formulations for their immunomodulatory properties. In the future it should be demonstrated whether the immunomodulatory capacities from the multispecies probiotic formulation with the desired profile will be effective (in adolescents, followed by application in children).

Introduction

The development of a well-balanced innate and adaptive immune system during early human development is of paramount clinical importance, not only to confer protection against infections but also to avoid immune-mediated diseases. Allergy is one of those immune mediated multifactorial diseases, the clinical expression of which is determined by the complex interplay between genetic factors of the host and environmental factors. The incidence of allergic diseases has increased greatly during the last decades in countries with a Western lifestyle. Among the factors potentially involved in the increased prevalence of allergic diseases, reduced microbial exposure during childhood, as put forward in the hygiene hypothesis, has been proposed.[1,2] The interaction of the developing immune system with (intestinal) microbiota appears to play a decisive role for the generation of a balanced immune system later in life.[3] The cellular and molecular compounds which determine the interaction between microbiota and developing mucosal immune system therefore have received considerable interest over the past years.[4,5] It has been shown that colonization of the infant gut by microorganisms over the first year of life is crucial for development of a balanced immune system and that early alterations in the gastrointestinal microbiota and gut composition of beneficial bacterial species protects development of allergy and atopic disease.[4,6,7]

Administration of probiotics, defined as living microorganisms that, when administered in adequate amounts, confer a health benefit on the host,[8] has shown to be able to reduce the incidence of atopic dermatitis, cow's milk allergy and the severity of allergic manifestations.[3,9-11] The first landmark study used which was supplemented to mothers prenatally and subsequently 6 months after birth to the newborns.[12] This study demonstrated that administration of this probiotic was associated with a significant reduction in the cumulative incidence of eczema during the first 7 years of life.[13,14] A number of other studies, including ours, also show preventive effects of probiotics on development of eczema.[15-17] The aggregate results of all studies however are non-significant, highlighting the fact that there is no 'generic' benefit common to all probiotics.[8] Therefore, a Cochrane review on this subject concluded that there is insufficient evidence to recommend the addition of probiotics to infant feeds for prevention of allergic disease or food hypersensitivity.[16]

With respect to management of atopic dermatitis by probiotics, most studies show no or only a modest effect.[3,15,17,18] Recent studies however show promising results.[19,20] For studies with probiotics, the outcome of meta-analysis including Cochrane reviews should be interpreted with care. The reason for this is the great heterogeneity between studies as many different probiotic strains are used, and for a meta-analysis all the probiotics are regarded as equal. In reality, most probiotics differ in their immunomodulatory capacity, as well as in their interaction with other gut microbiota. Also, some studies use single-strain probiotics, while other use multispecies probiotics or synbiotics.[15]

The functionality and efficacy of mono-strain probiotics may differ from that of multi-strain or multispecies probiotics. The term multispecies probiotics is used for preparations containing strains that belong to one or preferentially more genera.[21] A combination of strain-specific properties may result in synergistic and symbiotic probiotic effects or enhanced activity due to mutual symbiosis.[8,22,23] In this respect however, it is still unclear which species within a multistrain preparation have a synergistic relationship that might enhance the preparation's effectiveness.[24] As far as we know, the first study that made use of a specifically designed multispecies probiotic formulation for clinical application was the PandA study.[11] Probiotic strains were tested , after which a multispecies probiotic mixture (Ecologic®Panda) was designed.[25] application of the mixture showed a preventive effect on the incidence of eczema in high-risk children.[11] Based on this same principle, and as a first step in designing a multispecies probiotic preparation for management of atopic dermatitis, we have set out to screen a panel of 19 different probiotic strains for their ability to modulate the differentiation of T-helper lymphocytes.[26,27] The various strains were co-cultured with peripheral blood mononuclear cells (PBMCs) of healthy adults and the expression of transcription factors specific for $CD4^+$ T cell subsets was determined. The quantitative expression of transcription factors Foxp3, Tbet, GATA3, and RORγT, specific for regulatory T-cells (Treg), T helper 1 cells (Th1), T helper 2 cells (Th2) and T helper 17 cells (Th17), respectively[27] was influenced by probiotic bacteria. The capacity of individual probiotic strains to modulate $CD4^+$ T cell differentiation varied greatly and turned out be even more strain-specific than genus-specific.[28] Having established the immunomodulatory potential of individual probiotic strains, the next step was to design several candidate mixtures, containing the strains with a desired profile, and test overall immunomodulation. In this study we have compared these candidate mixtures with monostrain and multispecies combinations which have been previously used in clinical trials.

Materials and Methods

Selection of multispecies probiotic combinations

Eight differentspecies and strainsnine different and(all obtained from Winclove Bio Industries B.V., Amsterdam, The Netherlands) were tested for their ability to modulate in vitro differentiation of $CD4^+$ T cells.[28] Based on the immunomodulatory profile of individual strains and the desired characteristics of the ultimate product, four different probiotic formulations were designed (see the Results section).

The four different multispecies probiotic combinations were compared to Ecologic® Panda (containing W23, W52 and W58) and to the probiotic strain M-16V. Ecologic® Panda was tested as the complete product (apart from the carrier material (rice starch) and vitamin/ mineral mix added to the product to make it clinically appropriate); the mixture of 3 selected strains was cultured and used for the tests. The final concentrations of Ecologic® Panda and M-16V (10^8)

are comparable to the concentrations used previously.[11] All individual probiotic strains used in this study carry the European Union qualified presumption of safety (QPS) status.

Preparation of bacteria

From frozen stocks, pure strains were cultured in de Man, Rogosa and Sharpe broth (MRS, Merck, Darmstadt, Germany) at 37°C under anaerobic conditions for 24-48 hours (dependent on their growth characteristics). The bacteria were harvested by centrifugation (3000 for 15 minutes) during stationary growth phase. The pellets with bacteria were then washed three times in PBS, the concentration was determined by colony forming unit (CFU) counting. For the culture experiments, bacteria were diluted to a final working concentration of $1x10^8$ CFU/ml in RPMI 1640. All mixtures were prepared by combining the bacteria in equal amounts into an end product with a total viable cell count of $1x10^8$ CFU/ml. These stock suspensions were aliquoted and stored at -20°C.

Preparation of peripheral blood mononuclear cells

Sodium-heparinized blood was obtained by venapuncture from three healthy adult donors with no history of atopic eczema, asthma or allergies. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation (580 2300 rpm for 20 minutes) over a Ficoll density gradient (Pharmacia, Uppsala, Sweden). After washing, the cells were counted and resuspended at a concentration of $5x10^6$ cells/ml in RPMI 1640 tissue culture medium (Life Invitrogen). PBMCs were subsequently co-cultured with bacteria in a 1:10 ratio (PBMCs:CFU, based on viable cell counts) in RPMI 1640 (Gibco, Breda, The Netherlands) supplemented with 1% glutamine (Gibco), 0,5% Penicillin-Streptomycin (Gibco) to prevent bacterial overgrowth and 10% human AB serum.[28] There were no signs of acidification (the pH was measured by using a Phenol Red pH indicator). The methods used for measurement of transcription factors is described elsewhere.[28]

Cytokine production

The effect of the probiotic combinations directly on the PBMCs, as well as potential effects on cytokine production induced by phytohemagglutinin (PHA, Murex Biotech, Dartford, United Kingdom) were evaluated. Cell cultures were set up in duplicate in 96-well round bottom polystyrene microtitre plates (Nunc A/S, Roskilde, Denmark). All cultures contained $0.5x10^6$ PBMCs. PBMCs were cultured with medium only or stimulated with PHA in a final concentration of 35 μg/ml. (Mixtures of) probiotic bacteria were added in a lymphocyte:bacteria ratio of 1:1. Negative control cultures contained unstimulated PBMCs.

Cultures were incubated at 37°C in 5% CO_2 and 100% relative humidity. The cell-free culture supernatants were collected after 24hrs and 72hrs and stored at -80°C until analyses of the cytokines.

Cytokine profiles were measured by the multiplex immuno assay of Luminex, as described before (de Jager , 2003). Interleukin (IL)-4, IL-5 and IL-13 cytokines (Th2), IFN-g (Th1) and IL-10 (Treg) levels were measured.

Statistical analyses

Statistical analyses were performed by using the Kruskal-Wallis test and Mann-Whitney-U test for demonstrating differences between cytokine production in response to different probiotic combinations. Differences were considered significant when ≤ 0.05. Data were analyzed with SPSS, version 19 (SPSS Inc., Chicago, IL, USA).

Results

Selection of multispecies probiotic combinations

The immunomodulating capacity of 19 different probiotic bacterial strains of commonly used species has been investigated in terms of induction of transcription factors characteristic for $CD4^+$ T cell subsets.[28] Based on the results of that screening, and the desired immunomodulatory profile of the final product (induction of $Foxp3^+$ regulatory T cells and $Tbet^+$ Th1 cells but not $GATA3^+$ Th2 cells), four different probiotic combinations were prepared. Mixture 1 -- ATCC SD5219, ATCC SD5220, W62; Mixture 2 -- ATCC SD5219, ATCC SD5220, W62, W25, W57, W22 and W19. Mixture 3 -- ATCC SD5219, ATCC SD5220, W62, W25, W57 and W19, and Mixture 4 -- ATCC SD5219, ATCC SD5220, W62, W22, W19.

Table 1 shows the composition of the 4 mixtures and the fold induction of transcription factors. These mixtures were subsequently tested for their capacity to modulate cytokine production profiles and were compared with Ecologic®Panda and M-16V. Latter two products have shown beneficial effects when clinically applied in prevention and management of atopic dermatitis, respectively.[11,30,31]

Cytokine profiles were measured in culture supernatants of isolated PBMCs of three healthy donors with use of the multiplex cytokine assay.

Table 1. Fold induction of mRNA of the transcription factors and composition of the mixtures of probiotics. Data on induction of transcription factors are taken from de Roock *et al* [28]

Strain[1]		Fold induction of transcription factors[2]				Mixture			
		Foxp3	Tbet	GATA3	RORγT	1	2	3	4
B. coagulans	W64	1	4.8*	3.4*	2.5*				
B. bifidum	W28	1.2	3.0*	3.1*	2.8*				
B. breve	W25	0.4	3.0*	1.9	2.0*		+	+	
B. lactis	ATCC SD5219	2.3*	1.0	1.5	0.3	+	+	+	+
B. lactis	ATCC SD5220	2.1*	1.2	2.1	0.2	+	+	+	+
L. acidophilus	W22	0.5	3.5*	1.5	1.0		+		+
L. acidophilus	W74c	1.2	0.8	2.8*	6.0*				
L. plantarum	W21	3.0*	2.5	1.7	1.8				
L. plantarum	W62	2.0*	1.0	1.5	1.8	+	+	+	+
L. salivarius	W57	2.7*	0.7	2.2	5.2*		+	+	
Lc. lactis	W19	0.6	8.0*	1.7	0.5		+	+	+
S. thermophilus	W67	1.7*	0.4	2.8*	0.4				

1 B. = Bifidobacterium, L. = Lactobacillus, Lc. = Lactococcus, S. = Streptococcus.

2 Foxp3 is expressed in Treg cells, Tbet in Th1 cells, GATA3 in Th2 cells and RORγT in Th17 cells. Transcription factor mRNA was determined in $CD4^+$ T cells in PBMCs co-cultured with probiotic strains and compared to cells cultured in medium alone. Significant differences with medium indicated with an asterisk.

Downregulation of in vitro Th2 cytokine production

In PBMCs cultured in medium only, no, or only trace amounts of Th2 cytokines are produced (Figure 1, panels A and B). In PBMC cultures stimulated with PHA, at 24 hours the mean production of IL-4 was 12.85 ± 5.76 pg/ml, of IL-5 76.24 ± 29,65 pg/ml and IL-13 175.14 ± 97.43 pg/ml. In these cultures, all 4 mixtures as well as Ecologic®Panda and M-16V were able to significantly reduce the production of IL-4, IL-5, and IL-13. Reduction of IL-4 ranged from minimal 40% () to maximal 64% (mixture 3), = 0.012, of IL-5 (ranging from minimal 61% () to maximal 71% (mixture 2), = 0.007) and of IL-13 (ranging from minimal 46% to maximal 64% (mixture 2), = 0.044). All 4 mixtures were equally effective in this respect compared to the medium (see Figure 1, panel C).

In PBMC cultures stimulated with PHA for 72 hours, the production of IL-4 was decreased compared to that after 24 hours (3.85 pg/ml), while IL-5 and IL-13 were enhanced (118.14 pg/ml and 282.5 pg/ml, respectively).

Compared to the PHA stimulated cultures, at 72 hours the probiotic mixtures as well as Ecologic®Panda and M-16V reduced the production of IL-4, IL-5 and IL-13 to a similar or even higher degree than at 24 hours. For IL-4, the reduction ranged from 61% () to 92% (mixture 3), = 0.006. For IL-5, the reduction ranged from 67% () to 81% (mixture 2), = 0.007. For IL-13, the production ranged from minimal 42% () to maximal 69% (mixture 2), = 0.007.

