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John Penders

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Gut Microbiota and Atopic Manifestations in Infancy

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In dierbare herinnering aan mijn moeder

Contents

	List of abbreviations	9
Chapter 1	General introduction	11
Chapter 2	The role of the intestinal microbiota in the development of atopic disorders	21
Chapter 3	Quantification of *Bifidobacterium spp.*, *E. coli* and *C. difficile* in faecal samples of breastfed and formula-fed infants by real-time PCR	45
Chapter 4	Factors influencing the composition of the intestinal microbiota in early infancy	61
Chapter 5	Molecular fingerprinting of the intestinal microbiota of infants in whom atopic eczema was or was not developing	83
Chapter 6	Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study	97
Chapter 7	General discussion	117
	Summary	133
	Samenvatting	139
	List of publications	145
	Dankwoord	149
	About the author	153

List of abbreviations

AD	atopic dermatitis
AEDS	atopic dermatitis/eczema syndrome
APC(reg)	(regulatory) antigen presenting cell
ATCC	American Type Culture Collection
CARD	caspase recruitment domain protein
CFU	colony forming units
CI	confidence interval
Coef	coefficient
C_t	threshold cycle
DC	dendritic cell
DGGE	denaturing gradient gel electrophoresis
FISH	fluorescence in situ hybridisation
GALT	gut associated lymphoid tissue
IBD	inflammatory bowel disease
IBS	inflammatory bowel syndrome
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ISAAC	International Study of Asthma and Allergies in childhood
LPS	lipopolysaccharide
LT	*Escherichia coli* heat-labile enterotoxin
NTC	no template control
NTCC	National Collection of Type Cultures
OR	odds ratio
PCR	polymerase chain reaction
RCMV	rat cytomegalovirus
rDNA	ribosomal DNA
rRNA	ribosomal RNA
Spp.	species
SPT	skin prick test
TGF	transforming growth factor
TGGE	temperature gradient gel electrophoresis
Th1	T helper 1
Th2	T helper 2
TLR	toll-like receptor
T_m	melting temperature
Treg	regulatory T cell
UK-WP	U.K. Working Party

Chapter 1

General introduction

Chapter 1

Background

Allergic asthma, hay fever (and other airway allergies), eczema and food allergy are collectively known as 'atopic disorders'. A largely unexplained increase in the prevalence of these diseases was noted during the past decades, prominently in industrialised countries and especially in children [1]. Recently, this increase seems to level off in some [2, 3], but not all countries [4, 5]. The burden created by atopic diseases is huge affecting the individuals' physical and social health and causing an immense financial burden to the family and to the nation's health-care budget [6]. The development of atopic diseases depends on both genetic and environmental factors and it is likely to be an interaction of these, particularly in early life, which determines the atopic status of a person [7]. However, as the human genotype would not have changed substantially over a short time period, it is generally believed that the causes for the rise in atopic diseases are found among factors in the environment [8].

Immunologic aspects of atopic diseases

Naive T helper cells can differentiate into various effector phenotypes such as Th1 and Th2 cells characterized by its cytokine-secretion profile. Th1 cells mediate cellular immunity against intracellular bacteria and viruses and Th2 cells regulate humoral immunity and immunity against extracellular parasites. Both Th1 and Th2 immune responses can be downregulated by another subset of T cells termed regulatory/suppressor T cells (Treg)[9].

Atopic diseases are chronic inflammatory disorders caused by aberrant T helper 2 (Th2)-type immune responses against common 'innocuous' environmental antigens (allergens) in susceptible individuals [10]. Th2 cells produce interleukin (IL)-4, IL-13 and IL-5, which together regulate the allergic response. IL-4 directs the differentiation of T cells towards a Th2 cytokine profile and acts as a growth factor for the expression of these cells.

In addition, IL-4 and IL-13 regulate the synthesis of IgE by B cells, whereas IL-5 is a necessary factor for the differentiation, recruitment and activation of eosinophils [11, 12]. IgE binds with high affinity to receptors (FcεRI) found on mast cells leading to immediate and delayed release of inflammatory mediators, initiating hypersensitivity and late-phase reactions [13].

Hypothesis

Research has recently shifted attention from exposure to allergens that may cause sensitisation and/or provoke atopic diseases to factors that may programme the initial susceptibility to atopic diseases. This also in part involves a shift of attention from potential risk factors, such as indoor and outdoor pollution that do not seem to contribute substantially to the rise in atopy, to protective factors and the possible loss of protective factors in westernized countries. [8, 14, 15]. If a given environmental factor is considered to be of importance in the increased prevalence of atopy, it should have the ability to stimulate the immune system. In addition, its occurrence in nature should explain the difference in the prevalence of atopic diseases between the rich and the poor, between urban and rural areas, and between the East and the West [16]. Many now consider atopic diseases a result of an immature immune system, possibly caused by insufficient microbial stimulation of the immune system as a result of westernization (less childhood viral infections, altered gut microbiota) [8] (Chapter 2).

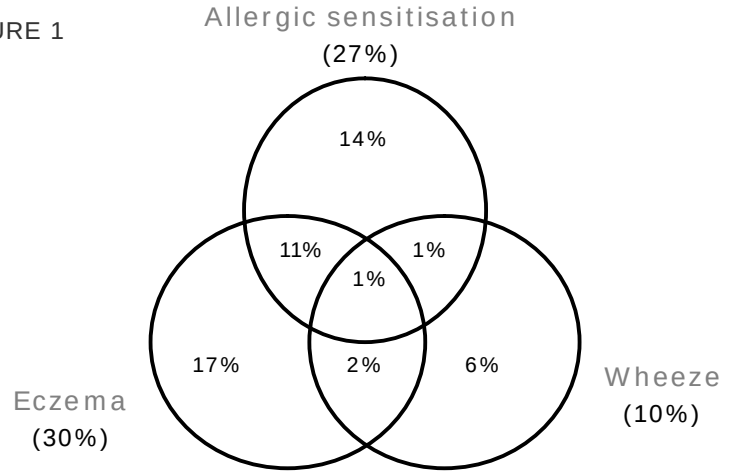
Infant atopic manifestations

The most common atopic manifestation in early infancy is eczema, a chronic, relapsing disease of the skin associated with pruritus, which usually has its onset during the first 6 months of life [6]. Eczema is the most common skin disease in children [17]. Other atopic diseases like allergic asthma or hay fever mostly manifest at older ages [18].

The proportion of infants with eczema, recurrent wheeze and allergic sensitisation in the KOALA Birth Cohort Study is shown in Figure 1. Although eczema has been thought of as principally an atopic disease, this figure shows that far from all children with eczema have raised specific IgE-levels. It is well known that a subgroup of patients suffer from a skin disease with features that clinically resemble the skin lesions and distribution pattern of atopic eczema, without being sensitised [19, 20]. This has led to a continuing controversy as to whether allergic sensitisation (ie, skin prick test positivity or increase of specific IgE levels to common environmental allergens) is an essential feature of childhood eczema [21]. Several theories have been proposed to explain why not all infants with eczema are sensitised. First, there may exist two different variants of eczema, the intrinsic or non-atopic form and the IgE-mediated extrinsic or atopic form [22]. Alternatively, allergic sensitisation may as well be a predictor (or even an epiphenomenon)

of disease activity, since sensitised infants more often have severe eczema compared to non-sensitised infants [23]. Also, sensitisation may be a consequence of eczema rather than a cause. The damaged skin barrier may act as an important route of antigen sensitisation [19, 24].

FIGURE 1



Venn diagram showing the proportion of infants with allergic sensitisation (specific serum IgE to hen's egg, cow's milk, peanut, birch, grass pollen, cat, dog and/or house dust mite), eczema, recurrent wheeze or any combination of the three conditions during the first two years of life in the KOALA study. Subset areas presented above are not proportional to the actual relative subset size.

However, it should also be noted that both the number of allergens tested for, and at what age(s) measurements are performed will have a direct effect on the proportion of infants with eczema that are considered as being sensitised. It has been shown that at least half of the patients with eczema without sensitisation against common allergens, produce IgE against rarer or formerly unidentified microbial allergens, such as *Staphylococcus aureus*, or autoantigens, not included in the standard batteries of skin prick testing (SPT) and specific IgE [25-27]. The age at which measurements are performed is especially important regarding sensitisation to food allergens, which in contrast to aero-allergens is often transient [20].