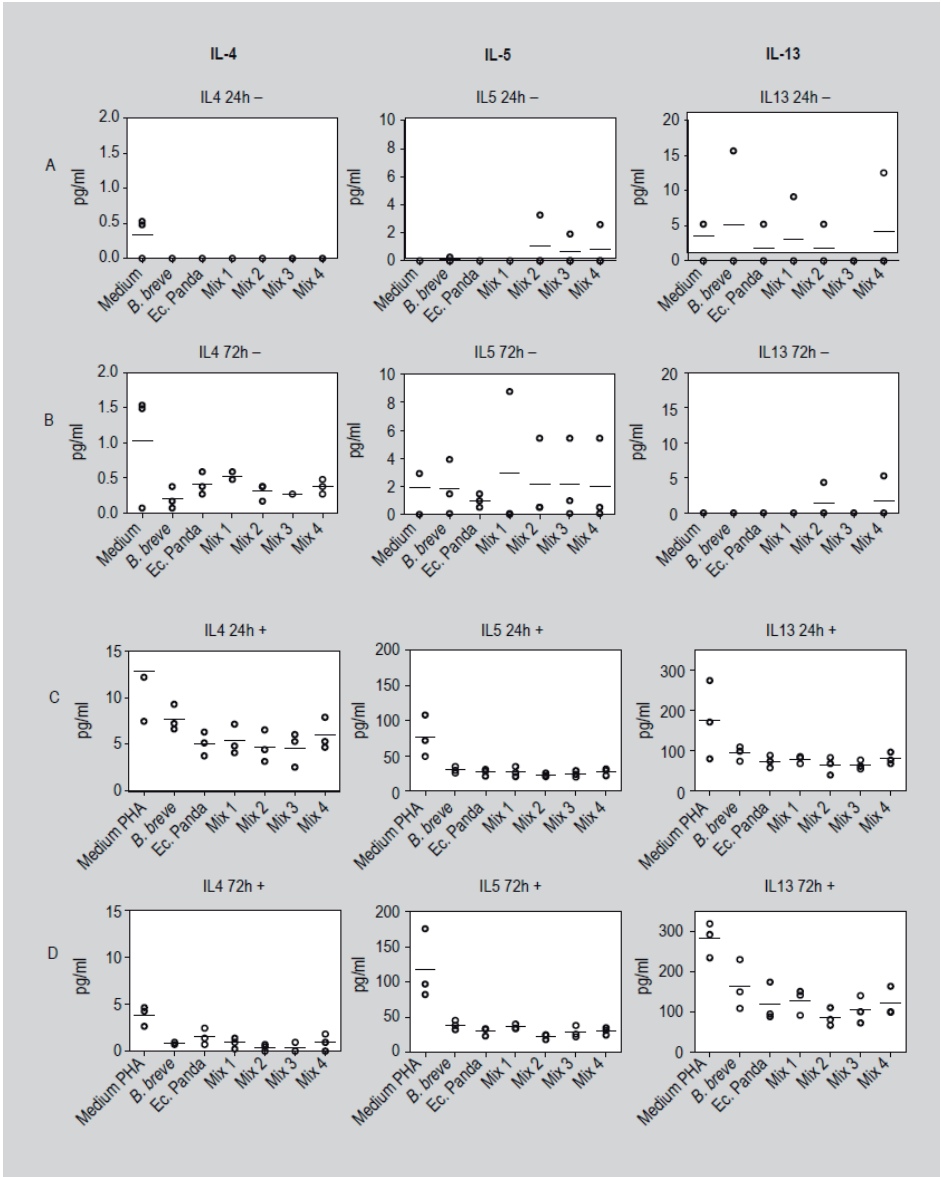

Figure 1. ***In vitro*** **induction of Th2 cytokines by mixtures of multispecies probiotics.**
Peripheral blood mononuclear cells were cultured in medium stimulated with PHA ("medium PHA") or in medium only ("medium"). Cultures were supplemented with Bifidobacterium breve M-16V, Ecologic®Panda probiotics or with four different mixtures of probiotics (see text for composition). IL-4, IL-5 and IL-13 were measured by multiplex cytokine assay in culture supernatants harvested after 24 hrs and 72 hrs. Data are presented as aligned dot-plots indicating the mean values of duplicate cultures of three individual donors and the horizontal bars represents the overall means. '24h' and '72h' indicate hours of stimulation, added with '+' for medium stimulated with PHA and with ' –' for medium only (not stimulated with PHA).

Upregulation of in vitro Th1 cytokine production

In supernatants of otherwise unstimulated PBMC cultures (Figure 2, panel A), all probiotic mixtures were able to induce significant amounts of IFN-g compared to PBMCs which were cultured in medium only (for the four mixtures together compared to unstimulated medium, =0.027 at 24 hours and = 0.009 at 72 hours). The data in Figure 2, panel A also show that at 72 hours mixtures 1 and 4 as well as Ecologic®Panda and M-16V induced an over 200-fold increase in IFN-g production compared to the medium. Mixture 2 and mixture 3 where significant less active (= 0.004) in this respect. In supernatants of cultures stimulated with PHA, a substantial amount of IFN-g was induced (approximately 500 pg/ml, Figure 2, panel B). All probiotic formulations showed only a moderate further increase of IFN-g compared to the medium at 24 hours. Also at 72 hours, for all mixtures the increase in IFN-g production was only 30-40% compared to the medium; mixtures 2 and 3 under these circumstances behaved similar to the other probiotic combinations.

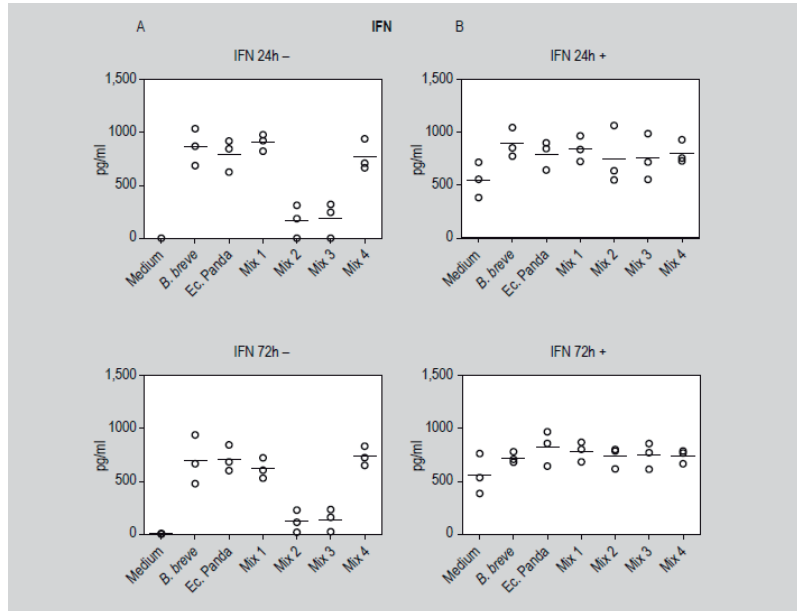

Figure 2. ***In vitro*** **induction of interferon-g (IFN-g) by mixtures of multispecies probiotics.**
See legend to Figure 1 for further explanation.

Influence of the probiotic mixtures on Treg cytokine production

In all 4 mixtures as well as Ecologic®Panda and M-16V, substantial amounts (> 19,000 pg/ml) of IL-10 were produced in PBMC cultures, either stimulated with PHA or not (see Figure 3, panels A and B). In supernatants of otherwise unstimulated cultures, a significant higher increase of IL-10 production by mixture 2 and 3 was shown, compared to the other formulations (=0.001 for mixture 2 and 3 compared to the other formulations, at both 24 and 72 hours (Figure 3, panel A). In PHA stimulated cultures (Figure 3, panel B), the production of IL-10 was further increased by all 6 probiotic formulations. At 24 hours the differences did not reach statistical significance but at 72 hours this was highly significant (0.001).

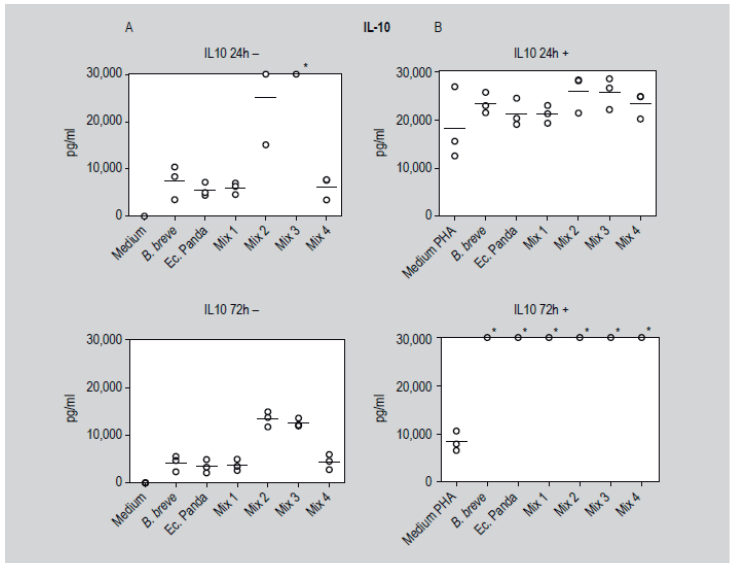

Figure 3. ***In vitro*** **induction of IL-10 by mixtures of multispecies probiotics.**
See legend to Figure 1 for further explanation. The * indicates values above the upper detection limit of 30,000 pg/ml.

Discussion

Immunomodulation is one of the mechanisms, next to direct antimicrobial effects and improvement of mucosal barrier function, by which probiotics are thought to exert their beneficial effects. The immunomodulating activity of probiotics includes reducing the Th2 responses and stimulation of Treg and to a lesser extent Th1 cell responses.[31] The immune system of the human newborn is biased towards Th2.[32] During the first months of life, microbial stimuli lead to outgrowth of Th1 cells and a balanced cellular immune system. For the design of a probiotic mixture intended to be used for primary prevention of allergy and atopic dermatitis, as done in the PandA study, emphasis was therefore put on stimulation of Th1 development, as well as Treg.[25] Based on the data obtained with individual strains, those with good IL-10-inducing capacity as well as efficient inhibition of IL-5 and IL-13 (Th2 cytokines) were selected to be used as a multi-species probiotic combination in the clinical trial. Development of a probiotic product for management of atopic dermatitis is essentially different. In allergic patients, a dysbalance of Th1 and Th2 cells already is present which cannot be restored by activation of Th1 cells. As Treg cells have the ability to restore the balance between Th1 and Th2, a high impact on IL-10 production is wanted in the designed product. At present, the role of Th17 cells in allergic disease is unclear and neither positive nor negative effects have been documented.[33,34] Therefore, the ability of a given probiotic strain to induce expression of the transcription factor RORγT was not used as a criterion to include or exclude that strain from the candidate mixtures.

Atopic dermatitis in infants can be considered as an abnormal, over-reactive response of the immune system to allergic stimuli from the environment. The increased Th2 stimulation is thought to reflect a dysregulation of innate immunity. In this study, PHA was used as a polyclonal activator of cytokine production, and the immunomodulatory capacity of the probiotic strains was tested in this model. Whether the probiotic strains are equally effective in an allergen specific immune response cannot be concluded yet. Possibly this probiotic mixture also is able to modulate cytokine activation by specific allergens that elicit a specific response of the adaptive immune system. Subtle differential alterations in cytokine responses during development of the immune system early in life are associated with subsequent atopy.[35]

As far as the capacity to modulate cytokine production and in particular IL-10 induction is concerned, no significant differences were found between mixture 2 and mixture 3. The difference in composition between mixture 2 and 3 is that mixture 2 contained one additional strain () which can produce D-lactate. Within lactobacilli, two groups can be distinguished; homofermentative (only D/L-lactate is produced) and heterofermentative (D/L-lactic acid, CO_2, and ethanol or acetic acid are produced). Bifidobacteria and lactobacilli can be used safely in children of all ages, although use of obligate D-lactate-producing lactobacilli should be discouraged in newborns because of potential toxic effects resulting from their inability as yet to completely metabolize D-lactate.[21,36]

As the intended use of the selected mixture is the management of existing atopic dermatitis in young children (aged 4-15 months), mixture 3, which included ATCC SD5219, ATCC SD5220, W25, W62, W57 and W19 (Ecologic®Panda II), has the most optimal immunomodulatory capacity. De Roock *et al.* studied the CD4 T cell responses to 19 different gut derived lactic acid bacteria, including the strains of the four designed formulations.[28] The data suggest that W57 to a large extent is responsible for the strong IL-10 production of formulations 2 and 3 (both formulations contain this strain).

In the future, a randomized placebo-controlled trial in children with mild to moderate atopic dermatitis should demonstrate whether the characteristics translate into an modulation of intestinal microbiota, normalization of the Th2 dominated immune responses and, most importantly, relief of clinical symptoms. Testing the efficacy of the described formulations in appropriate animal models could be an intermediate step before directly testing novel combinations of probiotics in humans. Unfortunately, animal models for atopic dermatitis are rather artificial and do not represent the situation in humans. A further complicating factor is that an optimal combination of bacteria to regulate the human immune system might not be optimal for the murine immune system.

Conclusion

Modification of the intestinal microbiota by use of probiotics can potentially offer a way of prevention or management of allergic diseases, including atopic dermatitis. Many different probiotic species and strains have been used for that purpose with variable results. assessment of the immunomodulatory capacity of candidate probiotic strains previously has been shown to be a relevant screen prior to its clinical application. For this study therefore, four different probiotic combinations were designed (based on characteristics of individual strains), intended to be used for management of atopic dermatitis. The candidate formulations were tested for modulation of cytokine production profiles and compared with Ecologic®Panda and M-16V which have been shown previously to be effective in prevention and management of atopic dermatitis, respectively. The formulation which included two strains of (ATCC SD5219 and ATCC SD5220) W25, W62 W57 and W19 was found to be superior in reducing Th2 cytokines and induction of IL-10 as compared to the other formulations.

Extrapolating results is not always possible in studies with probiotics (e.g. Flinterman [37]). However, as recommended by De Roock .,[28] prior to clinical application monitoring of the immunological effects of the strains (using a high throughput method) is requested. It should be demonstrated whether the immunomodulatory capacities from the most suitable multispecies probiotic formulation will be effective . Depending on regulatory guidelines, novel probiotic

combinations should first be evaluated in adult patients before they can be applied in children. Thereafter, during a randomized placebo-controlled trial in children with mild to moderate atopic dermatitis the formulation could be applied and this should demonstrate whether the immunomodulatory capacities from this multispecies probiotic formulation will be effective .

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10

MATERNAL USE OF PROBIOTICS DURING PREGNANCY

AND EFFECTS ON THEIR OFFSPRING'S HEALTH

IN AN UNSELECTED POPULATION

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Abstract

Probiotics are used by women in the perinatal period and may improve balance of microbiota, with possible health benefits for both mother and baby. Characteristics and (health) behavior patterns of mothers using probiotics during pregnancy, and health effects on their offspring, were investigated. Differences between mothers using probiotics during pregnancy and those who did not, were assessed. In total 341/2491 (13.7%) mothers reported use of probiotics during pregnancy. There were no significant differences in maternal features (gestation, age, ethnicity, education) between users and non-users. Logistic regression analyses showed that consumption of probiotics was significantly associated with use of homeopathic products (OR 1.65, 95% CI 1.17-2.33, *p*= 0.005), maternal history of smoking (OR 1.72, 95% CI 1.25-2.37, *p*= 0.001) and paternal history of smoking (OR 1.39, 95% CI 1.01-1.89, *p*= 0.05). Common disease symptoms during the first year of life in the offspring did not differ between both groups. *Conclusion:* The use of probiotics or other health related products without doctor's prescription during pregnancy might point to compensation for types of less favorable behavior. Probiotic use during pregnancy does not seem to induce positive health effects in the offspring in an unselected population.

Introduction

Adequate nutrition is of major importance for one's health and well-being, especially during preconception and pregnancy.[1-5] Women become more aware of the health aspects of nutrition during pregnancy and seek for more nutrition-related information. Compared to the period before conception and pregnancy, pregnant women are more interested in healthy food and may be more receptive to behavior change and lifestyle interventions.[6-9]

The increased nutrient requirements during pregnancy are mostly covered by a balanced diet, but dietary supplements are often taken to improve maternal or fetal health status.[10] Maternal (health) behavior and micronutrient status during pregnancy have been linked to the health status of the child.[2,3,5,11,12] Moreover, aberrant microbiota compositions have been detected during critical periods when early programming occurs, including pregnancy and early neonatal life.[3,12,13] Manipulation of the maternal microbiota composition through the use of probiotics may have subsequent consequences for the health of the offspring, as the presence of bacteria in human milk implicates that modulation of maternal gut microbiota during pregnancy and lactation could have an effect on infant health.[14-17] Improvement of maternal intestinal microbiota composition, relief of possible gastrointestinal complaints, reduced infant's risk of developing atopic dermatitis, atopic sensitization and gastrointestinal symptoms as well as changes in fetal and infant's growth have been reported as positive health effects of probiotics.[12,18-21] In western societies, a substantial percentage of pregnant women appear to use probiotic supplements.[11,22] Because of the potential positive effects for the health of the woman and her neonate, pregnancy is an opportune time for probiotic use. Both beliefs and knowledge seem to strongly affect the mother's behavior.[23] Review of the literature shows that ingestion of probiotics (combination of strains of *Lactobacillus* and *Bifidobacteria*) for a limited period of time (late) during pregnancy appears to be low risk as it does not increase the rate of adverse pregnancy outcomes and seems to be well tolerated.[24]

The aim of our study was to investigate characteristics and health behavior patterns of mothers who use probiotics during pregnancy. As a secondary aim we studied the effects of maternal use of probiotics on the offspring's health during the first year of life.

Materials and methods

Study design and study population

Subjects of the present study were mothers, with their child, participating in the ongoing Wheezing Illnesses Study LEidsche Rijn (WHISTLER) study. WHISTLER is a large prospective birth cohort study that started in December 2001.[25] Baseline pre-pregnancy data of these parents were available from the Utrecht Health Project.[26] At the infant's age of 3–8 weeks, information on pre- and post-natal risk factors is obtained by questionnaires and the infant's birth weight and height, as well as gestational age and gender are recorded at an outpatient visit. Health parameters during the infant's first year of life are followed in the WHISTLER study through linkage with the computerized medical files recorded by general practitioners.

Definitions of outcomes

General characteristics and behavior patterns of the mother (and father) were extracted from the WHISTLER database. History of smoking was defined as smoking "ever", prior to pregnancy (without limitation in months/years ago). A positive history of parental allergy was defined as questionnaire-reported allergy to pollen, house dust mite, pets, or food. Maternal higher education was defined as higher vocational or university education. Maternal paid occupation was defined as having a paid job (yes or no) at time of completing the questionnaire.

At the visit shortly after birth, maternal use of probiotics was asked as follows: did you use probiotics during pregnancy, either as in a probiotic milk or yoghurt product and/or probiotic-containing supplements? If yes, how many portions did you (on average) use per week? One portion was defined as use of one sachet or one capsule, or use of one serving of a known probiotic-containing milk or yoghurt product. Active maternal smoking during pregnancy was considered present if the mother smoked at least one cigarette per day during pregnancy. Exposure to smoke during pregnancy was defined present when the mother smoked herself and/or if she reported being exposed to environmental cigarette smoke for at least 2 hours per week. Use of supplements without doctor's prescription was defined as maternal reported use of at least one of the following, during the past 3 months:

1. vitamins, minerals, iron substitutes or resistance increasing substitutes;
2. substitutes for other gastrointestinal complaints;
3. substitutes against cough and cold;
4. laxatives or sedatives.

Use of homeopathic or herbal products during the past 3 months was also recorded. Eating fruits and/ or vegetables on a regular basis was considered as a parameter for a healthy lifestyle and the variables were defined as eating five or more pieces of fruit a week and preparing fresh vegetables seven or more times a week.

To analyze the effects of the use of probiotics on the offspring's health we used follow-up data from the WHISTLER study. In this study data on respiratory symptoms, disease episodes and day care attendance are recorded during the first year of life using monthly questionnaires. Furthermore, GP diagnoses on upper respiratory tract infections, lower respiratory tract infections, gastrointestinal tract infections and constitutional eczema are recorded using International Classification of Primary Care (ICPC) codes.

Analysis

We compared mothers that used probiotics during pregnancy to non-users. In order to assess differences between groups, chi-square tests and independent samples *t* tests were used where appropriate. For all the analyses, firstly the univariable association with use of probiotics during pregnancy was estimated using logistic regression. Secondly, we extended to multivariable logistic regression to adjust for maternal characteristics or behavior patterns that were significantly associated with use of probiotics or showed a trend towards significance in the univariable analysis. A cut-off *p* value of <0.30 in the univariable association was used to insert variables into the multivariate model. Results are presented as odds ratios, with 95% confidence intervals and *p* values. Associations were considered statistically significant if *p* values were ≤0.05. Analyses were run using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Data of 2491 mothers were used for analysis of their life style and behavior during pregnancy. Group characteristics and maternal attitudes between mothers who did and did not use probiotics during pregnancy are shown in Table 1. Of the total group, 13.7% of the mothers reported use of probiotics during pregnancy. The mean usage per week was 3.5 portions (ranging from 1-15), in which there were no differences between the first and the second half of their pregnancy (data not shown). No differences were shown between both groups for gestational age, birth weight, maternal age at time of delivery, education and ethnicity of the mother. Probiotic using mothers more often had a history of smoking, compared to non-users (p= 0.003). There also was a significant association between fathers with a history of smoking and the use of probiotics by the mother (p= 0.01). Maternal use of probiotics during pregnancy was significantly associated with use of other supplements and substitutes without doctor's prescription (p= 0.02) and the use of homeopathic products (p<0.001).

Table 2 shows characteristics of the offspring of both probiotic using and non-using mothers. In the probiotic group mothers more frequently gave birth to a boy (p= 0.04). Otherwise no differences between the groups were demonstrated.

The results of the multivariable analysis on the association between the use of probiotics and other parental characteristics are shown in Table 3. The use of probiotics during pregnancy was increased in mothers who reported use of homeopathic substitutes or herbal medicines (OR= 1.65, 95% CI 1.17-2.33, p= 0.005). Use of probiotics by the mother was also significantly associated with a higher frequency of history of smoking of both mother (OR 1.72, 95% CI 1.25-2.37, p= 0.001) and father (OR 1.39, 95% CI 1.01-1.89, p= 0.05).

Table 4 shows the results of the multivariable analysis on the association of maternal use of probiotics during pregnancy and offspring's disease symptoms during the first year of life. These symptoms did not differ between infants from probiotic using mothers and non-using mothers in this population.

Table 1. Characteristics of the study group (parents)

	TOTAL group	**Probiotic use**	**Non-probiotics use**	***p-value***
	n = 2491	(13.7%)	(86.3%)	
Maternal age at time of delivery (mean, in yrs) (SD)	32.7 (3.9)	32.8 (3.7)	32.7 (4.0)	0.63[a]
Maternal weight (mean, in kg) (SD)	71.4 (12.5)	70.2 (12.4)	71.5 (12.3)	0.12[a]
Ethnicity mother (% western)	89.9	91.0	89.7	0.51[b]
Maternal higher education (%)	67.3	70.0	66.9	0.31[b]
Maternal paid occupation (%)	89.8	89.9	89.8	0.99[b]
Maternal history of smoking (prior to pregnancy 'ever') (%)	35.4	43.3	34.1	**0.003**[b]
Maternal smoking during pregnancy (%)	6.2	7.0	6.1	0.51[b]
Maternal smoke exposure during pregnancy (%)	14.9	17.0	14.5	0.23[b]
Current smoking mother (%)	8.2	9.8	7.9	0.30[b]
Paternal history of smoking ('ever') (%)	40.6	48.3	39.4	**0.01**[b]
Current smoking father (%)	17.5	16.5	17.7	0.64[b]
Pet ownership during pregnancy (%)	39.3	41.6	38.9	0.34[b]
Use of alcohol (in general) (%)	79.6	81.2	79.4	0.50[b]
Use of substitutes/ supplements without doctor's prescription during the past 3 months (%)[c]	75.9	81.5	75.0	**0.02**[b]
Use of homeopathic substitutes/herbal medicines during the past 3 months (%)	22.3	30.5	20.9	**<0.001**[b]
Use of fruits (5 or more pieces a week) (%)	64.8	68.0	64.3	0.24[b]
Use of fresh vegetables (7 or more times a week) (%)	40.9	44.0	40.4	0.25[b]
Maternal allergy (%)[d]	35.1	34.1	35.2	0.71[b]
Maternal allergy (%)[e]	48.2	50.9	47.7	0.33[b]
Paternal allergy (%)[e]	43.7	41.6	44.0	0.47[b]
Children's day care visit during the first 6 months of life (%)	65.3	70.8	64.4	**0.03**[b]

p values in bold are statistically significant. [a] = *t* test, [b] = Chi square test
[c] = e.g. vitamins, minerals, iron substitutes, resistant increasing substitutes, substitutes for other gastrointestinal complaints, substitutes against cough and cold, laxatives, sedatives
[d] = allergy to pollen, dust, house mite, pets
[e] = allergy to pollen, dust, house mite, pets, food, or 'other'

Table 2. Characteristics of the study group (infants)

	Total group	Probiotic use group	Non-probiotic use group	*p-value*
	n = 2491	(13.7%)	(86.3%)	
Gestational age (mean, in weeks) (SD)	39.4 (1.4)	39.3 (1.5)	39.4 (1.4)	0.13 [a]
Birth weight (mean, in grams) (SD)	3526 (514)	3529 (539)	3525 (510)	0.87 [a]
Gender (% boys within the group)	49.3	54.5	48.5	**0.04** [b]
Siblings (% with at least one)	54.0	50.9	54.5	0.22 [b]
Upper respiratory tract infections [c] (%)	46.9	46.6	47.0	0.88 [b]
Lower respiratory tract infections [c] (%)	9.0	11.0	8.6	0.15 [b]
Gastrointestinal tract infections [c] (%)	16.8	17.3	16.7	0.79 [b]
Constitutional eczema [c] (%)	12.9	14.3	12.7	0.39 [b]

p values in bold are statistically significant
[a] = *t* test, [b] = Chi square test, [c] = during the first year of life, doctor's diagnosis

Table 3. Associations between parental characteristics and use of probiotics during pregnancy

	Multivariable analysis	
	OR (95% CI)	***p-value***
Maternal history of smoking (prior to pregnancy 'ever')	1.72 (1.25 – 2.37)	**0.001**
Maternal smoke exposure during pregnancy	1.06 (0.67 – 1.70)	0.79
Use of substitutes/ supplements without doctor's prescription during the past 3 months*	1.05 (0.70 – 1.58)	0.82
Use of homeopathic substitutes/herbal medicines during the past 3 months	1.65 (1.17 – 2.33)	**0.005**
Use of fruits (5 or more pieces a week)	1.14 (0.82 – 1.60)	0.44
Use of fresh vegetables (7 or more times a week)	0.96 (0.70 – 1.32)	0.81
Paternal history of smoking ('ever')	1.39 (1.01 – 1.89)	**0.05**
Maternal higher education	1.27 (0.88 – 1.83)	0.20
Children's day care visit during the first 6 months of life	1.31 (0.92 - 1.88)	0.13

p values in bold are statistically significant
* See Table 1 for definition

Table 4. Associations between maternal use of probiotics during pregnancy and infant characteristics

	Multivariable analysis	
	OR (95% CI)	***p-value***
Upper respiratory tract infections [a]	0.97 (0.77-1.22)	0.79
Lower respiratory tract infections [a]	1.31 (0.90-1.91)	0.16
Gastrointestinal tract infections [a]	1.03 (0.76-1.40)	0.86
Constitutional eczema [a]	1.15 (0.82-1.60)	0.42

[a] = during the first year of life, doctor's diagnosis

Discussion

This study shows that about 1 out of 7 mothers in our population used probiotics during pregnancy. Use of probiotics during pregnancy was independently associated with use of homeopathic products, and with a history of smoking of both mother and father. To our knowledge, to date, no other studies analyzed the association between maternal use of probiotic supplements and other behavior patterns during pregnancy. The number of mothers that reported consumption of probiotics during pregnancy in our cohort corresponds reasonably with previous estimates.[11,22] In our study mothers that used probiotics during pregnancy were not characterized by specific maternal features (gestation, age, ethnicity, education) compared to mothers that did not use probiotics during pregnancy, although literature shows that generally the adequacy of micronutrient intake during pregnancy is related to environmental, cultural and demographic variables.[2,11,27]

To many, probiotics, homeopathic products and nutritional and dietary supplements belong to the category of complementary medicines. Pregnancy is a time to become more aware of a healthy lifestyle including healthy nutrition. Taking any form of supplement may be part of such a (change in) lifestyle. We hypothesized that next to the health-promoting properties that are suggested for probiotics, mothers may use them during pregnancy to compensate for adverse (prior) habits of themselves or their partners, for instance smoking.