Since hay fever and asthma usually occur in later childhood, recurrent wheeze is often used as an indicator of atopic airway disease in young infants [28, 29]. Wheezing is a relatively common symptom in infancy, however not all wheezing bronchitis is or will become asthma. Instead, wheezing illness in young children represent a heterogeneous group of conditions [30]. Nevertheless, follow-up studies indicate that wheezing in infancy is an important risk factor for asthma later in life [28].

Allergic sensitisation in children below the age of 4 occurs predominantly against foods. By the age of 2 to 4 years, most children outgrow their food allergy (to cow's milk and hen's egg). After 6 years of age, allergic sensitisation to inhalant allergens such as house dust mite and pollen is more frequently diagnosed than food sensitisation [31].

Terminology of atopic diseases

The terminology used to characterize allergic and allergy like reactions is still confusing [32]. Atopic eczema and atopic dermatitis are terms that have been used synonymously [33]. Many terms were coming and going such as: 'extrinsic/intrinsic atopic dermatitis', 'atopic eczema/dermatitis syndrome' (AEDS) but also 'atopiform dermatitis'[34-37]. Recently, a review committee by the World Allergy Organization has published its recommended terminology [32]. Now, dermatitis is used as the 'umbrella' term for a local inflammation of the skin. 'Eczema' is proposed as replacement for the term 'atopic eczema/dermatitis syndrome'. In particular, 'atopic eczema' is 'eczema' in a person of the atopic constitution i.e. based on the presence of IgE sensitisation. In the present thesis we have acknowledged to use the term 'eczema' instead of 'atopic eczema' if IgE sensitisation has not been determined. However, inconsistent with the new nomenclature, we still use the term 'atopic dermatitis' when considering the UK Working Party Criteria according to Williams et al [38-40], since Williams and colleagues when introducing these criteria originally used the term atopic dermatitis. Further, we have chosen to use the term 'atopic manifestations' when reporting on symptoms of eczema and recurrent wheeze and/or the presence of specific IgE sensitisation.

The KOALA Birth Cohort Study

The KOALA Birth Cohort Study aims at identifying factors that influence the clinical expression of atopic diseases with a main focus on lifestyle (e.g., anthroposophy, vaccinations, antibiotics, dietary habits), breastfeeding and breast milk composition, intestinal microbiota composition, infections during the first year of life, and gene–environment interactions. The recruitment of pregnant women started in October 2000. First, participants with 'conventional lifestyles' (n = 2343) were retrieved from an ongoing prospective cohort study (n = 7020) on pregnancy-related pelvic girdle pain [41]. In addition, pregnant women (n = 491) with 'alternative lifestyles' with regard to child rearing practices, dietary habits (organic, vegetarian), vaccination schemes and/or use of antibiotics, were recruited through organic food shops, anthroposophic doctors and midwives, Steiner schools, and dedicated magazines. All participants were enrolled between 14 and 18 weeks of gestation and completed an intake questionnaire on family history of atopy and infant care intentions. Documentation of other relevant

variables started in the pregnant mother and covered the first and third trimester as well as early childhood by repeated questionnaires at 14–18, 30, and 34 wk of gestation and 3, 7, 12, and 24 months post-partum. A subgroup of participants, including both conventional and alternative lifestyles, was asked to consent to maternal blood sampling, breast milk and a faecal sample of the infant at 1 month post-partum, capillary blood at age 1 yr, venous blood and physical examination of manifestation of atopic dermatitis during home visits at the age of 2 yr (using the UK working party criteria), and buccal swabs for DNA isolation from child–parent trios[42].

Aim and outline of the thesis

The aim of this thesis was to examine the intestinal microbiota composition in early infancy, using molecular techniques, and to evaluate the role of this gut microbiota in the subsequent development of atopic manifestations.

Chapter 2 gives an overview of the observational studies on the role of the gut microbiota in the aetiology of atopic diseases, with a special focus on the methods used to quantify the intestinal microbiota.

To study the gut microbiota in a culture-independent way, we developed several real-time PCR assays. Chapter 3 describes the development and validation of real-time PCR assays for the quantification of *Bifidobacterium spp., Clostridium difficile* and *Escherichia coli,* and the application of these assays on faecal samples of 50 exclusively breastfed and 50 exclusively formula-fed infants. These and other real-time PCR assays have then been applied to study the composition of the gut microbiota of over one thousand one-month-old infants participating in the KOALA Study. The exploration of a broad range of environmental factors potentially contributing to the composition of the gut microbiota in early infancy has been described in Chapter 4.

In a case-control study, nested within the KOALA birth cohort, we examined the composition of faecal samples of infants in whom atopic eczema was or was not going to develop within the first year of life. In this study we used another molecular approach, 16S rDNA based PCR combined with denaturing gradient gel electrophoresis, to examine the composition of the gut microbiota (Chapter 5). Chapter 6 presents a study on the association between the gut microbiota composition, as determined by real-time PCR, and the development of atopic manifestations within the first two years of life. Chapter 7 discusses the results of this thesis and brings us back to the question what is still to be discovered with regard to the intestinal microbiota composition and its role in the aetiology of atopic diseases.

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Chapter 2

The role of the intestinal microbiota in the development of atopic disorders

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Accepted pending revision. Allergy

Chapter 2

Abstract

The prevalence of atopic diseases, including eczema, allergic rhinoconjunctivitis and asthma, has increased worldwide, predominantly in westernized countries. Recent epidemiological studies and experimental research suggest that microbial stimulation of the immune system influences the development of tolerance to innocuous allergens. The gastrointestinal microbiota composition may be of particular interest, since it provides an early and major source of immune stimulation and seems to be a prerequisite for the development of oral tolerance. In this review the observational studies on the association between the gut microbiota and atopic diseases are discussed. Although most studies indicated an association between the gut microbiota composition and atopic sensitisation or symptoms, no specific harmful or protective microbes can be identified yet. Some important methodological issues that have to be considered are; the microbiological methods used (traditional culture vs. molecular techniques), the timing of examing the gut microbiota, the definition of atopic outcomes and the possibility of reverse causation.

In conclusion, the microbiota hypothesis in atopic diseases is a promising hypothesis and deserves further attention. To gain more insight on the role of the gut microbiota in the aetiology of atopy, large-scale prospective birth cohort studies using molecular methods to study the gut microbiota are needed.

Introduction

The role of the gut microbiota in health and disease has received considerable scientific interest recently. Especially the development of new culture independent techniques has re-emerged the interest in intestinal microbial ecology. The gut microbiota has been linked to the risk of gastrointestinal diseases like inflammatory bowel diseases (IBD) [1-4], irritable bowel syndrome (IBS) [5, 6] and necrotizing enterocolitis [7-9]. However the role of the gut microbiota in health and disease may go even beyond the gut as it has also been linked to atopic diseases. This review aims to give a comprehensive overview of observational studies on the association between the gut microbiota composition and atopic disorders, with a special focus on the methods used to characterize this microbiota.

The commensal microbiota of the gastrointestinal tract

The fetal intestine is sterile and bathed in swallowed amniotic fluid. Following delivery, the colonisation of the intestines by a variety of microorganisms begins [10]. Gastrointestinal colonisation involves a succession of bacterial populations waxing and waning as the diet changes and the host develops [11]. This assemblage of bacteria inhabiting the gut is usually referred to as the commensal intestinal microbiota. Each human adult harbors approximately 10^{14} bacteria in the gut, which is about 10 times the number of cells making up the human body [12]. There are at least 400-500 different bacterial species and these species can again be divided into different strains, highlighting the enormous complexity of this ecosystem. Furthermore the composition of this microbiota differs depending on the location in the gut. The concentration of bacteria ranges from 10^3 colony forming units/ml (CFU/ml) in the stomach, where the number of ingested bacteria is dramatically reduced by contact with gastric acid, to 10^{11} – 10^{12} CFU/ml in the colon [13]. The colonic microbiota is dominated by obligatory anaerobes such as *Bacteroides* spp., *Clostridium* spp., bifidobacteria, eubacteria, and fusobacteria. Facultative anaerobes occur in 100-1000 fold lower numbers and include lactobacilli, enterococci, streptococci and enterobacteriaceae [12, 14]. In addition to variations in the composition of the microbiota along the axis of the gastrointestinal tract, surface adherent and luminal microbial populations also differ [15]. Bacteria may be free-living in the lumen or attached to the mucus, mucosa surface, food particles or digestive residues.
The attached bacteria produce microcolonies, leading to the development of biofilms which initially may be composed of only one bacterial species, but frequently develop into a complex community composed of different bacterial species [16].