We showed comparable disease symptoms during the first year of life in the offspring from probiotic using and non-using mothers. Reviews and a meta-analysis demonstrated that current evidence on the effects of probiotics on the offspring's health is fairly inconclusive.[13,28,29] Our data do not add evidence for a beneficial effect.
The main strength of this study was the sample size which was large enough to estimate

correlates of probiotics use during pregnancy. Our data have been prospectively documented and all extensive parental characteristics and behavior patterns could be aggregated from the database. Former studies of our group have demonstrated that the results may be generalized to other populations.[30]

However, there are also some limitations. Use of supplements, and especially probiotics, may have been underreported due to non-recall or format of the questions, as has been reported in the literature.[31] Nevertheless, we cannot conceive that non-recall of probiotics use would be related to use of other supplements or history of smoking, and therefore is unlikely to have caused real bias. Also, neither the type of probiotic supplement nor regularity of intake was specified and we were not able to investigate the use of probiotics by the mothers before and after pregnancy, which would have helped to discriminate mothers based on their using habits. There is emerging evidence that the effect of probiotics is strain specific and timing, administration route and the applied dose do affect the outcomes. We consider the current reported conclusions valid and reliable because of the standardized manner of data collection, correction for potential confounders and presence of the unselected population. Moreover, we consider our population size sufficiently large to render our results statistically robust. Thirdly, as reported earlier, in the study population of the Utrecht Health Project and WHISTLER study, a vast percentage of participants completed higher vocational or university education.[32,33] High socio-economic status and ethnicity might have played a role in parents' decision to participate, which results in a not entirely unselected study population. This effect will be mediated in the population but has to be taken into account when results are generalized to lower class (young) families.

Conclusion

This study shows that about 1 out of 7 mothers in our population use probiotics during pregnancy. Probiotic using mothers are not characterized by specific maternal features (gestation, age, ethnicity, education) compared to non-users. Use of probiotics during pregnancy is independently associated with use of homeopathic products and with parental history of smoking. According to common doctors' diagnosed disease symptoms in the offspring the first year of life, no differences between groups are observed. Using probiotics and/or other health related products without doctor's prescription during pregnancy, for their health promoting properties, might point to compensation for types of less favorable behavior such as parental smoking. Caregivers and people concerned with pregnant women should be aware of this effect when discussing (nutritional) behavior.

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11

GENERAL DISCUSSION

General discussion & future perspectives

The aim of this thesis was to investigate and describe the development of the intestinal microbiota in infancy. This included studying the effects of probiotics in early childhood on the composition and functionality of infant microbiota.

The main findings of this thesis are:

- Microbiota development of breast-fed and formula-fed infants proceeds according to similar developmental stages, with distinct microbial marker species.
- Early life antibiotic treatment disturbs the microbiota development in infants, mostly evident in Bacteroidetes species.
- Regardless of antibiotic treatment, neonatal microbiota development can be classified into two distinct clusters (with high abundance of Bacteroidetes, versus high abundance of Firmicutes, Actinobacteria, Fusobacteria and Verrucomicrobia) with different progression over time.
- Regardless of intervention or atopic disease status, children have a shared microbiota development over time determined by age that continues to develop between two and six years of age.
- Supplementation with a probiotic mixture consisting of *B. bifidum*, *B. lactis* and *Lc. lactis* (Ecologic®Panda), to children at high-risk for atopic disease, has no preventive effect on the development of asthma and allergic rhinitis at age 6 years. Besides, it has minor effects on gut microbiota composition during the supplementation period and no long lasting differences are identified. However, the supplementation seems to lead to higher levels of lactate and short chain fatty acids (including butyrate) and lower levels of lactose and succinate at the age of 3 months. This might explain the temporary preventive effect of probiotics on the development of eczema.
- Modulation of *in vitro* cytokine production profiles can be used to differentiate between selected probiotic formulations for their immunomodulatory properties.
- The use of probiotics or other health related products without doctor's prescription during pregnancy might point to compensatory behavior and less favorable life style factors. Probiotic use during pregnancy does not seem to induce positive health effects in the offspring in an unselected population.

Evolving techniques to characterize the gut microbiota: significance, pros and cons.

In this thesis, as in general, microbiome analysis focuses on identifying bacterial species. Fecal microbiota analysis is relevant because the vast majority of microbial communities populating our body surfaces inhabit the gastrointestinal tract. Bacteria form the predominant group of the gut microbiome. Almost all (>99%) of DNA in the human intestine belongs to bacteria, reflecting their predominance in the gut.[1] Besides, bacteria have the most comprehensively documented phylogenetic data sets and classification systems.[2]

The majority of the molecular microbiological techniques is culture-independent (i.e. do not require amplification of the material by *in vitro*-culture) techniques, focuses on one or a few marker genes and is based on the extraction of DNA and amplification of 16S ribosomal RNA (rRNA) component of the small subunit of prokaryotic ribosomes. Other techniques use an entire metagenomic approach, making it possible to comprehensively explore the biological nature of complex communities (including eukaryotic, fungal and DNA viruses that do not possess a 16S rRNA gene).[3] Both culture-based and 16S-based approaches have crucial limitations for further studies, particularly for functional analysis.

In this thesis we describe four different identifying techniques, all based on rRNA gene analysis, that allow detailed characterization of the multitude of bacterial species and strains present in a sample: quantitative polymerase chain reaction (qPCR), fluorescent in-situ hybridization combined with microscopy (FISH-MS), pyrosequencing and IS-pro.

The PCR allows isolation of DNA fragments from genomic DNA by selective amplification and quantification of 16S rRNA, being the most accurate culture-independent measure of the total microbial load. qPCR not only amplifies DNA but also reliably quantifies the amount of DNA present. It can be used as a stand-alone technique or in combination with other techniques. Introduction of qPCR for characterization of microbiota has been a huge technical advance across the medical field, as it allows for phylogenetic discrimination, is a fast technique and allows designing primers to target specific species. This technique however, can also be labor intensive and technically challenging, and it has two important limitations; first, each step in the (physical, chemical and biological) process signifies a potential source of bias. For example, differential lysis of microbial cells can affect the final perceptible microbiota composition and a primer pair can be highly specific for a given bacterial species but at the same time lack sensitivity for distinctive taxa.[4,5] Second, PCR is unable to identify unknown species because primers must be designed to target all (known) phyla. The detection of bacteria therefore crucially depends on the choice of primers used.[6] When qPCR is performed in combination with other (molecular or semi-quantitative) techniques it can provide more detailed information on the diversity and abundance of the gut microbiota.[4,7,8]

Another powerful technique is FISH, which is a semi-quantitative and fast probe hybridization technique, lacking a PCR bias as described above. It utilizes copy numbers of specific repetitive elements in genomic DNA of whole cell populations. This technique uses fluorescently labelled rRNA targeted group- and species-specific oligonucleotide probes that hybridize complementary target 16S rRNA sequences.[4] When hybridization occurs, fluorescent bacteria can be enumerated using either (automated) microscopy or flow cytometry. For microbiota analysis, FISH can be performed directly on a stool sample. It is mainly applied for comparison of gut microbiota between healthy and disease states. A limitation of this technique is that identification and quantification of species present is dependent on available probe sequences. Probes can be designed to target specific phyla or species, but (as in qPCR) unknown species cannot be identified.[4,9]

Quantitative phylogenetic identification by sequencing techniques enabled to identify many individual genera and species that by biochemical methods could not be identified. After successful extraction of bacterial DNA, several sequencing methodologies are available. For DNA-based microbiome studies, two types of analysis are mostly performed. Targeted amplicon studies focus on one or more marker genes, while shotgun metagenomic studies use an entire metagenomics approach by randomly attaining genomic sequences.[5]

Pyrosequencing is one of the most commonly used marker gene sequencing techniques. It uses specific genetic targets, such as hypervariable regions within bacterial 16S rRNA genes, which are amplified by PCR and then subjected to DNA pyrosequencing. Subsequently, informative target selection is combined with signature sequence matching (by use of comprehensive databases).[10] Disadvantages of sequencing techniques are the intense data analysis, costs (although these are declining because of the widespread implementation), and biases that are introduced by preferential PCR amplification of marker genes.[4]

IS-pro is a fairly new marker gene technique that combines bacterial species by virtue of the length of the 16S-23S ribosomal RNA interspace region with instant taxonomic classification by labeling of PCR-primers. For amplification of IS regions, phylum-specific, fluorescently labeled primers are designed, corresponding to conserved regions within the 16S rRNA and 23S rRNA genes.[11] The procedure consists of two separate phylum-specific PCRs, a combination of which provides very broad coverage for bacterial identification at the phylum level. This technique has been particularly designed for application in a clinical setting. It has been validated for clinical diagnostics, which makes it fully reproducible and the high-throughput nature of IS-pro makes analysis of a large number of samples feasible.[12]

As in other PCR based techniques, biases as described above may be introduced and direct comparison of relative abundances addressing all phyla together therefore is hampered. Furthermore, IS-pro does not generate 16S sequence data but instead amplifies the 16S-23S

IS region for bacterial identification. While IS-pro can generally differentiate bacterial taxa to species level, direct comparison to results of other studies performed with 16S sequencing may be less straightforward. However, the validity of the technique has been extensively validated for reproducibility on fecal samples and has been shown to correlate well to sequence data.[11,13] In chapter 2 we showed that the three microbiota profiling approaches used (qPCR, FISH-MS and 454-pyrosequencing), consistently indicate that microbiota development proceeds according to similar developmental stages. Deviations detected by pyrosequencing could be confirmed by qPCR. Subsequently (in chapter 4, 5 and 7), we performed microbiota analysis by IS-pro, a technique that has not yet been used on a large scale. Future availability, (clinical) applicability and accessibility of the microbiota analysis technique formed the basis of this choice for IS-pro. It is in line with expectations that the analysis of intestinal microbiota offers a novel way to diagnose pathologies: fecal microbiota may be used as a diagnostic tool, in an outpatient setting, aiming at a personalized treatment strategy. For example, recently it was demonstrated that the diagnosis of diverticulitis could be done by microbiome analysis (by use of IS-pro) with relatively good accuracy.[14]

Results described in chapters 2, 4, 5 and 7 of this thesis illustrate the variability and heterogeneity between study results, mainly introduced by making use of different techniques, as validation of the techniques (analysis and interpretation) is often lacking. Possible confounding factors and optimal sampling protocols have not yet been investigated fully and explained sufficiently. This has to be taken into account when results, described in the consecutive chapters, are compared and put into perspective.

Although the validity of IS-pro has been underlined by sequence data and identification up to the species level is validated, direct comparison to results of other studies performed with 16S sequencing may be hampered. Phylum level patterns, while informative, can be argued not to show the picture as detailed as analyses at genus-level or species-level can do. If pyrosequencing was used instead of IS-pro, the clustering into two different 'settler types' (described in chapter 4 and 5) might have been more deeply explored or validated. It could have facilitated comparing the results from chapter 2, 4 and 5. Next to that, the sequential microbiota signatures shown in the small population (eight infants) in chapter 2 might have been found as well in the much larger population of INCA infants when analyzed by pyrosequencing. At the same time, adding IS-pro as a fourth technique for stool sample analysis in chapter 2 could have been interesting to draw additional conclusions on sequential signatures in microbiota development, mainly at the phylum level. At least more broadly based statements could have been made regarding microbiota signatures (chapter 2) and presence of two demarcated subgroups at one week of age (chapter 4 and 5) by making use of one (same) technique. Lack of sufficient stool samples and the costs- time aspects made it impossible to implement these comparisons in this study.
Choosing which technique to use for investigating microbiota composition depends on the desired application and should be determined the depth of analysis required as well as by

the cost. The most optimal approach should aim at estimating, as well as possible, the exact proportion and function of the major, dominating phyla within the intestinal community, combined with a very sensitive method that can detect the extreme rare species inhabiting specific niches and main metabolites present (with)in the gastrointestinal tract. It may be clear that the method of choice is more determined by the specific research questions rather than characteristics of the technology itself.

Gut microbiota development: host, genetic and environmental factors and their interplay.

A number of early life environmental exposures have been associated with the evolvement of infantile microbiota into an adult-like gut microbiota composition, with delivery mode and feeding type (particularly cessation of breastfeeding) being the two key factors in term born infants.[15,16] In chapter 2, 4 and 5 of this thesis, we elucidated part of the associations between delivery mode, feeding type and microbiota composition. In chapter 2 we initially demonstrated that individuality, meaning the unique and personal microbiota development of each individual, is the major driver of microbiota composition in term born, vaginally delivered infants. A time-resolved developmental signature in the microbial colonization patterns during the first 3 months of life was seen, characterized by sequential colonization by I) intrauterine/vaginal birth derived first colonizers, II) skin derived taxa, III) domination of *Bifidobacteriaceae*, and IV) the first appearance of adult-like taxa. All three profiling approaches used in that study indicated that microbiota development of breast- and formula-fed infants proceeds according to similar developmental stages with distinct microbial marker species. Incidence and dominance of skin and human breast milk derived microbes were increased in the gut microbiota of breast-fed infants compared to formula-fed infants. Latter finding was confirmed in chapter 5: a clear effect of feeding type (breast-fed vs formula-fed) was shown, with breast-fed children harboring more skin derived bacteria than formula-fed children.

A striking difference between the studies described in chapter 2 and 5 respectively was the fact that we showed an abundance of *Bifidobacteriaceae* in the feces of the infants described in chapter 2 versus low numbers in the feces of the INCA children. Other studies have also shown conflicting results on the amount of *Bifidobacteriaceae* in infants: several studies have reported that bifidobacteria dominate the intestinal community of breast-fed neonates in early life,[17,18] but paucity of *Bifidobacterial* species by culture-independent investigations has also been described.[19,20] These differences may be due to techniques employed, such as DNA isolation protocols and PCR primers.[6,18] While bifidobacteria are a key genus in the gastrointestinal tract, it is well known that Gram-positive organisms (such as bifidobacteria) can be underrepresented in microbial profiling studies due to the presence of their thick cell wall.[21] The detection of Bifidobacterial sequences present in DNA have been shown to be optimized by designing

'bifidobacteria-optimized' universal primers.[22] Careful selection of primers is key in order to ensure effective detection of bifidobacteria, without under/or overestimation.

According to developmental stages in microbiota development, in chapter 4 and 5 we described the presence of two clearly demarcated subgroups in one-week-old infants from the INCA cohort: term infants are clustered into *Bacteroidetes*-dominant or *Firmicutes-Actinobacteria-Fusobacteria-Verrucomicrobia* (FAFV)-dominant microbiota, termed settler types B and F, irrespective of antibiotic treatment. Settler type B consists exclusively of vaginally delivered children, while settler type F consists of children delivered vaginally and by caesarean section. Although mainly investigated at the phylum level, both subgroups are quite divergent at one week of age, but migrate towards each other over time. This might points towards the time-resolved developmental signature in microbial colonization patterns, suggested in chapter 2 as well as chapter 7, indicating that children have a shared microbiota development over time determined by age that continued to develop between two and six years. Moreover, in chapter 5, infants' microbiota composition was linked to their mothers' microbiota composition, showing that infants of settler type B reveal a high similarity to gut microbiota of their mothers during the first 3 months of life, while those in settler type F reveal a low similarity to that of their mothers. Based on the present data, we hypothesize that not delivery mode per se does have the greatest impact on initial development of the microbiota, but that exposition to maternal fecal microbiota during delivery is a prerequisite for acquisition of *Bacteroidetes* dominant microbiota. Because epidemiological evidence suggests that acquiring bacteria from the *Bacteroidetes* phylum (both abundance and diversity of these species) is associated with an improved risk of asthma and allergic diseases as well as type 1 diabetes,[23–28] the specific conditions during labor and delivery may be important; duration of ruptured membranes, total duration of delivery, maternal bowel movements during labor, and environment (home versus hospital (delivery room, operating room), to name a few. Future investigations are required in order to elucidate which of these factors contribute most to the initial microbiota development. Associations with disease states later in life might prove easier to establish by classifying children into these settler types.

In contrast to previous insights that acquisition of the intestinal microbiota begins at birth, there is now sufficient evidence that the development of the microbiota begins well before the infant is born. *In utero,* the unborn fetus is suggested to be exposed to microbes in the amniotic fluid, however, the way in which these microbes gain access to the uterus remains unknown.[29] Moreover, how distinct immune cell activation and maturation pathways are affected by microbial exposures *in utero* and in early life needs to be further explained, which is underlined in several recent reviews.[30–33] Most of the immune cell types appear during the first trimester of pregnancy and then expand significantly until birth. This includes the mucosal immune system of the gut, which is fully developed by 28 weeks of gestation.[34] The effector functions of innate and adaptive immune cells are, however, inadequate during the entire fetal

period and activation does not occur until the infant is born.[30,34] During the first year of life, the mucosal immune system maturates and protective immunity is established, irrespective of gestational age at birth.[15,34] The process of expansion and priming of adaptive cell populations is, next to differences in host genomes, greatly influenced by environmental exposures during infancy and early childhood. Confounding environmental exposures during these critical periods of development may permanently alter immune defenses.[30,32] In animal models, the pattern of intestinal colonization during the first 3 months of life,[35] or even during the first day,[36] is suggested to be a programming event during which functional immune regulation is induced. The first 100 days of life seem to represent an early-life critical window in which gut microbial dysbiosis is linked to aberrant immune development. Developmental changes in the infant gut microbiota during the first 3 months could therefore have profound, long-term effects on composition and function of the adult microbiota, which subsequently influences metabolism[37,38] and can explain later life occurrences, including obesity,[39] diabetes,[40] and immune-mediated diseases.[41] At the same time, the physiological plasticity during the early post-natal period may make this period suitable for future therapeutic interventions that aim at minimizing the effect of environmental perturbations, thereby positively impacting on human health. It could be hypothesized that, at this point, (in humans) the optimal way to look for and demonstrate alterations in functional immune regulation, possibly caused by early programming, has not yet been established. Recent investigations point towards the role of innate lymphoid cells (ILCs), and particularly group 3 ILCs, in regulating interactions between host and bacteria. ILCs limit dysregulated CD4+ T cell responses to commensal bacteria, thereby maintaining an effectual separation between inflammatory microbial-derived products and the host immune system.[42]

In chapters 2, 4, and 5 similarities as well as differences in microbiota development were shown, together with disturbance of microbiota composition after antibiotic treatment during this so-called critical window. So far it is unknown whether this will induce long-lasting effects, clinical, immunological and/or metabolic. Studies in young adult males who received a 4 day broad spectrum antibiotic course did not show clinically relevant changes in insulin sensitivity, insulin release or release of other gut hormones (during a period of 6 weeks).[43] However, it may very well be that application of antibiotics during critical periods of life, such as early infancy, can induce more far-reaching effects on the gut microbiota. This supports the set-up of our INCA study, in which composition as well as function of the gut microbiota after antibiotic treatment is examined, thereby discriminating between duration, types and dosages of antibiotics given to varying populations (as much as possible). This approach will help unraveling the associations between antibiotic treatment, microbiota development and (infant) health. Extended stool sample analysis, combined with blood sample analysis for immune markers and follow-up of the population regarding clinical effects, will confirm or deny long-term consequences and proof the hypothesis of possible (altered) functional immune regulation during the first 3 months of life.

Modulation of the microbiota by probiotics and effects on disease development.

Chapter 6 stresses the temporarily effect of probiotics, by revealing a beneficial clinical effect of the probiotic bacteria on the development of eczema at the age of two years, but, in line with reports in literature, no preventive effect on the development of asthma and allergic rhinitis at the aged of six years in children at high-risk for atopic disease. In chapter 7 we showed there is some impact on microbiota composition of early life intervention with (*in vitro* selected) multispecies probiotics during supplementation (i.e. during the first year of life), but no long-term effect on later composition of gut microbiota was seen. This is in accordance with literature, showing there is only limited evidence that demonstrated changes in gut microbiota can induce a significant long-term clinical effect.[44,45] However, small subtle but important differences may have been present that were not traced by the technique applied here. Next to that, the duration and timing of treatment with probiotics might also play a crucial role in possible long-term effects. In chapter 6 and 7 the intervention period with multispecies probiotics was one year, which is relatively long compared to other studies that merely have a much shorter intervention period (of 3 or 6 months postnatally), but showed correspondingly results. There are still no studies that investigated the optimal timing and length for an intervention period. Also, the exact *in vivo* mechanism of action of probiotics in shaping the immune response still needs to be determined. Location, timing, and context are essential co-variables regarding microbial interactions with immunity.[32] Above mentioned variables need to be further elucidated prior to making probiotics supplementation more, and consistently, successful. Furthermore, strengthening the understanding of possible working mechanisms of gut bacterial metabolites -particularly short chain fatty acids (SCFAs), produced by gut inhabitants as well as supplemented bacteria- will help to explain associations between gut microbiota and clinical states. In chapter 8 the early role of these metabolites was illustrated, as supplementation of multispecies probiotics seems to induce higher levels of lactate and SCFAs (including butyrate), and lower levels of lactose and succinate in three-months-old infants who later develop eczema. It remains to be established if and how initial microbiota settler types contribute to development of eczema, to SCFAs profiles, and response to treatment with probiotics.

Exemplifying whether the *in vitro* immunomodulatory capacities of a multispecies probiotic formulation will be effective *in vivo* is also necessary prior to targeted clinical application; chapter 9 demonstrates that modulation of *in vitro* cytokine production profiles can be used to differentiate between selected probiotic formulations for their immunomodulatory properties, but so far it is unknown if these in vitro selected probiotics will have better preventive or therapeutic effects on atopic diseases. Use of probiotics during pregnancy, without targeted use, does not seem to induce positive, long-term health effects. Chapter 10 showed that about 1 out of 7 mothers in an unselected population in the Netherlands used probiotics during pregnancy, presumably for their health promoting properties, but common doctors' diagnosed

disease symptoms in the offspring during the first year of life did not differ between probiotics users and non-users. This supports the fact that strain-specificity, dosage and the role for timing of administering probiotics are of particular importance for perhaps inducing (long-term) effects and these factors need to be clarified before probiotic supplementation during pregnancy can be generally recommended. Besides, how the gut microbiota contributes to immunological modulations during pregnancy must await further characterization of the gut microbiota in pregnant women and the neonatal offspring.[46] The exact mechanisms by which microbes colonize the neonatal gut from different sources (e.g. breastmilk) should be determined. This will help to depict the way microbes communicate and interact with the host[18,47,48] and support successful probiotic supplementation in the future.

Future perspectives: in gut we trust?

The field of microbiome research has been (and still is) moving rapidly from identifying what is in the gut, to trying to define what these microbes are doing and how they have impact on health and disease. This is endorsed by the tremendous increase in scientific publications on the (human) microbiome in a broad context. Although we are still at the beginning, the prospects for developing a mechanistic understanding of the factors that underlie the plasticity of the microbiome, and for manipulating the microbiome to improve health, can be considered positive. There is a multitude of functional effects of the gut microbes, which seem to be mainly beneficial and positive for human health. Efforts should be made to further investigate and describe what specifically constitutes a 'healthy gut', and causal relationships between gut microbiota and disease states need to be further explored prior to manipulating the gut microbiome for the purpose of preventing and treating diseases. For example, recently the importance of gut microbiota composition for the effects of food on glycemic response was demonstrated, at the same time underscoring the great inter-individual variation in response to environmental stimuli.[49] Nutritional effects were shown to be individually different, which emphasizes the need for accurate personalized predictions of these effects in a variety of multi-factorial diseases. Subsequently, nutritional modifications can be practically applied and more extensively integrated into the clinical setting.

Whether, but particularly how, early variations in the intestinal microbiota composition in infants can be linked with gut microbiota development and allergic/systemic conditions later in life, continues to be an important subject of investigation. Studies described in this thesis at least support the suspected short-term associations, though do not point towards clear-cut answers. Future research should focus on further elucidating microbial interactions with immunity. Not all bacteria within the microbiota are known to affect the immune system; rather, specific bacteria have particular immunomodulatory effects[32] and the organisms that produce strong uniform effects should be investigated, combined with the mechanisms by which they communicate with

host cells (e.g. metabolites) and regulate specific immune functions.[50] Consequently, rationally designed microbe-based ('probiotic') therapies with specific microbes can be developed, aiming at targeted modification of the microbiota of infants. By doing so, prevalence of important diseases like asthma,[51] obesity and diabetes,[52] but also neuropsychiatric disorders[53] might be reduced, and autoimmunity and/or immunodeficiency may be modulated.[31]

The clinical data which will come out of the INCA study (i.e. frequency and severity of respiratory tract infections, allergic symptoms) will allow to relate initial microbiota development with health and well-being. It is an illusion to expect that our INCA study will provide answers to all above questions. We can only hope that it will expand our knowledge and help to unravel the intricate associations between microbiota development, maturation of the immune system, consequences of modulation of the emerging ecosystem, and (infant) health.

Concluding remarks

The development of the gut microbiota in early life is a complex process, regulated by endogenous as well as exogenous factors, which are being unraveled at the moment but also need additional in-depth investigations. Ultimately, everyone develops his or hers own unique, individual microbiota. The vast majority of those microbiota are associated with maintenance of health. In all those cases it is important not to disturb this developmental process in early life (e.g. with antibiotics). When the microbiota develops in an aberrant way, associated with disease, early intervention (e.g. with pre- or probiotics) is warranted. These interventions must be optimized by personalization and targeted application, an approach which now is within reach: the microbiome has the greatest potential to be translated into the clinic in the very short term. The challenge will be to bring this approach into clinical practice.

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12

TO CONCLUDE

Summary

A bacterial population, weighing more than 1.5 kilogram inhabits the adult human intestinal tract: the gut microbiota, comprising approximately 10^{14} bacteria. The gut microbiota is the ecological community consisting of commensal, symbiotic and pathogenic microorganisms that resides in a defined environment. This environment is established directly after birth when the human infant develops an initial microbiota by becoming colonized with a wide variety of microorganisms. The neonatal period comprises a critical period for microbiota development, also because this is the period during which the immune system matures. The long-term composition and function of the newborn's gut microbiota is suggested to be programmed during this period, thereby laying the foundation for future health and influencing the risk of developing disease later in life. Metchnikoff already hypothesized in the early 20th century that replacing or diminishing 'putrefactive' bacteria in the gut with lactic acid bacteria could normalize bowel health and prolong life. After a lag period of almost a century, the outburst of discoveries in the microbiome field in the course of the last decade changed our perspective on human biology, both in terms of health and disease.

The adult human distal gut microbial community is typically dominated by two bacterial phyla (divisions): the *Firmicutes* and the *Bacteroidetes*, that constitute over 90% of the gut microbiota. However, also other phyla such as *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and *Verrucomicrobia* have a significant influence on the microbial configuration. Studies strongly suggest that dysbiosis contributes to the development of various kinds of disease, such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), obesity, allergy, asthma and diabetes.

This thesis addresses the development of the intestinal microbiota in infancy, investigated by different molecular approaches, and includes studies describing consequences of early life modulation of microbiota, by supplementation of probiotics, on composition and functionality of the microbiota.

PART I: INTESTINAL MICROBIOTA IN INFANCY

After birth, infants are rapidly colonized by microbes from different environments they are exposed to. The classical early colonizers of the infant gut are facultative anaerobes of the phylum *Proteobacteria*, such as *Escherichia coli* and other *Enterobacteriaceae*. These organisms pave the way for strictly anaerobic bacteria by depleting the initial available oxygen in a matter of days. The rapid colonization by commensal bacteria has great impact for the development of the infant's (mucosal) immune system. The development of the gut microbiota continues during the first years of life and its composition is considered to resemble the adult gastrointestinal tract by the age of three. Mode of delivery, gestational age and feeding mode especially affect the infant's microbiota composition. Besides, administration of antibiotics in early life and supplementation with prebiotics or probiotics can have a major effect on the intestinal microbiota composition.