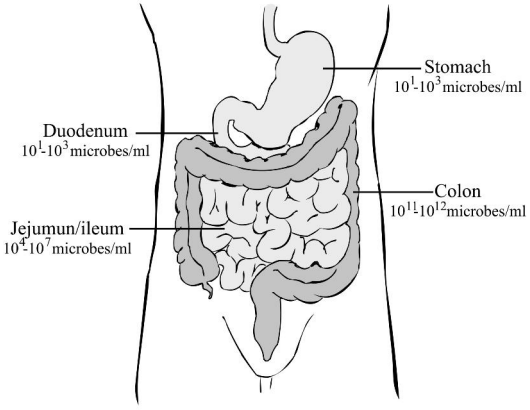
TABLE 1

The most important genera in the human intestinal tract

Obligatory anaerobic genera		Facultative anaerobic genera	
Bifidobacteria	G+	Lactobacilli	G+
Clostridia	G+	Enterococci	G+
Eubacteria	G+	Streptococci	G+
Bacteroides	G-	Staphylococci	G+
Fusobacteria	G-	Enterobacteria	G-

G-, gram-negative; G+, gram-positive

FIGURE 1



Bacterial densities increase throughout the gut, with highest concentrations found in the colon

Factors influencing the intestinal microbiota composition can be divided into host factors (such as pH, transit time, bile acids, pancreatic enzymes and the mucus composition), non-host factors (such as nutrients, medication and environmental factors) and bacterial factors (such as adhesion capacity, enzymes and metabolic capacities) [17]. The bacteria in the gut interact with their human host, and although some bacteria are potentially pathogenic and can become a source of infection and sepsis, this host-bacterial interaction is mainly symbiotic. The host provides a nutrient rich environment and the bacteria can infer important health benefits for the human host [18]. Probably the most important function of the gut microbiota is the so-called colonisation resistance. By competing for nutrients and adhesion sites, but also by the production of antibacterial substances (bacteriocins), the indigenous gut microbiota makes it difficult for entering, potentially pathogenic, bacteria to colonize. Other important functions are the

fermentation of non-digestible dietary residues and endogenous mucus, salvage of energy as short chain fatty acids, production of vitamin K and absorption of ions [18]. Furthermore the gut associated lymphoid tissue (GALT) is the largest immune organ of the human body, which is exposed to an enormous dietary and bacterial antigenic load. Studies on germ-free animals have shown that the gut microbiota plays an important role in the development of the gastrointestinal immune system. Germ-free animals have amongst others decreased Peyer´s patch size, decreased number of IgA producing lymphocytes in the lamina propria, decreased number of intraepithelial T cells and a delayed immune response after antigenic challenge compared to conventional animals [12, 19-23].

From culture to genotype analysis

Research on the intestinal microbiota composition has relied almost exclusively on the quantitative culture of microbes from faecal samples. Enumeration of particular microbial genera or species relies on the use of selective culture media. The analysis of the composition of the normal microbiota using these media is undoubtedly biased by the inability to culture all of the microbes present in samples (about 40-80 % of bacteria as seen by microscopy cannot be cultivated), the fact that few selective media are absolutely selective and that these media do not always equally support the growth of different species comprising a population. Even when cultivatable, the identification to species level using biochemical tests requires experience and is subject to intuitive interpretations [18, 24]. Another major disadvantage in using classical culturing techniques in large epidemiological studies is that samples require immediate processing [25]. The relative inexpensiveness and wide availability on the other hand make that culturing is still the most applied technique.

The development of molecular techniques to investigate ecological microbial communities has provided the microbiologist with a vast array of new techniques to study the human intestinal microbiota. With these techniques unculturable species are detectable, anaerobic handling and expertise is not required and samples can be kept frozen for later analysis [25]. Analysis of bacterial communities using molecular techniques has so far targeted 16S rRNA gene sequences because small ribosomal sub-unit RNA (16S rRNA in the case of bacteria) contains regions of highly conserved nucleotide base sequences interspersed with hypervariable regions [26]. These hypervariable regions contain the signatures of phylogenetic groups, and, sometimes even species.

Fluorescence in situ hybridization (FISH), PCR combined with denaturing or temperature gradient gel electrophoreses (PCR-DGGE/PCR-TGGE) and real-time PCR are molecular techniques which have found their application in studying the gut microbiota [27].

These techniques all have their advantages and limitations. FISH is based on the use of fluorescent oligonucleotide probes targeting 16S ribosomal RNA sequences of intact bacterial cells. Technical difficulties can influence the accuracy of the results. Probes must reach their target sequence, which is inside the bacterial cell, by passing the cell wall. Gram-positive bacterial cells such as lactobacilli, for example, are more difficult to permeabilise than others [28]. Furthermore this method is rather insensitive with detection limits of 10^6 bacterial cells per gram. This technique is particularly useful to visualize the spatial distribution of microbes within the intestinal ecosystem.
Another molecular method is PCR combined with denaturing or temperature gradient gel electrophoresis (PCR-TGGE/DGGE). In this method of analysis, bacterial DNA is extracted from the faecal sample and fragments of the 16S rRNA gene are amplified by PCR, subsequently the 16S molecular species within the resulting mixture are separated by denaturing or temperature gradient gel electrophoresis. The double stranded 16S fragments migrate trough the polyacrylamide gel until each kind of fragment is partially denaturated by the prevailing temperature or chemical conditions [26, 28]. The advantage of PCR-DGGE/TGGE is that it generates a bacterial fingerprint of the dominant bacteria in a sample. Knowledge about the bacterial composition is unnecessary. The technique is however not quantitative, rather insensitive and very laborious making it unsuitable for analysis of large numbers of samples.
The quantitative real-time PCR method monitors the amount of PCR products of DNA as they are amplified by the use of fluorescent oligonucleotide probes. The fluorescence intensity emitted during the amplification process reflects the amplicon concentration in real time. From the change of amplicon concentration throughout the amplification cycles, the initial concentration of the target DNA/RNA can be estimated [29, 30]. Real-time PCR lends itself well as a tool for the quantification of intestinal populations since it combines the specificity of fluorescent oligonucleotide probes with the sensitivity of PCR [28]. Care should however be taken regarding the method used for DNA/RNA extraction, since DNA/RNA may not be extracted with equal efficiency from all bacteria [13].

The microbiota hypothesis in atopy

Only a few years ago it was first hypothesized that the gut microbiota may also be involved in the aetiology of atopic diseases. Atopic diseases are chronic inflammatory disorders caused by aberrant Th2-type immune responses against common 'innocuous 'environmental antigens (allergens) in susceptible individuals [31]. The worldwide rise in atopic diseases (eczema, food allergy, hay fever and asthma) was most predominant in the westernized countries and occurred in such a pace that this could never be solely explained by changes in genetic make-up [32, 33]. Therefore the

causes of the atopic epidemic are generally believed to be of environmental origin. In 1989 Strachan hypothesized that this increase in atopic disease was the result of a lack of infections in early infancy. This hypothesis was based upon Strachan's observations that infants with higher number of siblings were at decreased risk for developing atopy [34]. Although sibship size [35, 36], and other indirect markers of microbial exposure such as rural and farm living (especially contact with livestock) [37, 38] were consistently shown to be associated with a decreased risk of developing allergies, studies on the association between viral and bacterial infections and allergy were less consistent [39, 40]. In 1998 Wold suggested that rather than a decrease in viral or bacterial infections, an altered normal intestinal colonisation pattern in infancy, which fails to induce immunological tolerance, could be responsible for the increase in allergies [41]. This idea of a potential role of the gut microbiota was based on the observations that (i) it is difficult to achieve oral tolerance in germ-free animals [42]; (ii) administration of lipopolysaccharide (a constituent of the outer membrane of gram-negative bacteria) together with food antigens increases the tolerizing effect of feeding [43]; (iii) and bacterial toxins may break oral tolerance [44]. Since then, several observational studies on the gut microbiota composition and allergy have been conducted.