For the identification of intestinal bacterial species, bacterial cultures and biochemical typing techniques were the gold standards for many years. Culture-independent (molecular) approaches however have changed this field completely. The majority of techniques is currently based on sequence analysis of the 16S rRNA gene, which is highly conserved between bacterial species, but varies in a manner that allows species identification. In this thesis, as in general, microbiome analysis focuses on identifying bacterial species. Bacteria form the predominant group of the gut microbiome: more than 99% of DNA in the human intestine is bacterial. We have used four different identifying techniques, all based on rRNA gene analysis, that allow detailed characterization of the multitude of bacterial species and strains present in a sample: quantitative polymerase chain reaction (qPCR), fluorescent in-situ hybridization combined with microscopy (FISH-MS), pyrosequencing and IS-pro. Variability and heterogeneity exists between studies that describe microbiota and development, mainly introduced by making use of different techniques. Validation of the techniques is often lacking, and possible confounding factors and optimal sampling protocols have not yet been investigated sufficiently. This has to be taken into account when results, described in the consecutive chapters, are compared and put into perspective.

In **Chapter 2** microbial signatures of the fecal microbiota of breast-fed infants and formula-fed infants during the first 3 months of life were analyzed by a combination of 454 barcoded-pyrosequencing, fluorescent in situ hybridization with microscopy (FISH-MS), and quantitative PCR (qPCR). We demonstrated that individuality, meaning the unique and personal microbiota development of each individual, is the major driver of microbiota composition in term born, vaginally delivered infants (p=0.002) and was significantly more pronounced in breast-fed infants than in formula-fed infants. All three profiling approaches used, indicated that microbiota development of breast- and formula-fed infants proceeds according to similar developmental stages with distinct microbial marker species. Thus a time-resolved developmental signature in the microbial colonization patterns during the first 3 months of life was observed. Incidence and dominance of skin and human breast milk derived microbes were increased in the gut microbiome of breast-fed infants compared to formula-fed infants.

Since evidence is increasing that exposure to antibiotics in early life is associated with profound effects on the gut microbiome and various disorders later in life, we performed an observational cohort study to investigate the potential clinical and microbial consequences of antibiotic use in early life. The study protocol of the so-called INCA-study (INtestinal microbiota Composition after Antibiotic treatment in early life) is presented in **Chapter 3**. A total of 450 term born infants, of whom 150 are exposed to antibiotic treatment in early life and 300 are not (control group), are included in this ongoing observational cohort study with a one-year follow-up. Clinical outcomes, including coughing, wheezing, fever >38°C, runny nose, glue ear, rash, diarrhea and >3 crying hours a day, are recorded daily by parents and examined by previously defined doctor's diagnosis. At baseline, parents fill out an online questionnaire containing

questions on demographics, comorbidity and use of medication. A blood sample is taken at the end of the study to investigate the infant's vaccination response and sensitization for food and inhalant allergens. Fecal samples are obtained at eight time points during the first year of life. Potential differences in microbial profiles of infants treated with antibiotics versus healthy controls are determined by use of 16S-23S rRNA gene analysis (IS-pro). Both clinical and microbial effects of antibiotic treatment may be demonstrated. **Chapter 4** discusses the results of the INCA study with respect to microbiota composition during the first 3 months of life between infants exposed to antibiotics as compared to the control infants who didn't receive antibiotics. Fecal samples of 45 vaginally term-born and exclusively breastfed infants, of which 21 received antibiotics and 24 were controls, were obtained at one week, one month and 3 months of age. IS-pro was used for bacterial profiling. Antibiotic treatment in the first week of life resulted in a lower abundance, diversity and a delay in acquisition of species of the *Bacteroidetes* phylum. *Escherichia coli*, a common cause of neonatal sepsis, was more prevalent and more stable over time in controls compared to antibiotic-treated infants. The latter group had a less stable microbial composition over time. We concluded that early life antibiotic treatment disturbs the microbiota development in infants, mostly evident in *Bacteroidetes* species. The long-term health implications of these effects are yet unknown, and await the completion of the INCA study. When the data would show that early antibiotic treatment has major long-term consequences, this is of clinical importance. Intervention strategies consisting of the use of pre- or probiotics may mitigate potential negative health effects by influencing the ecological balance of the microbiota. Moreover, chapter 4 shows that regardless of antibiotic treatment, one-week-old infants could be clustered into *Bacteroidetes*-dominant (settler type B) or *Firmicutes-Actinobacteria-Fusobacteria-Verrucomicrobia* (FAFV) dominant microbiota (settler type F). By classifying children according to their settler type, instead of by delivery mode, we hypothesized that developmental patterns and maternal inheritance of microbiota might become more clear. **Chapter 5** investigates the presence of the settler types and their relation with delivery mode and maternal microbiota composition during the first 3 months of life, in healthy infants included in the INCA study. Furthermore, it discusses the early development of the two settler types in relation to breast-feeding versus formula-feeding. In total, 77 neonates (45 vaginally delivered and 32 by caesarean section) were included. 32 children were exclusively breast-fed and 45 were exclusively bottle fed. All fecal analyses were performed with IS-pro. A substantial proportion of vaginally delivered children harbored a microbiota very much similar to that of children delivered by caesarean section. At week one of age, the previously indicated settler types could be confirmed. Interestingly, the settler type B was found exclusively in vaginally delivered children, while the settler type F was present in children delivered vaginally and by caesarean section. Microbiota of children in settler type B showed a high similarity to gut microbiota of their mothers at all time points, while those in settler type F showed a low similarity to that of their mothers at all time points. Both subgroups were quite divergent at one week of age, but converged over time. This might point towards the time-resolved developmental signature in microbial colonization patterns, suggested in chapter 2 as well as

chapter 7, indicating that children have a shared microbiota development over time, determined by age, that continues to develop between two and six years. By classifying children into settler types instead of delivery mode, group effects may become clear and associations with disease later in life might prove easier to establish. Labor characteristics (such as hygiene, duration of ruptured membranes, total duration of delivery, maternal bowel movements during labor, and environment) may be of crucial importance as acquiring bacteria from the *Bacteroidetes* phylum (by exposition to maternal fecal microbiota during delivery as prerequisite for acquisition of *Bacteroidetes* dominant microbiota) seems quite important.

PART II: EFFECTS OF USING PROBIOTICS IN EARLY CHILDHOOD

The development of allergic diseases has been linked to an altered gut microbiota composition, reduced microbial exposure and reduced bacterial diversity in childhood. As such, aberrancies in the microbial colonization patterns or distortion of the microbial ecology early in life might predispose the infant to diseases such as T-helper 2 (Th2) mediated diseases like allergy, wheezing and asthma, or auto-inflammatory T-helper 1 (Th1) diseases, like inflammatory bowel disease, diabetes and obesity. Modulation of the infant microbiota is likely to restore ecological balance. Administration of probiotic bacteria may be a potential approach to prevent allergic disease, by modification of the intestinal microbiota and establishing and/or restoring the physiological balance between Th1 and Th2 cells. The term 'probiotic' describes microorganisms which exert health benefits beyond basic nutrition, and probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.

A previously conducted randomized controlled trial in the Netherlands (PandA-study) showed that administration of multispecies probiotics, in children at high-risk for atopic disease, led to significantly less children developing eczema compared to controls at the age of 3 months, which seemed to be sustained during the first 2 years of life. **Chapter 6** assesses the long-term follow-up results in children included in the PandA study, aiming to determine the potential preventive effect on the prevalence of asthma, allergic rhinitis and eczema at the age of 6 years. The PandA study was a double-blind, randomized, placebo-controlled trial, in which a mixture of probiotic bacteria was perinatally administered to pregnant women and their offspring. In the current single-blind follow-up trial, 84 high-risk children aged 6 years completed the follow-up with a questionnaire, physical examination, lung function testing and specific IgE measurements. Asthma was diagnosed in 5/39 (12.8%) of participants in the probiotic group and in 8/44 (18.2%) of the placebo group (p=0.50). Allergic rhinitis was diagnosed in 5/39 (12.8%) of participants in the probiotic group and in 1/44 (2.3%) of the placebo group (p=0.09). The severity of allergic rhinitis and the prevalence of eczema were comparable between both groups. No differences were found in lung function parameters nor in sensitization between both groups. Therefore, Ecologic® Panda cannot be recommended for long-term primary prevention of asthma and allergic rhinitis. Prolonged gut microbiota management beyond 1 year of age may be required to achieve a long-lasting impact. In **chapter 7** the long-term effects

of added probiotics on the composition and diversity of gut microbiota in infants included in the PandA study are discussed. Fecal samples were collected from 99 children over a 6-year period with the following time points: first week, second week, first month, three months, first year, eighteen months, two years and six years. Bacterial profiling was performed by IS-pro. The presence of the supplemented probiotic strains in fecal samples was confirmed, and the probiotic strains had a higher abundance and prevalence in the probiotic group during supplementation. Only minor and short-term differences in composition of microbiota were found between the probiotic and placebo group and between children with or without atopy. The diversity of Bacteroidetes was significantly higher after two weeks in the placebo group, and after two years atopic children had a significantly higher Proteobacteria diversity ($p < 0.05$). Between two and six years, microbiota composition at phylum level evolved towards an adult-like configuration. These results suggest that perinatal supplementation with Ecologic® Panda, to children at high-risk for atopic disease, has only minor effects on gut microbiota composition during the supplementation period, and does not cause long-lasting differences. Regardless of intervention or atopic disease status, children have a shared microbiota development over time.
Chapter 8 focuses on gut metabolic activity of the microbial population, as one of the suggested mechanisms of action of probiotics is modulation of the activity of the resident microbiota. Metabolites in fecal samples of 3-month old children (of the PandA population) were measured by 1H-NMR to investigate possible gut metabolic alterations. Lower amounts of short-chain fatty acids (SCFAs), succinate, phenylalanine and alanine were found in fecal samples of children later developing eczema, whereas the amounts of glucose, galactose, lactate and lactose were higher compared to the children not developing eczema. These differences were already present at the age of 3 months, while eczema did not develop in the majority of children before the age of 1 year. These results highlight the role bacterial metabolites may play in the development of the immune system, even before clinical expression of allergic disease becomes manifest.
Chapter 9 discusses screening of a panel of 19 different probiotic strains for their ability to modulate the in vitro differentiation of T-helper lymphocytes, as preamble to application in vivo. Multispecies probiotic combinations were formulated and tested for their effects on in vitro cytokine production by human mononuclear cells and were compared to products that already have shown beneficial effects in vivo. All 4 tested combinations of probiotics showed a 40-71% decrease of Th2 cytokine production and a variable increase of Th1 (interferon-γ) and Treg cytokine (IL-10) production compared to the medium control. One specific probiotic mixture was superior in its stimulating effect on IL-10 production. We conclude that modulation of in vitro cytokine production profiles can be used to differentiate between selected probiotic formulations for their immunomodulatory properties.
Not only an aberrant microbiota composition of newborns has been associated with clinical consequences later in life, also manipulation of the maternal microbiota through the use of probiotics may have subsequent consequences for the health of their offspring. In **chapter 10**, characteristics of mothers who used probiotics during pregnancy are described, as well as the effectiveness of maternal use of probiotics on the offspring's health during the first year of life.

In total 341/2491 (13.7%) mothers reported use of probiotics during pregnancy. There were no significant differences in gestation, age, ethnicity, or education between users and non-users. Logistic regression analyses showed that consumption of probiotics was significantly associated with use of homeopathic products (OR 1.65, 95% CI 1.17-2.33, p= 0.005), maternal history of smoking (OR 1.72, 95% CI 1.25-2.37, p = 0.001) and paternal history of smoking (OR 1.39, 95% CI 1.01-1.89, p= 0.05). No differences between groups were observed regarding doctors' diagnosed disease symptoms in the offspring during the first year of life. Using probiotics and/or other health related products without doctor's prescription during pregnancy, for their assumed health promoting properties, might point to compensation for types of less favorable behavior such as parental smoking. Caregivers and people concerned with pregnant women should be aware of this effect when discussing (nutritional) behavior.

The closing **chapter 11** provides a general discussion with the implications for future research and clinical practice. The development of the gut microbiota in early life is a complex process, regulated by endogenous as well as exogenous factors, which are being unraveled at the moment but also need additional in-depth investigations. Ultimately, everyone develops his or her own unique, individual microbiota. The vast majority of those microbiota are associated with maintenance of health. In all those cases it is important not to disturb this developmental process in early life (e.g. with antibiotics). When the microbiota develops in an aberrant way, associated with disease, early intervention (e.g. with pre- or probiotics) is warranted. These interventions must be optimized by personalization and targeted application, an approach which now is within reach: the microbiome has the greatest potential to be translated into the clinic in the very short term. The challenge will be to bring this approach into clinical practice.

Samenvatting

In de darm van een volwassen mens bevindt zich een populatie bacteriën van circa 1,5 kg: de darmmicrobiota, die bestaat uit ongeveer 10^{14} bacteriën. Met darmmicrobiota wordt een ecosysteem van bacteriën bedoeld, bestaande uit commensalen (onschadelijke gast-organismen), symbionten (organismen die met wederzijdse acceptatie naast elkaar leven) en pathogenen (ziekteverwekkers). Direct na de geboorte raakt de darm gekoloniseerd door een grote verscheidenheid aan bacteriën. De periode net na de geboorte is een belangrijke periode in de ontwikkeling, onder meer vanwege het feit dat het immuunsysteem van de zuigeling in deze periode uitrijpt. Men gaat ervan uit dat de samenstelling van de darmmicrobiota, en de functie die hij vervult op lange termijn, in deze periode wordt bepaald. Daarmee wordt het fundament gelegd voor gezondheid in de toekomst en heeft de darmmicrobiota invloed op het risico van bijvoorbeeld het ontwikkelen van allergische aandoeningen later in het leven.

Het eerste wetenschappelijk onderzoek naar het mogelijke verband tussen darmbacteriën en gezondheid werd al vroeg in de 20e eeuw uitgevoerd door dr. Elie Metchnikoff, een Russische microbioloog en Nobelprijswinnaar. Hij beschreef dat het verminderen of vervangen van 'rottende' bacteriën in de darm, door lactobacillen (melkzuurproducerende bacteriën), een levensverlengend effect kon hebben. Nu, bijna een eeuw later, heeft de enorme hoeveelheid aan ontdekkingen en doorbraken in het onderzoeksveld van het (darm)microbioom ons perspectief op gezondheid en ziekte veranderd.