Potential immunological mechanisms

The initial immunological explanation for the hygiene hypothesis was a lack of microbial antigen-induced immune deviation from the Th2 cytokine profile to a Th1 type profile, resulting in the development of enhanced Th2 cell responses to allergens [45-47]. However, this explanation did not take into account that the prevalence of Th1-associated diseases, such as Crohn's disease, type 1 diabetes and multiple sclerosis, were also increasing and that chronic parasitic worm (helminth) infections which induce strong Th-2 responses and high IgE levels are not associated with an increased risk of allergy [48]. An alternative interpretation conceives anti-inflammatory immune responses to be of fundamental importance in the development of mucosal and systemic tolerance [49]. These immunosuppressive mechanisms are orchestrated by regulatory T cell classes (Treg cells) that control (largely via the production of IL-10 and/or TGF-β) both Th1 and Th2 responses and hence the development of both atopic and autoimmune diseases. [49, 50]. Indeed the importance of a delicate balance between allergen-specific Treg cells and allergen specific Th2 cells in healthy and allergic immune responses to common environmental allergens was demonstrated in a study conducted by Akdis and colleagues [51]. Relatively harmless organisms, including bifidobacteria, lactobacilli, but also helminths and saprophytic mycobacteria, may skew immune responses towards immunoregulation by inducing Treg cells, rather than eliciting a proinflammatory immune response. The

ſmicrobiota hypothesis 'proposes that the loss of exposure to these harmless microorganisms in the westernized environment might explain the increase in immunedysregulatory disorders [52, 53]. The epidemiological findings and the experimental evidence available so far suggest that both the reduced immune suppression by Treg cells and the lack of immune deviation from a Th2 to Th1 profile are involved [54].

Methods of literature review

Observational studies comparing healthy and allergic subjects (defined as: subjects with atopic eczema/dermatitis, food allergy, wheeze, allergic rhinitis, asthma and/or sensitisation) regarding the (quantitative) presence of intestinal bacteria, were included in this review. Intervention studies on the effects of probiotics, prebiotics and synbiotics in the treatment or prevention of allergic diseases are beyond the scope of this review. The publications were analyzed for population size, design of the study, atopic outcomes, and methods used for analyzing the faecal samples.
To identify all observational studies on the association between the gut microbiota composition and atopic disorders, a literature search was performed by searching the databases of Medline, EMBASE and PubMed up to May 2006. The following keywords and limits were used: (intestines [mesh] OR intestin* OR gut OR gastrointestin* OR enteric) AND (flora OR microbiolog* OR microflora OR bacteria OR bacterial OR colonisation OR colonisation OR microbes OR microbial OR microbiota) AND (hypersensitivity OR atopic dermatitis OR allergic OR allergy OR atopic OR atopy OR eczema OR rhinitis OR asthma) AND (English[la]). Additional reports were found searching the reference list of pertinent articles.

Results

Using the search criteria as described above, we identified 14 observational studies on the association between the gut microbiota and atopic diseases. Six studies were exclusively based on traditional bacteriological culture techniques to study the microbial composition of faecal samples, another five studies (additionally) used molecular techniques. The remaining three studies were not based on the examination of faecal samples, but tested for IgG seropositivity to certain intestinal microbes. All studies, except one, are presented and compared in table 2 according to the type of atopic disease under study, the study population, design and methodology to examine the intestinal microbiota. One study [55] was not incorporated in table 2, because it lacked statistical testing and a reference group.

Culture-dependent studies

Already in the early 1980s, Russian scientists linked food allergy to abnormal intestinal microbiota [55]. The authors examined 60 infants with dermatological syndrome, caused by food allergy and reported dysbacteriosis of different degree in all cases. This dysbacteriosis was characterized by a deficiency of bifidobacteria and lactobacilli combined with an increase of enterobacteriaceae. The study however lacked statistical testing and it was not clear how a deficiency of lactic acid bacteria and an increase of enterobacteriaceae was defined since data of a reference group of infants without dermatological symptoms was not mentioned in the paper.

It was not until the late 1990's when the group of Bjorksten instigated the research on the potential role of the gut microbiota in the aetiology of allergic diseases. Bjorksten's group studied the gut microbiota of 1-year-old healthy infants living in Estonia with a low prevalence and Sweden with a high prevalence of childhood allergy [56]. Especially lactobacilli and eubacteria were more prevalent in Estonian infants. Swedish infants harbored higher counts of clostridia, especially *Clostridium difficile* was more common than in the Estonian infants. These observations led to the first case-control study comparing the gut microbiota composition of allergic infants with that of healthy infants (Table 2). In this cross-sectional study 2-year-old allergic and non-allergic infants from Estonia and Sweden were compared [57]. Allergic infants were less often colonised by lactobacilli compared to non-allergic infants in both countries. In contrast, the allergic children harbored higher counts of facultative aerobic micro-organisms, especially coliforms in the Estonian and *Staphylococcus aureus* in the Swedisch children. The number of study subjects was relatively low (27 allergic and 35 non-allergic subjects); therefore differences other than country of origin could not be taken into account. This was the first study to demonstrate differences in the gut microbiota between healthy and allergic infants; however since it was cross-sectional a conclusion on what comes first could not be drawn. Therefore in a following study, the same research group aimed at prospectively relating the intestinal microbiota composition to the development of allergy in 20 Swedish and 24 Estonian infants [58]. Faecal samples were collected at several moments throughout the first year of life and infants were clinically followed for the presence of allergic symptoms and sensitisation until the age of two years. The prevalence of colonisation by bifidobacteria was consistently lower throughout the first year of life in infants who developed allergy compared to those who did not. Other differences were only present at one moment during the follow up. Especially the observation of differences between healthy and allergic infants at only one moment has to be interpreted with caution since comparing both prevalence and counts of multiple bacterial groups at several different time points introduces a considerably risk of false positive findings due to multiple

testing. Furthermore numbers were too small for statistical analysis for the two countries separately.
In a Japanese case-control study, among minors with atopic dermatitis and healthy control subjects, counts of bifidobacteria were lower in cases than in healthy controls. The proportion of bifidobacteria in total bacterial count was negatively correlated with the severity of atopic dermatitis. Furthermore the prevalence of colonisation with staphylococci was higher in the atopic dermatitis group than in the control group [59].
In another Japanese case-control study [60], faecal samples of 11 adult atopic dermatitis patients and 14 healthy adults were compared. Although proportions of the predominant bacteria in total bacteria were almost the same in both groups, those of Enterobacteriaceae were significantly higher in AD patients than in healthy adults.
Sepp and colleagues studied the association between the intestinal microbiota and immunoglobulin E responses in 5-year-old Estonian infants. Counts of clostridia were higher in children with specific IgE antibodies to food and/or inhalant allergens, furthermore the relative share of clostridia was higher and that of bifidobacteria lower in allergic than in non-allergic children [61].
These studies all provided valuable information and especially the studies from Estonia and Sweden formed the basis of this research area. However the fact that these studies were solely based on culture-dependent techniques makes them vulnerable to bias.

Culture-independent studies

In a study on microflora-associated characteristics it was amongst others shown that sensitised infants had higher levels of i-caproic acid in their stools compared to non-sensitised infants. This rather uncommon short chain fatty acid was suggested to indicate the presence of *C. difficile*.[62]
Molecular techniques to quantify intestinal bacteria were first used in two studies conducted in Finland [63, 64]. In a case-control study using fluorescence in situ hybridization (FISH) to characterize the gut microbiota, no differences in concentrations of specific genera were found between healthy infants and infants suffering atopic dermatitis. Higher bacteroides counts and lower counts of bifidobacteria were however associated with more severe manifestations of dermatitis. [63] In a cohort study prospectively following 76 high-risk infants during their first year of life [64], bacterial fatty acid profiles in faecal samples collected at age 3 weeks significantly differed between infants in whom atopy (positive skin prick test) was or was not developing. Since bacterial cultivation failed to identify the bacteria possibly responsible for these differences, FISH was additionally used in a subpopulation of the cohort. Quantification of bacteria using this molecular technique demonstrated that the atopic infants tended to have fewer

bifidobacteria and had more clostridia. In contrast, in a recent study among Singapore infants numbers of clostridia, as determined by FISH, were lower in infants with eczema compared to healthy controls [65]. It should however be noted that the former study only quantified the *Clostridium histolyticum* subgroup, whereas the latter study also included the *Clostridium lituseburense* subgroup. The Singapore study also found bifidobacterial numbers to be lower in infants with eczema, whereas numbers of lactic acid bacteria were higher compared to healthy controls.

The association between the intestinal microbiota composition and recurrent wheeze has up to now only been investigated in two nested case-control studies. Both studies were conducted within the context of the prospective National Asthma Campaign Manchester Asthma and Allergy Study (^{NAC}MAAS). The use of PCR combined with denaturing gradient gel electrophoreses (DGGE) showed no differences in the dominant faecal microbiota and the bifidobacterial and lactobacilli composition between sensitised wheezy and non-sensitised non-wheezy 4-year-olds [66]. In a second study within the ^{NAC}MAAS cohort one-year-old sensitised wheezy infants had significantly higher serum IgG levels against *Clostridium difficile* compared to non-sensitised non-wheezy infants. [67]

The use of IgG serology to determine exposure to intestinal microbes was also used in two large population-based cross-sectional studies [68, 69]. In one of these studies conducted in Copenhagen, both sensitised subjects and subjects suffering from allergic rhinitis were more often IgG-seropositive to intestinal bacterial pathogens, especially *C. difficile,* compared to subjects without sensitisation and allergic rhinitis respectively [68]. The other study comprised 787 Japanese schoolchildren and examined the association between IgG titers to *Escherichia coli*, *Bacteroides vulgatus*, *Enterococcus faecalis*, and *Bifidobacterium longum* and allergic symptoms [69]. Infants with two or more allergic symptoms (asthma, rhinitis, eczema and/or food allergy) had significantly higher IgG titers to *B. vulgates*.

TABLE 2

Overview of case-control and cohort studies on the association between intestinal microbiota composition and allergy

Ref.	Year	Definition of allergy	Study population	Design	Bacteriological/ serological analyis	Results for atopic compared to non-atopic subjects
[57]	Bjorksten 1999	Atopic dermatitis (Hanifin & Rajka) and at least 1 positive SPT	27 allergic and 35 non-allergic 2-year old children	Cross-sectional, nested case-control	Bacteriological culture and biochemical identification	Lower prevalence of bifidobacteria (not significant after stratification for country) and lactobacilli Higher counts of coliforms (in atopic compared to non-atopic infants from Estonia) and *S. aureus* (in atopic compared to non-atopic infants from Sweden)
[58]	Bjorksten 2001	Atopic dermatitis (Hanifin & Rajka) and/or at least 1 positive SPT at age 3,6,12 or 24 months	44 newborns (18 became cases)	Prospective birth cohort (until age 2 years)	Bacteriological culture and biochemical identification at age 1 week and 1, 3, 6 and 12 months	Lower prevalence of enterococci (during 1st month) Higher counts of clostridia (at 3 months) Higher prevalence of *S. aureus* (at 6 months) Lower counts of bacteroides (at 12 months) Lower prevalence of bifidobacteria (during 1st yr)
[63]	Kirjavainen 2001	Atopic dermatitis (Hanifin & Rajka)	27 allergic and 10 non-allergic children (5-13 months)	Cross-sectional, case-control	Bacteriological culture and FISH	No differences in concentrations of specific genera (bacteroides, lactobacilli/enterococci, C*lostridium difficile, E. coli*), although some genera were associated with severity of atopy
[64]	Kalliomaki 2001	At least 1 positive SPT at age 12 months	76 newborns at high risk of allergy (22 became cases)	Prospective birth cohort (until age 1 year)	Gas-liquid chromatography, Bacteriological culture at age 3 weeks and 3 months and FISH (only at 3 weeks)	Different bacterial fatty acid profiles at 3 weeks. No differences in prevalence of colonisation (at 3 weeks and 3 months as determined by culture) Lower bifidobacteria : clostridia ratio (at 3 weeks as determined by FISH)

[62]	Bottcher 2000	At least one positive SPT at age 12 months	25 allergic and 43 non-allergic 13-month-old infants	Cross-sectional, nested case-control	Gas chomatography	Lower levels of propionic, i-butyric, butyric, i-valeric and valeric acid and higher levels of i-caproic acid. Higher relative distribution of acetic and i-caproic acid
[59]	Watanabe 2003	Atopic dermatitis (Japanese Dermatological Association)	30 allergic and 68 non-allergic children	Cross-sectional, case-control	Bacteriolocical culture and biochemical identification	Higher prevalence of *S. aureus* Lower counts of bifidobacteria Significant trend between percentage bifidobacteria of total flora and severity of atopic eczema.
[60]	Matsumoto 2004	Severe Atopic dermatitis	11 allergic and 14 non-allergic adults	Cross-sectional, case-control	Bacteriological culture and measurement of sIgA in faeces	Lower total counts and total anaerobes. Higher proportion of enterobacteriaceae. Higer content of sIgA.
[65]	Mah 2006	Atopic dermatitis (Hanifin & Rajka)	21 allergic and 28 non-allergic 3-year old children	Cross-sectional	Bacteriolocical culture and biochemical identification (for aerobes) and FISH (for anaerobes)	Higher counts of LAB (especially enterococci) Lower counts of bifidobacteria and clostridia.
[67]	Woodcock 2002	Recurrent wheeze ($\geq$ 3 episodes) and at least 1 positive SPT	10 allergic and 10 non-allergic 1-year-old children	Cross-sectional (nested) case-control	Serum IgG levels to *C. difficile*	Higher levels of *C. difficile*-specific IgG levels

TABLE 2 continued

Ref.	Year	Definition of allergy	Study population	Design	Bacteriological/ serological analyis	Results for atopic compared to non-atopic subjects
[66]	Murray 2005	Recurrent wheeze (≥ 3 episodes) and at least 1 positive SPT	33 allergic and 33 non-allergic 4-year old children	Cross-sectional, nested case-control	PCR-DGGE and FISH	No difference in prevalence of colonisation with lactobacilli and bifidobacteria. No differences in bifidobacterial counts (lactobacilli not enumerated), or in bifidobacterial species composition.
[68]	Linneberg 2003	Specific serum IgE and/or allergic rhinitis	1112 subjects (15-69 years)	Cross-sectional population-based	IgG seropositivity to several microorganisms	Higher prevalence of specific IgE and allergic rhinitis in subjects seropositive to *C. difficile*
[69]	Fukuda 2004	Asthma, rhinitis, eczema and/or food allergy	787 junior high school children	Cross-sectional population-based	Serum IgG levels to several bacterial species	Higher IgG levels against *B. vulgates* in subjects with 2 or more symptoms
[61]	Sepp 2005	Atopic dermatitis, bronchial asthma and/or allergic rhinitis	19 allergic and 19 non-allergic 5-year-old children	Cross-sectional case-control	Bacteriological culture and biochemical identification	Lower prevalence and proportion of bifidobacteria, higher proportion of clostridia. Higher count of clostridia in infants with specific IgE antibodies

SPT; Skin Prick Test
FISH; Fluorescence in situ hybridisation
Prevalence, defined as percentage of infants colonised with a certain bacterial group or species
Counts, defined as mean or median log10 colony forming units per gram of faeces
Proportion, defined as mean or median percentage a certain bacterial group or species contributes to the total bacterial counts
GLC; Gas-liquid chromatography

Discussion

The microbiota hypothesis in atopic diseases has appeared to be a promising hypothesis. Eleven out of the 13 observational studies conducted so far indicated an association between the gut microbiota composition and atopic sensitisation or symptoms. No specific harmful or protective microbes can however be identified yet.

Lactobacilli belong to the candidate microorganisms to stimulate immunoregulation by stimulating regulatory T cell responses [70]. However in the current observational studies there is little support for a protective role of lactobacilli, only the first studies in Swedish and Estonian infants reported a higher prevalence of colonisation by lactobacilli among non-allergic infants in both countries [57]. It should however be noted that different species of lactobacilli induce distinct and even opposing dendritic cell (DC) responses with regard to their Th1/Th2/Treg-driving capacity [71]. Since none of the studies identified lactobacilli to the species level, such species dependent effects could be overlooked.

Bifidobacterium spp. is also a genus which is known for its supposed health beneficial properties and these bacteria may also induce immunoregulatory pathways [72]. Of the 9 studies which included the quantification of bifidobacteria, five studies found support for a protective effect of this genus. However differences in prevalence and counts of bifidobacteria between allergic and healthy infants as found by traditional culture have to be interpreted with caution, since especially the cultivation of these bacteria is subject to bias [73]. As for lactobacilli the effects of bifidobacteria may be species-dependent. Intestinal *Bifidobacterium* species have been shown to induce varying cytokine production by cells of the innate immune system [74]. Furthermore allergic infants have been reported to harbor different *Bifidobacterium* species compared to healthy infants [75]. This difference was however not confirmed for sensitised wheezy infants compared to non-sensitised non-wheezy infants [66].