Bij een volwassen mens wordt de darmmicrobiota gedomineerd door twee bacteriële phyla (taxonomische rangen, afdelingen), die samen meer dan 90% van de darmmicrobiota uitmaken: de *Firmicuten* en de *Bacteroïdeten*. Daarnaast hebben de andere phyla *Proteobacteriën*, *Actinobacteriën*, *Fusobacteriën* en *Verrucomicroben* een belangrijke invloed op de microbiële samenstelling. Onderzoek duidt erop dat een verstoorde samenstelling van de darmmicrobiota bijdraagt aan de ontwikkeling van ziekten, waaronder ontstekingsziekten van de darm (IBD), prikkelbare darmsyndroom (IBS), ernstig overgewicht (obesitas), allergie, astma en diabetes.

In dit proefschrift wordt de ontwikkeling van de darmmicrobiota vroeg in het leven beschreven, waarbij voor het bestuderen van de samenstelling van de darmmicrobiota verschillende moleculaire technieken zijn gebruikt. Tevens omvat het proefschrift studies die de gevolgen beschrijven van vroege verandering in de darmmicrobiota als gevolg van de suppletie van probiotica (voedselsupplementen in de vorm van levende micro-organismen), op de samenstelling en de functionaliteit van de darmmicrobiota.

DEEL I: DE DARMMICROBIOTA VROEG IN HET LEVEN

Na de geboorte wordt een baby in snel tempo gekoloniseerd door bacteriën uit verschillende milieus waaraan hij of zij wordt blootgesteld. De eerste bacteriën die de darm koloniseren zijn de

facultatief anaëroben (bacteriën die geen zuurstof nodig hebben om te overleven), onderdeel van de *Proteobacteriën*, zoals *Escherichia coli* en andere soorten van de groep *Enterobacteriaceae.* Deze organismen maken de weg vrij voor strikt anaërobe bacteriën, doordat de beschikbare zuurstof in de darm binnen een paar dagen op raakt. De snelle kolonisatie van commensale bacteriën heeft grote gevolgen voor de ontwikkeling van het mucosale (ofwel de afweer van de slijmvliezen) immuunsysteem van de zuigeling. De ontwikkeling van de darmmicrobiota blijft doorgaan gedurende de eerste levensjaren, en rond de leeftijd van drie jaar is de samenstelling van de microbiota min of meer zoals bij een volwassene. De factoren die van cruciaal belang zijn voor de samenstelling van de darmmicrobiota zijn 1. geboren worden via een vaginale bevalling of keizersnede, 2. zwangerschapsduur (op tijd geboren of te vroeg) en 3. het krijgen van borstvoeding dan wel flesvoeding. Daarnaast kunnen de toediening van antibiotica vroeg in het leven en de suppletie met prebiotica (onverteerbare koolhydraten of voedingsvezels) of probiotica een groot effect hebben op de samenstelling van de darmmicrobiota.

Voor het herkennen en identificeren van darmbacteriën waren de bacteriële kweek en de biochemische typeringstechnieken gedurende vele decennia de 'gouden standaard'. Moleculaire technieken, die onafhankelijk zijn van en toegepast kunnen worden zonder de bacteriële kweek, hebben ons inzicht in de bacteriële populatie in de darm volledig veranderd. Dat komt omdat de meeste bacteriën uit de darm helemaal niet gekweekt kunnen worden. Het merendeel van de technieken is tegenwoordig gebaseerd op aflezen van erfelijk materiaal, waarbij gebruik wordt gemaakt van sequentieanalyse van het 16S rRNA gen, een gen dat vóórkomt in alle bacteriën, maar dat tegelijkertijd varieert tussen verschillende bacteriesoorten en daarmee identificatie van de soort mogelijk maakt.

De analyse van de darmmicrobiota richt zich in dit proefschrift op het identificeren van bacteriesoorten. Binnen de darmmicrobiota vormen bacteriën de belangrijkste populatie: meer dan 99% van het erfelijk materiaal, het DNA, in de menselijke darm is bacterieel. Wij hebben gebruik gemaakt van vier technieken, allemaal gebaseerd op de analyse van het rRNA gen, die gedetailleerd het aantal bacteriële species en stammen (een bacteriecultuur die uit één cel (bacterie) is ontstaan) in een monster kunnen weergeven: kwantitatieve polymerase kettingreactie (qPCR), fluorescerende in situ hybridisatie (een techniek om met behulp van een probe (een bekend DNA-fragment) overeenkomstige stukjes DNA aan te tonen) gecombineerd met microscopie (FISH-MS), zogenaamde 'pyrosequencing' (waarbij bekende opeenvolgende sequenties op het rRNA gen worden herkend, gekopieerd en afgelezen uit het DNA) en een techniek die gebruik maakt van de tussenruimte (interspace) tussen rRNA genen: IS-pro. Er is sprake van een grote mate aan variabiliteit en heterogeniteit tussen studies die de samenstelling en ontwikkeling van de darmmicrobiota beschrijven, voornamelijk doordat gebruik wordt gemaakt van verschillende analysetechnieken en manieren om de resultaten weer te geven. Onderbouwing van de gebruikte technieken ontbreekt dikwijls, en mogelijk verstorende factoren en optimale protocollen om monsters te verwerken zijn nog onvoldoende onderzocht. Hiermee dient rekening te worden gehouden bij de interpretatie en onderlinge vergelijking van de resultaten die in

de opeenvolgende hoofdstukken worden beschreven.
In **hoofdstuk 2** werden de kenmerken van de darmmicrobiota geanalyseerd van borstgevoede, dan wel flesgevoede zuigelingen gedurende de eerste 3 maanden van hun leven. Een combinatie van 454 barcode-pyrosequencing, fluorescerende in situ hybridisatie met microscopie (FISH-MS), en kwantitatieve PCR (qPCR) werd hiervoor gebruikt. We hebben aangetoond dat in op tijd en vaginaal geboren zuigelingen een individueel bepaalde, unieke ontwikkeling van de darmmicrobiota de belangrijkste factor is die de samenstelling beïnvloedt ($p = 0,002$). Individualiteit als beïnvloedende factor was significant hoger bij zuigelingen die borstvoeding krijgen dan bij fles gevoede zuigelingen. Alle drie toegepaste analysetechnieken toonden aan dat de ontwikkeling van de darmmicrobiota van borst- en fles gevoede pasgeborenen verloopt volgens vergelijkbare, discrete ontwikkelingsstadia, waarbij in ieder stadium verschillende microbiële soorten (bacteriën) belangrijk zijn. Er werd dus een tijdsgebonden, algemeen ontwikkelings- en kolonisatiepatroon gezien gedurende de eerste 3 maanden van het leven. In de darmmicrobiota van borstgevoede pasgeborenen kwamen bacteriën afkomstig van de huid en uit de moedermelk vaker voor en was hun aanwezigheid meer overheersend dan in de darmmicrobiota van flesgevoede pasgeborenen.

Het wordt meer en meer duidelijk dat vroege blootstelling aan antibiotica gepaard kan gaan met ingrijpende gevolgen voor de samenstelling en functie van de darmmicrobiota, alsmede ten aanzien van het ontstaan van verschillende aandoeningen later in het leven. Vanwege dit mogelijke verband hebben we een observationele cohortstudie opgezet en uitgevoerd, die de klinische en microbiële gevolgen van het gebruik van antibiotica, vroeg in het leven, bestudeert. Het studieprotocol van deze zogenaamde INCA-studie ('samenstelling van de darmmicrobiota na behandeling met antibiotica, vroeg in het leven') wordt beschreven in **hoofdstuk 3**. Er zijn in totaal 450 op tijd geboren kinderen geïncludeerd in deze studie, die gedurende hun eerste levensjaar gevolgd werden. Van de totale groep zijn er 150 blootgesteld aan een behandeling met antibiotica direct na de bevalling: de antibioticagroep, en 300 niet: de controlegroep. Het vóórkomen van klinische symptomen, waaronder hoesten, piepende ademhaling, koorts >38 ˚C, loopneus, ontstoken oor, huiduitslag, diarree en meer dan 3 huiluren per dag, is gedurende één jaar wekelijks door ouders bijgehouden en is geverifieerd door het vóórkomen van vooraf gedefinieerde en door een arts gestelde diagnoses te onderzoeken. Bij aanvang van de studie vulden ouders een online vragenlijst in waarmee demografische gegevens, comorbiditeit en het gebruik van medicatie in kaart gebracht werden. Aan het einde van de studie, op het moment dat het kind één jaar was, werd een bloedmonster afgenomen om een mogelijke overgevoeligheid van het immuunsysteem voor voedings- en inhalatieallergenen (zoals boompollen, graspollen, huisstofmijt, kippenei-eiwit, pinda) te onderzoeken, alsmede de reactie van het immuunsysteem te bepalen op de vaccinaties uit het Rijksvaccinatieprogramma. Poepmonsters werden verzameld door ouders op acht afgesproken tijdstippen gedurende het eerste levensjaar van het kind. Middels IS-pro werden mogelijke verschillen in de samenstelling van de darmmicrobiota onderzocht, waarbij de antibioticagroep vergeleken werd met de

controlegroep.

Hoofdstuk 4 beschrijft de samenstelling van de darmmicrobiota tijdens de eerste 3 maanden van het leven, in kinderen die na de bevalling met antibiotica behandeld werden vergeleken met 'controle-kinderen' die geen antibiotische behandeling kregen. Poepmonsters van 45 zuigelingen, geboren via een vaginale bevalling en die uitsluitend borstgevoed waren, werden daarbij onderzocht. Van hen waren er 21 met antibiotica behandeld en 24 niet. Soorten bacteriën van het phylum *Bacteroïdeten* blijken na vroege behandeling met antibiotica in mindere mate en lagere diversiteit voor te komen. Daarnaast vindt de verwerving van bacteriën uit dit phylum vertraagd plaats. In de controlegroep kwam *Escherichia coli,* een vaak voorkomende verwekker van neonatale sepsis (een ernstige ontstekingsreactie van het hele lichaam), frequenter voor en zijn aanwezigheid was stabieler. In de antibioticagroep was de populatie bacteriën minder stabiel. We concludeerden dat behandeling met antibiotica kort na de geboorte de ontwikkeling van de darmmicrobiota bij pasgeborenen verstoort, wat vooral zichtbaar is in de bacteriesoorten van het phylum *Bacteroïdeten*. Om een uitspraak te kunnen doen over mogelijke gevolgen op de lange termijn, ten aanzien van gezondheid en ziekte, zullen we moeten wachten op de afronding van de INCA-studie. Als de verkregen data aantonen dat vroege blootstelling aan antibiotica lange-termijn gevolgen heeft op de samenstelling van de darmmicrobiota, dan is dit klinisch van belang. Toepassing van pre- of probiotica zou mogelijk negatieve effecten op de gezondheid kunnen verminderen door het positief beïnvloeden van het ecologisch 'evenwicht' van de darmmicrobiota. Daarnaast beschrijven we in hoofdstuk 4 dat we in de darmmicrobiota van zuigelingen, op de leeftijd van één week, twee clusters kunnen onderscheiden: één cluster waarin *Bacteroïdeten* dominant zijn (we noemen dit 'settler type B'), en één cluster dat gedomineerd wordt door *Firmicuten* ('settler type F'). Door vervolgens de kinderen te classificeren naar dit 'settler type', in plaats van op basis van wijze van geboorte (vaginaal dan wel via keizersnede), denken we dat er meer duidelijkheid zou kunnen komen in veranderde ontwikkelingspatronen van de darmmicrobiota en in de invloed die de moeder hierop heeft.

In **hoofdstuk 5** wordt de aanwezigheid van de 'settler types' bestudeerd in een groep controlekinderen uit de INCA-studie gedurende de eerste 3 levensmaanden, en de relatie van het 'settler type' met de wijze van geboorte en met de samenstelling van de darmmicrobiota van moeder. Verder wordt ingegaan op de vroege ontwikkeling van de twee 'settler types' bij borstvoeding en flesvoeding. In totaal zijn 77 pasgeborenen (45 vaginaal geboren en 32 via een keizersnede) geïncludeerd. Daarvan kregen 32 kinderen uitsluitend borstvoeding en 45 uitsluitend flesvoeding. Alle analyses werden uitgevoerd met IS-pro. Voor een aanzienlijk deel van de vaginaal geboren kinderen was de samenstelling van de darmmicrobiota zeer vergelijkbaar met die van kinderen die middels een keizersnede ter wereld kwamen. Op de leeftijd van één week kon de eerder beschreven aanwezigheid van 'settler types' worden bevestigd. Daarbij is een interessante bevinding dat 'settler type B' uitsluitend voorkomt bij vaginaal geboren kinderen, terwijl 'settler type F' voorkomt bij zowel vaginaal als middels keizersnede geboren kinderen. De darmmicrobiota van zuigelingen met 'settler type B' was in

grote mate overeenkomstig die van hun moeder, op alle tijdstippen, terwijl de darmmicrobiota van zuigelingen met 'settler type F' veel minder overeenstemming toonde met die van hun moeder (op alle tijdstippen). Qua microbiële samenstelling liepen de twee groepen sterk uiteen op de leeftijd van één week oud, maar gingen ze na verloop van tijd meer op elkaar lijken. Dit kan duiden op een algemeen ontwikkelingspatroon van microbiële kolonisatie, zoals ook beschreven wordt in hoofdstuk 2 en hoofdstuk 7, dat per individu verschilt en leeftijdsgebonden is en zich verder ontwikkelt tussen de leeftijd van 2 en 6 jaar.

Door zuigelingen te classificeren naar 'settler types' werden groepseffecten duidelijk. Dit zou het aantonen van een mogelijk verband tussen samenstelling van de darmmicrobiota en (ontwikkeling van) ziekte later in het leven kunnen vereenvoudigen. Onze hypothese hierbij is dat factoren tijdens en rondom de bevalling (zoals hygiëne, duur van gebroken vliezen, totale duur van de bevalling, of moeder wel of niet ontlasting heeft tijdens de bevalling, en de omgeving waarin de bevalling plaatsvindt) van cruciaal belang zijn voor het verwerven van bacteriën van het phylum *Bacteroïdeten*.