While some bacteria are associated with a reduced risk of atopic diseases, there are also bacteria which have been reported to be associated with an increased risk of atopic diseases. Although findings are far from consistent, clostrida and staphylococci are potential candidates since these have been associated with an increased risk of atopic diseases in several studies. Different outcomes, differences in study design, differences in the microbes under study and the methods used to identify them, however, all make the current studies difficult to compare. Several methodological issues that have to be considered when interpreting these studies will now be discussed.

Critical window period

Many studies have emphasized that the timing of exposure to environmental factors may be essential to promote beneficial or harmful effects regarding the development of atopic diseases. The most important 'window of opportunity' for immune education seems to be in early infancy, when the maturation of the immune system is not yet completed and is still building up immune tolerance against food and microbial antigens [34, 35, 76, 77]. Several studies have prospectively examined the postnatal maturation of T-helper cell responses in atopy-prone infants and infants who did not develop atopy. Prescott et al. demonstrated that continuation of Th2 responses (IL-4, IL-13) and decreased capacity for Th1 responses (IFN-γ) to house dust mite allergen was associated with the development of allergy, whereas non-atopic patients showed strong Th2 responses only at birth, but declining Th2 and increasing Th1 responses starting within the first 6 months of life [78]. Van der Velden and colleagues observed a significantly increased production of IL-4 at 6 months of age in the children developing atopy compared to the non-atopic children, whereas this difference was resolved at 12 months of age. This transient increase in IL-4 production, pointing to an active development of Th2 cells (as shown by a similarly increased production of IL-5 and IL-13), correlated in time with the first signs of clinical symptoms characteristic of an atopic disease (atopic dermatitis (AD), asthma-like disease and food or upper-airway allergy). Therefore, the first 6 months of life may represent a critical time window for the initiation of immunological changes reflecting allergic sensitisation [79].

This would imply that the initial gut microbiota composition, rather than the composition later on in life, may be considered a determinant in the development and maintenance of normal gut barrier functions, oral tolerance and a disease-free state of the host [77]. Indeed the importance of timing for the effect of the gut microbiota was suggested in a number of studies on germ-free mice. Sudo et al. demonstrated that oral administration of ovalbumin to germ-free mice induced Th2-type cytokine- and antigen-specific IgE production, whereas reconstitution of the intestinal microflora with bifidobacteria during the neonatal period, but not in older age, resulted in oral tolerance induction [42]. In another study it was shown that colonisation of germ-free mice with a human gut microbiota protected them against *Escherichia coli* heat-labile enterotoxin(LT)-mediated abrogation of oral tolerance to an unrelated co-ingested protein. This protection was only achieved if the gut microbiota was present from birth on [80].

Based upon this critical window period, it thus seems unlikely that perturbations in the gut microbiota as present beyond infancy may still have an effect on the aetiology of atopic diseases. It is more likely that these perturbations reflect disturbances in the gut microbiota already present in early life. The most powerful studies on the gut microbiota and atopic

disorders are therefore studies quantifying the microbial composition in early life.

Atopic outcomes

One of the difficulties in assessing the role of the gut microbiota, as well as other determinants, for the inception of allergic and wheezing illnesses is the heterogeneity of atopic conditions [81] It is generally known that far from all children with a clinical phenotype of (atopic) eczema are actually sensitised against allergens [82-84]. This has led to the idea that there may exist at least two variants of eczema, an atopic and a non-atopic variant [84, 85]. If this idea holds true, studies focusing solely on a clinical phenotype of atopic dermatitis may underestimate associations since the effect of the intestinal microbiota may be different for these two types of eczema. This may as well apply for other atopic symptoms, since non-IgE mediated rhinitis, wheeze and asthma has also been reported [86, 87]. Furthermore eczema, asthma and allergic rhinitis tend to cluster in the same individuals and families, the exact relationship over time is however far from clear. The question whether eczema is a risk factor for asthma in a progressive atopic march or that a comanifestation exists of 2 phenotypes at an early age, remains to be resolved [82, 88].

The studies conducted so far on the association between the gut microbiota and atopic outcomes have focused on a variety of outcomes: 6 studies were focused on both allergic sensitisation and clinical symptoms (of which 2 on atopic dermatitis/eczema, 2 on recurrent wheeze, 1 on allergic rhinitis and 1 on several atopic symptoms), 2 studies were focused on allergic sensitisation only, and 5 studies have focused on clinical symptoms only (4 atopic dermatitis/eczema and 1 several atopic outcomes). The variety of outcomes used in the different studies is one of the main reasons why comparing results is difficult. If future longitudinal population studies would examine the association of the gut microbiota composition with both allergic sensitisation and different atopic phenotypes, this could provide further insight into the role of the gut microbiota in the aetiology of childhood atopy.

Reverse causation

Most studies conducted so far were cross-sectional. Such studies obviously cannot exclude the possibility that differences in the gut microbiota between healthy and allergic subjects are the result of the disease itself or the consequence of the use of medication or changes in diet or lifestyle by allergic subjects. Longitudinal studies on the other hand can demonstrate that perturbations in the gut microbiota actually precede the development of atopic manifestations, however they still cannot exclude that early differences in intestinal microbiota are a consequence of a underlying predisposition towards atopy, rather than the cause of disease [66]. It is thus possible that

differences in the gut microbiota and differences in the development of immunity are consequences of one or more unknown other factors, associated with the atopic genotype [58]. Only experimental studies in animals or humans can completely settle this issue. Clinical trials using probiotics in the treatment or prevention of atopy for example are capable of demonstrating a causative effect of bacteria and can gain more insight in the underlying mechanisms. However these studies can give no information as to whether perturbations in the indigenous microbiota itself can explain the epidemic of atopic diseases.
Observational studies can at least partly deal with the possibility that differences in the gut microbiota are caused by factors associated with the atopic genotype, namely by controlling for a positive family history of atopic diseases. Until now only two studies were prospective. One of these prospective studies was based upon a high risk population (father, mother and/or older sibling(s) having an atopic disease [64], thereby indirectly taking the possible effect of a positive family history into account. In two cross-sectional studies, the potential effect of a positive family history was taken into account by matching for this factor [66, 67]. None of the studies up to now used multivariate analyses to control for family history of atopy and potential other confounders.

Future perspectives

The best way to gain more insight into the association between the gut microbiota and atopic diseases is probably to conduct large-scale prospective birth cohort studies, in which the gut microbiota can be studied in early infancy and infants can be followed up for the development of atopic symptoms and sensitisation. The introduction of molecular techniques has simplified the analyses of the gut microbiota in large studies since faecal samples can be frozen prior to the analyses. Furthermore these molecular techniques provide more accurate and sensitive results compared to traditional culture. In such large-scale prospective studies different atopic outcomes and sensitisation can be studied separately, thereby creating more insight into the aetiology of atopy and atopic disorders.
Currently within several prospective studies such as; the IMPACT Study [89], the Allergyflora project and the KOALA Birth Cohort Study [90], the association between intestinal colonisation in infancy and development of atopic diseases is examined. Results of these studies are expected to be available soon.

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Chapter 3

Quantification of *Bifidobacterium spp.*, *E. coli* and *C. difficile* in faecal samples of breastfed and formula-fed infants by real-time PCR

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Chapter 3

Abstract

To determine the influence of either exclusive breastfeeding or formula feeding on both composition and quantity of the gut microbiota in infants, we have developed real-time, quantitative PCR assays for the detection of *Bifidobacterium spp.* and *Clostridium difficile*. Furthermore, we have monitored the prevalence and counts of *Escherichia coli* by applying a previously described real-time PCR assay. We found all 100 infants tested to be colonised with *Bifidobacterium spp.* The bifidobacterial counts were comparable between the 50 breastfed and 50 formula-fed infants with median values of 10.56 $\log_{10}$ and 10.24 $\log_{10}$ CFU g^{-1} wet weight faeces, respectively. *C. difficile* was detected in 14% of the breastfed and 30% of the formula-fed infants. In addition, the *C. difficile* counts were significantly lower in breastfed infants than in the formula-fed group (median values of 3.28 $\log_{10}$ and 7.43 $\log_{10}$ CFU g^{-1}, respectively; $p = 0.03$). The prevalence of *E. coli* in the breastfed and formula-fed group was 80% and 94%, respectively. Also, the *E. coli* counts in colonised infants was significantly lower in the breastfed infants than in the formula-fed group (median values of 9.11 $\log_{10}$ and 9.57 $\log_{10}$ CFU g^{-1}, respectively; $p = 0.004$). We conclude that the prevalence and counts of *C. difficile* as well as *E. coli* are significantly lower in the gut microbiota of breastfed infants than in that of formula-fed infants, whereas the prevalence and counts of *Bifidobacterium spp.* is similar among both groups.

Introduction

The normal human microbiota is a complex ecosystem, consisting of at least several hundred different bacterial species. The gut microbiota plays an important role in human health by producing nutrients and by preventing colonisation of the gut by potential pathogenic microorganisms [1]. In addition, the intestinal microbiota is involved in maintaining the health of the host, for instance by influencing the immune system. This impact of the gut microbiota on the immune system, such as the development of oral tolerance, was most clearly shown in studies on germfree mice [2]. Also, diseases of the immune system, like allergic diseases and inflammatory bowel diseases (Crohn ś disease and ulcerative colitis) seem to be associated with gut microbiota composition [3-5].

Major changes in the intestinal microbial composition occur especially early in life. Until birth the gastrointestinal tract is sterile and subsequent development of the intestinal microbiota is influenced by factors like way of delivery (vaginal/caesarean), hygiene and antibiotic use, developmental stage of the gastrointestinal tract (premature/full term) and type of feeding (breast/formula) [6].

For decades, the quantification of bacterial genera and species present in the intestinal microbiota was based on traditional bacteriological culture and biochemical identification techniques. However, these methods are time-consuming and are limited by low sensitivities, the inability to detect nonculturable bacteria as well as unknown species, and the low levels of reproducibility due to the multitude of species to be identified and quantified [7]. Approximately 40-80% of the bacteria visible by direct microscopic examination of diluted faecal samples cannot be cultured [1]. For example, for bifidobacteria, several selective media have been developed. These media, however, do not equally support growth of all *Bifidobacterium* species. In particular the number of *B. adolescentis* are underestimated by selective plating. Moreover, it has been shown that the different media affect the total numbers of bifidobacteria recovered [8].

To study the gut microbiota composition in a culture-independent way, molecular methods have been developed which are based upon the detection of bacterial 16S ribosomal RNA (rRNA) genes [7, 9]. These methods include temperature gradient gel electrophoresis [10, 11], denaturing gradient gel electrophoresis [12] and fluorescent in situ hybridization [13, 14]. A drawback of these techniques is, however, that they are not very suitable for analysis of large numbers of samples. By contrast, real-time PCR is a technique that is highly suitable for high-throughput analyses. Other advantages of real-time PCR are that it can be performed quantitatively, and that it does not require post-PCR sample handling, which prevents potential

carry-over contamination and also results in much shorter assay times [15]. Recently several assays have been published for the detection of bacterial groups and species in faecal samples and gastrointestinal mucosa [7, 16-19]. Here, we describe the use of real-time PCR in the quantitative analysis of the presence of *Bifidobacterium spp.*, *C. difficile* and E. *coli* in faecal samples of breast- and formula-fed infants.

Material and methods

Study population

Infants were selected from the KOALA-study, an ongoing birth cohort study on the development of allergic diseases in the Netherlands. In this study faecal samples of over eleven hundred infants at the age of one month have been collected.

After exclusion of premature infants, infants born through caesarean section and infants who had received antibiotics during their first month of life, 50 exclusively breastfed and 50 exclusively formula-fed infants were randomly selected. All children were born at home or at the outpatient department of the hospital. Characteristics of the infants participating in this study are shown in table 1.

Bacterial strains and culture conditions

The bacterial strains listed below were obtained from the American Type Culture Collection (ATCC; Manassas, USA.), the National Collection of Type Cultures (NCTC; London, United Kingdom), Winclove Bio Industries BV (WBI; Amsterdam, the Netherlands) or were laboratory isolates at the University Hospital of Maastricht (azM; Maastricht, the Netherlands). The following reference strains were used in this study to evaluate the sensitivity and specificity of the PCR primer-probe sets: *Bacteroides distansonis* RE01B05-azM, *Bacteroides fragilis* ATCC 25285, *Bacteroides ovatus* ATCC 8483,

TABLE 1

Characteristics of babies participating in this study

	Breast-fed	Formula-fed
No. of babies	50	50
Male/female	22/28	26/24
Mean (SD) birth weight (g)	3572 (448)	3433 (451)
Mean (SD) length at birth (cm)	51 (3)	51 (2)
Mean (SD) duration of pregnancy (weeks)	40 (1)	39 (1)

Bacteroides thetaiotaomicron RE01B01-azM, *Bacteroides uniformis* RE01B04-azM, *Bacteroides vulgates* RE01B03-azM, *Bifidobacterium adolescentis* ATCC 15703, *Bifidobacterium bifidum* WBI 4, *Bifidobacterium breve* WBI 9, *Bifidobacterium infantis* ATCC 15697, *Bifidobacterium longum* WBI 2, *Clostridium bifermentans* ATCC 19299, *Clostridium difficile* ATCC 9689, *Clostridium histolyticum* ATCC 19401, *Clostridium novyi* ATCC 7658, *Clostridium perfringens* ATCC 13124, *Clostridium septicum* ATCC 6008, *Clostridium sordellii* ATCC 9714, *Clostridium tetani* NCTC 279, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC35218, *Eubacterium spp.* azM, *Lactobacillus casei* WBI 60, *Lactobacillus plantarum* WBI62, *Lactobacillus rhamnosus* WBI 40, *Leuconostoc spp.* azM, *Peptostreptococcus spp.* azM, *Streptococcus viridans* azM.
The numbers of colony forming units (CFU) were determined by plating different dilutions of an overnight culture (37°C) of the microorganisms on fastidious anaerobic agar plates (LabM LabGo, Lancashire, UK) for the bifidobacteria, *C. difficile* and other (facultative) anaerobic bacteria, and on blood agar plates (Oxoid CM271, Basingstoke, UK) for *E. coli* and other (facultative) aerobic bacteria using a spiral-plater (Spiral Systems Inc. Salm en Kip, Utrecht, the Netherlands) and incubating the plates anaerobicly or aerobicly respectively for 24-48h.

DNA purification from faeces

Faecal samples were tenfold diluted in peptone-water (Oxoid CM0009) containing 20% v/v glycerol (Merck, Darmstadt, Germany) and stored at –20°C. For DNA isolation 0.2 ml of the diluted samples was added to a 2-ml vial containing approximately 300 mg glass beads (diameter 0.1 mm) and 1.4 ml of ASL-buffer from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The bacterial samples were disrupted in a mini-bead beater (Biospec Products, Bartlesville, Okla.) at 5,000 rpm for 3 min. Subsequently, the bacterial DNA was isolated from the samples using the QIAamp DNA Stool Mini Kit, according to the instructions of the manufacturer. The DNA was eluted in a final volume of 200 µl.

DNA purification from bacterial cultures

DNA from the reference strains used in this study was isolated in a similar manner as the DNA from faecal samples. In short, 0.2 ml of a bacterial suspension was added to a 2-ml vial containing 300 mg glass beads and 1.4 ml ASL-buffer and bead-beated for 3 min., whereafter the samples were subjected to the DNA Stool Mini Kit.

Design of primers and probes

The primers and probes for the detection of bifidobacteria and *C. difficile* were based on 16S rRNA gene sequences, retrieved from the National Center

for Biotechnology Information databases using the program Entrez (http://www.ncbi.nlm.nih.gov). The bifidobacterial or *C. difficile* sequences were aligned with sequences from closely related species using the program ClustalW from the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw.htm). The alignments revealed sequences specific for either bifidobacteria or *C. difficile.* From these sequences, forward primers, reverse primers and TaqMan probes were designed using the Primer Express 2.0 software program (Applied Biosystems [ABI], Foster City, Calif.). Sequences and coordinates of the selected primers and probes are listed in Table 2. To check for specificity, the selected primers and probes were compared to all available 16S rRNA gene sequences using the BLAST database search program (www.ncbi.nlm.nih.gov/blast). Primers and probes were manufactured by Sigma Genosys.

TaqMan assay conditions

For the *Bifidobacterium*-TaqMan assay, amplifications were carried out in a total volume of 50 µl, containing 1x TaqMan universal PCR master mix (ABI), 300 nM of both primers, 150 nM of TaqMan probe, and 20 µl of purified target DNA (see above). For the *C. difficile*-TaqMan assay, amplifications were carried out in a total volume of 25 µl, containing 1x TaqMan universal PCR master mix (ABI), 900 nM of both primers, 200 nM of TaqMan probe, and 10 µl of purified target DNA.