DEEL II: EFFECTEN VAN HET GEBRUIK VAN PROBIOTICA VROEG IN HET LEVEN

De ontwikkeling van allergische aandoeningen hangt samen met een veranderde samenstelling van de darmmicrobiota, verminderde blootstelling aan bacteriën en verminderde bacteriële diversiteit in de kindertijd. Afwijkingen in de patronen van microbiële kolonisatie, waarbij het hele microbiële ecosysteem van de zuigeling beïnvloed wordt, kunnen leiden tot T-helper 2 cel (Th2) gemedieerde ziekten als allergieën, piepende ademhaling en astma, of auto-inflammatoire T-helper 1 cel (Th1) predisponerende ziekten als ontstekingsziekten van de darm (IBD), diabetes en ernstig overgewicht (obesitas). Aanpassing van de darmmicrobiota van de zuigeling kan waarschijnlijk het ecologisch evenwicht in het systeem herstellen. Toediening van probiotische bacteriën kan een strategie zijn om de ontwikkeling van allergische ziekten te voorkomen, door aanpassing van de compositie van de darmmicrobiota en/of herstel van het fysiologische evenwicht tussen Th1 en Th2-cellen. Probiotica zijn gedefinieerd als levende micro-organismen die, wanneer ingenomen in voldoende hoeveelheden, een gezondheidsbevorderend effect hebben.

Een eerder in Nederland uitgevoerde studie (de PandA-studie) toonde aan dat toediening van multispecies (mengsel van meerdere stammen) probiotica, bij kinderen met een hoog risico op allergische aandoeningen, ervoor zorgde dat aanzienlijk minder kinderen eczeem ontwikkelden in de probiotica-groep ten opzichte van de controlegroep op de leeftijd van 3 maanden. Dit positieve effect leek aan te houden gedurende de eerste 2 levensjaren. **Hoofdstuk 6** beschrijft de lange-termijn resultaten van kinderen uit de PandA-studie, gericht op het mogelijk preventieve effect van probiotica op vóórkomen van astma, hooikoorts en eczeem op de leeftijd van 6 jaar. De PandA-studie was een dubbelblinde, gerandomiseerde, placebo-gecontroleerde studie, waarin een mengsel van probiotische bacteriën werd toegediend aan zwangere vrouwen (vanaf 6 weken voor de uitgerekende datum) en hun nakomelingen (dagelijks, vanaf de geboorte,

gedurende het eerste levensjaar). In de huidige follow-up studie konden 84 kinderen, van 6 jaar, met een hoog risico op het ontwikkelen van allergische aandoeningen worden geïncludeerd. De follow-up bestond uit een vragenlijst, lichamelijk onderzoek, longfunctieonderzoek en specifieke metingen van antistoffen in het bloed. Astma werd gediagnosticeerd bij 5/39 (12,8%) van de kinderen in de probioticagroep en 8/44 (18,2%) van de kinderen in de placebogroep (p = 0,50). Hooikoorts werd gediagnosticeerd bij 5/39 (12,8%) van de kinderen in de probioticagroep en 1/44 (2,3%) van de kinderen in de placebogroep (p = 0,09). De ernst van de hooikoorts en de prevalentie van eczeem waren vergelijkbaar tussen beide groepen. Er werd geen verschil gevonden qua longfunctie, noch in sensibilisatie (gevoeligheid van het afweersysteem) tussen beide groepen. Er werd geconcludeerd dat suppletie van Ecologic®Panda (het mengsel van 3 verschillende probiotische bacteriën) niet kan worden aanbevolen ter voorkoming van astma en hooikoorts op de lange termijn.

Hoofdstuk 7 beschrijft de lange-termijn effecten die probiotica hebben op samenstelling en diversiteit van de darmmicrobiota, bij zuigelingen uit de PandA-studie. Poepmonsters werden verzameld van 99 kinderen gedurende een periode van 6 jaar, op diverse tijdstippen vanaf de geboorte. De samenstelling van de darmmicrobiota werd geanalyseerd met IS-pro. De aanwezigheid van de gesuppleerde probiotische stammen in poepmonsters werd bevestigd, en de probiotische stammen kwamen in hogere mate en vaker voor in de probioticagroep, tijdens suppletie. Slechts kleine verschillen in de samenstelling van de darmmicrobiota werden gevonden tussen de probiotica- en de placebogroep. Er werden ook maar kleine verschillen gevonden tussen kinderen met en zonder allergie. De diversiteit binnen het phylum *Bacteroïdeten* was significant hoger na twee weken in de placebogroep, en na twee jaar hadden allergische kinderen een significant hogere diversiteit van *Proteobacteriën* (p <0,05). Tussen 2 en 6 jaar vond de overgang plaats naar een volwassen-achtige samenstelling van de darmmicrobiota. Deze resultaten suggereren dat de suppletie met Ecologic®Panda slechts een kleine invloed heeft op de samenstelling van de darmmicrobiota tijdens de periode van suppletie, en dat deze suppletie geen langdurige verschillen veroorzaakt. Ongeacht interventie (probiotica of niet) en het wel of niet hebben van allergie, maken kinderen een algemeen geldende, maar wel individueel bepaalde, ontwikkeling van darmmicrobiota door.

In **hoofdstuk 8** is de metabole activiteit van de microbiële populatie in de darm onderzocht, omdat wordt verondersteld dat één van de werkingsmechanismen van probiotica is dat ze de activiteit van de in de darm aanwezig bacteriën kunnen beïnvloeden. Metabolieten in poepmonsters van kinderen van 3 maanden oud (van de PandA-studie populatie) werden gemeten met ^{1}H-NMR (een nucleaire magnetische resonantietechniek) om metabole veranderingen in de darm te bestuderen. Er werden minder korte-keten vetzuren gevonden bij kinderen die later eczeem ontwikkelden, en tegelijkertijd werd in de ontlasting van deze groep meer glucose, galactose, lactose en lactaat aangetoond in vergelijking met de kinderen die geen eczeem ontwikkelden. Deze verschillen waren op de leeftijd van 3 maanden al aanwezig, terwijl

de meeste kinderen niet vóór de leeftijd van 1 jaar (atopisch) eczeem ontwikkelen. Toediening van het probiotica mengsel leidde tot een verhoging van de korte-keten vetzuur productie. Deze resultaten onderschrijven de rol die bacteriële metabolieten kunnen spelen bij de ontwikkeling van het immuunsysteem, zelfs voordat een allergische aandoening zich klinisch openbaart.

Hoofdstuk 9 beschrijft de screening van een panel van 19 verschillende probiotische stammen op hun vermogen om de *in vitro*, dus buiten het lichaam, uitrijping van T-helpercel-lymfocyten (gespecialiseerde afweercellen) te beïnvloeden, vooruitlopend op de toepassing *in vivo*, dus in de mens. Verschillende combinaties van probiotische bacteriën werden gemaakt en effecten van deze mengsels op de productie van cytokinen (moleculen die een rol spelen in de afweer) door menselijke éénkernige cellen werden *in vitro* getest. De mengsels werden vervolgens vergeleken met producten waarmee al gunstige effecten *in vivo* waren aangetoond. Alle 4 de geteste combinaties van probiotica toonden een 40-71% afname van de cytokineproductie van Th2 cellen en een variabele toename van cytokineproductie van Th1 cellen en van regulatoire T-cellen (IL-10), in vergelijking met de controleproducten. Eén specifiek probiotisch mengsel stak boven de rest uit ten aanzien van zijn stimulerende effect op de IL-10 productie. We concludeerden dat beïnvloeding van de cytokineproductie *in vitro* kan worden gebruikt om onderscheid te maken tussen de potentie van probiotische mengsels met betrekking tot de mogelijkheid om (de uitrijping van) het immuunsysteem te beïnvloeden.

Niet alleen een afwijkende samenstelling van de darmmicrobiota van zuigelingen is geassocieerd met klinische gevolgen op latere leeftijd, ook manipulatie van de darmmicrobiota van moeder, door het gebruik van probiotica, kan gevolgen hebben voor de gezondheid van hun zuigeling. In **hoofdstuk 10** zijn kenmerken beschreven van moeders die probiotica gebruikten tijdens de zwangerschap. Daarnaast is het effect onderzocht van het gebruik van probiotica door moeder op de gezondheid van hun nakomelingen tijdens het eerste levensjaar. In totaal rapporteerden 341/2491 (13,7%) moeders het gebruik van probiotica tijdens de zwangerschap. Er waren geen significante verschillen ten aanzien van zwangerschapsduur, leeftijd van moeder, etniciteit, of opleidingsniveau tussen gebruikers en niet-gebruikers. Logistische regressieanalyse toonde aan dat het gebruik van probiotica significant geassocieerd was met het gebruik van homeopathische middelen (odds ratio 1,65, 95% betrouwbaarheidsinterval 1,17-2,33, p = 0,005), of moeder in het verleden had gerookt (odds ratio 1,72, 95% betrouwbaarheidsinterval 1,25-2,37, p = 0,001) en of vader in het verleden had gerookt (odds ratio 1,39, 95% betrouwbaarheidsinterval 1,01-1,89, p = 0,05). Er werden geen verschillen tussen de groepen gezien met betrekking tot veelvoorkomende ziektesymptomen bij de kinderen in het eerste levensjaar. Het gebruik van probiotica en/of andere gezondheidsgerelateerde producten zonder doktersvoorschrift tijdens de zwangerschap, wellicht vanwege het veronderstelde gunstige effect op gezondheid, zou kunnen wijzen op een compensatiemechanisme ten aanzien van minder gezond gedrag in het verleden, zoals roken door (één van de) ouders. Professionele zorgverleners dienen zich bewust te zijn van dit effect, wanneer zij voeding en het gedrag omtrent voeding met de zwangere bespreken.

Het afsluitende **hoofdstuk 11** vormt de algemene discussie, met daarin implicaties voor toekomstig onderzoek en voor de klinische praktijk. De ontwikkeling van de darmmicrobiota vroeg in het leven is een complex proces, dat gereguleerd wordt door zowel endogene als exogene factoren. Uiteindelijk ontwikkelt elk individu zijn of haar eigen unieke, individueel samengestelde darmmicrobiota. Het lijkt in ieder geval belangrijk dit ontwikkelingsproces, vroeg in het leven, niet te verstoren bijvoorbeeld door behandeling met antibiotica. Wanneer de darmmicrobiota zich 'afwijkend' ontwikkelt, is dit mogelijk geassocieerd met het vóórkomen van ziekten op latere leeftijd; vroegtijdige interventie (bijvoorbeeld door suppletie van pre- of probiotica) lijkt dan gerechtvaardigd. Deze interventies moeten dan wel gerichter worden toegepast. Deze aanpak is binnen handbereik: het onderzoek naar het microbioom heeft de potentie om op korte termijn te kunnen worden toegepast in de kliniek

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Lieve clubgenoten, (oud)huisgenoten, vrienden en vriendinnen, familie, hockeyteam: heel veel dank voor jullie betrokkenheid, steun en interesse rondom mijn onderzoek, maar vooral voor de mooie vriendschappen en de gezelligheid en humor buiten het werk om. Er komt nu hopelijk weer wat meer tijd om elkaar te zien!

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Lieve Sander en *Cees-Bart*, opperbeste zwagers! Jullie interesse en humor zijn geweldige afleiders (geweest). Dank daarvoor, houden zo! Ik vind het super om te merken hoe jullie met de kleintjes omgaan, jullie betrokkenheid en gekkigheid met ze waardeer ik enorm.

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Liefste Nine en *Loek*. Als deelnemers aan de INCA-studie stonden jullie voor het eerst in je leven een buisje bloed af..! *Nine*, dit is dan het boekje waar mama steeds aan moest werken. Het zal een kleine teleurstelling zijn dat het niet roze is, maar zoals beloofd mag je erin kleuren, tekenen, plakken en lettertjes zoeken. *Loek*, ik denk dat jij er vooral mee zal gooien en het lekker kapot gaat scheuren. Ook prima! Steeds weer geniet ik van jullie ontdekkingstocht en ik kijk uit naar wat meer quality-time met jullie. Ik verheug me op alles wat we samen gaan meemaken!

Liefste Mark. Een promotietraject verenigen met alle life events die je je kunt bedenken… dat is niet de meest makkelijke cocktail. Ik herinner me nog de dag dat je zei 'kap er toch mee'! Maar dat ik hier (natuurlijk) wel sta is echt ook dankzij jou. Als laatste genoemd worden in zo'n dankwoord, dat leek je al die tijd wel wat hè..!! Het is je meer dan gegund. Dank voor je steun en relativeringsvermogen, opgewekte karakter, humor, positiviteit en liefde. Samen leven met jou en de kinderen geeft het leven kleur, en daar kan ik geen genoeg van krijgen!

Curriculum vitae

Nicole Rutten was born on March 12th 1982 in Beuningen, the Netherlands. She grew up with an older and a younger sister. In 2000 she graduated from secondary school at the Stedelijk Gymnasium Nijmegen in Nijmegen. That year she started as a student Psychology at the Rijksuniversiteit Groningen, and after two years she draw a place to start studying Medicine at the same university. During her medical training she participated in a student team for organ donation and transplantation (supervisor Prof. dr. R.J. Ploeg). Her enthusiasm and interest led her go to Madison, Wisconsin, USA, where she performed elective clerkships in transplant surgery and pediatric endocrinology and did a research project on non-heart beating kidney donation (supervised by Dr. A.M. D'Alessandro). She performed her internships at the Deventer Hospital. During three months she worked at the Biharamulo Designated District Hospital in Tanzania, performing an optional internship in tropical medicine. She carried out her research internship at the St. Antonius Hospital in Nieuwegein, under supervision of pediatricians H. van Wieringen and Dr. W. Balemans. After graduating from Medical School in 2009 she stayed at the St. Antonius Hospital and worked as a resident at the department of Pediatrics (supervisor Dr. W. Balemans). In June 2010 she started working on the research described in this thesis under supervision of Dr. A.M. Vlieger, Prof. dr. ir. G.T. Rijkers and Prof. dr. C.K. van der Ent.

Nicole is married to Mark Slijfer, and together they have a daughter, named Nine (2012) and a son, named Loek (2014).