TABLE 2

Primers and probes used in this study

Target organism(s)	Target*	Sequence (5´-3´)	T_m (°C)	Reference
Bifidobacterium spp.				
Forward primer	42-63	GCGTGCTTAACACATGCAAGTC	59	this study
Reverse primer	167-147	CACCCGTTTCCAGGAGCTATT	59	this study
Probe	126-103	TCACGCATTACTCACCCGTTCGCC	70	this study
C. difficile				
Forward primer	62-100	TTGAGCGATTTACTTCGGTAAAGA	58	this study
Reverse primer	175-151	TGTACTGGCTCACCTTTGATATTCA	59	this study
Probe	105-126	CCACGCGTTACTCACCCGTCCG	69	this study
E. coli				
Forward primer	395-414	CATGCCGCGTGTATGAAGAA	59	[7]
Reverse primer	490-470	CGGGTAACGTCAATGAGCAAA	59	[7]
Probe	437-467	TATTAACTTTACTCCCTTCCTCCCC GCTGAA	68	[7]

*Numbering corresponding to *E .coli* 16S rRNA gene [36]

The conditions of the *E. coli*-TaqMan assay, which was developed by Huijsdens et al. [7], were adjusted so that amplifications were carried out in a total volume of 25 µl, containing 1x TaqMan universal PCR master mix (ABI), 900 nM of both primers, 200 nM of TaqMan probe, and 10 µl of purified target DNA. The amplification (2 min at 50°C, 10 min at 95°C, followed by 42 cycles of 15 s at 95°C and 1 min at 60°C) and detection were carried out on an ABI Prism 7000 sequence detection system (ABI).

Sensitivity, specificity and inhibition

To determine the sensitivity of the real-time TaqMan assay, DNA purified from serial tenfold dilutions of quantified *B. bifidum* and *C. difficile* cultures were subjected to PCR. Bacterial densities of the dilutions were determined by viable counts. Since the assay conditions for the detection of *E. coli* were slightly adjusted, the sensitivity of this assay was also determined in a similar way. To check whether the *Bifidobacterium*-TaqMan assay could detect DNA from bifidobacteria other than *B. bifidum*, PCR was performed using 1 to $1*10^6$ genome copies of *B. adolescentis, B. breve, B. infantis, B. longum* and *B. bifidum*.The specificity of both the *C. difficile* and *Bifidobacterium* assay was further investigated by testing DNA purified from a mixture of bacterial species other than the target species (10^4-10^5 CFU of each of the species per mixture), either in the absence or presence of *B. bifidum* or *C. difficile*. The bacteria that were selected are listed in the "bacterial strains and culture condition "section. In addition, 40 faecal samples that were either positive or negative in the *C. difficile* TaqMan assay were cultured on a selective medium (Oxoid CM0601B).

To analyze the presence of potential PCR inhibitory substances after DNA purification, faecal samples, as well as pure bacterial culture suspensions, were spiked with rat cytomegalovirus (RCMV) particles before DNA extraction. Each purified DNA sample was subjected to a RCMV DNA-specific TaqMan assay, which has previously been described [20].

Ultrafiltration of the PCR mix

To remove traces of *E. coli* DNA contaminating the universal PCR master mix, an ultrafiltration step as described by Yang and colleagues was used [21]. In brief, the Amicon Microcon YM-100 centrifugal filter device (Millipore Corporation, Bedford, Mass.) was utilized for filtering the PCR master mix prior to addition of template DNA. The PCR mix was spun through the YM-100 device at 100 x *g* for 30 min.

Statistical methods

The $\log_{10}$ CFU g^{-1} of *Bifidobacterium spp.*, *E. coli* and *C. difficile* were calculated for each stool sample from the threshold cycle (C_t)-values using the constructed standard curves. Since the data did not exhibit a normal

distribution we used the Mann-Whitney rank sum test to compare values between the breast- and formula-fed groups.

Ethical considerations

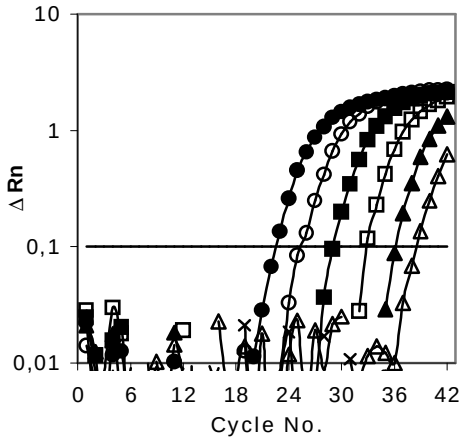
The KOALA-study was approved by the Ethical Committee of the University Hospital of Maastricht.

Results

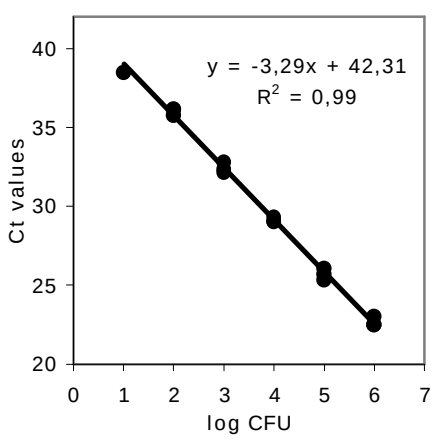
Sensitivity of the assays

The sensitivity of the real-time PCR assays was determined using serial tenfold dilutions of quantified cultures of *B. bifidum*, *C. difficile* and *E. coli*. As shown in Fig. 1(a), the *Bifidobacterium* TaqMan assay could reproducibly detect $\geq$ 10 CFU of *B. bifidum* per PCR reaction, which corresponds to $5*10^3$ CFU per gram faeces. From the average C_t values obtained with each dilution in three replicate amplifications, a standard curve was constructed (Fig. 1(b)). This curve shows that the *Bifidobacterium* TaqMan assay was exponential over a broad dynamic range, from 10 to at least 10^6 CFU starting material.

FIGURE 1 A



B



TaqMan assay of one of the three replicate runs of serial, tenfold dilutions of *B. bifidum* ranging from 1 to $1*10^6$ CFU per PCR reaction (A). ΔR_n indicates the normalized fluorescent reporter value, subtracted from the background value.
No template control (NTC) (-x-); $1*10^6$ CFU (- ●-); $1*10^5$ CFU (- ○-); $1*10^4$ CFU (- ■-); $1*10^3$ CFU (- □-); $1*10^2$ CFU (- ▲-); $1*10^1$ CFU (- △-); threshold (—).
Standard curve based upon three replicate runs of serial, tenfold dilutions of *B. bifidum* ranging from 1 to $1*10^6$ CFU per PCR reaction (B).

In addition, the slope of the standard curve was -3.3, which is similar to the optimal theoretical value. To investigate whether our assay was capable of detecting *Bifidobacterium* species other than *B. bifidum*, DNA was extracted from cultures of *B. infantis* ATCC 15697, *B. adolescentis* ATCC 15703, *B. breve* WBI 9 and *B. longum* WBI 2. For each of these species, the assay was performed on serial dilutions of purified DNA ranging from 1 to 10^6 genome copies. The bifidobacterial species tested were detected with a similar sensitivity as *B. bifidum* (data not shown).
The lower detection limit of the *C. difficile* TaqMan assay was 2 CFU per PCR reaction, corresponding to $2*10^3$ CFU per gram faeces. In addition, the amplification was exponential over a broad range of input material, from 2 to at least $2*10^5$ CFU per PCR.
The *E. coli* TaqMan could detect as little as 4 CFU per PCR reaction, which corresponds to $4*10^3$ CFU per gram faeces. The assay was exponential from 4 to at least $4*10^5$ CFU per PCR. Ultrafiltration of the PCR master mix resulted in complete removal of contaminating traces of *E. coli* DNA, whereas the sensitivity was not influenced.

Specificity of the assays

To determine the specificity of the assays, purified DNA from a mixture of non-target bacteria was subjected to the *Bifidobacterium spp.* and *C. difficile* assays, either in the presence or absence of DNA from the target bacteria. The TaqMan assays for *Bifidobacterium spp.* and *C. difficile* were highly specific for the target bacteria: no difference in amplification or quantification of *B. bifidum* or *C. difficile* was observed either in the presence or absence of 'nonspecific' bacteria in the mixtures. In addition, amplification was not observed when *B. bifidum* or *C. difficile* were omitted from these mixtures (data not shown). The specificity of the *E. coli* TaqMan assay has previously been demonstrated [7].
Additional validation of both the sensitivity and the specificity of the *C. difficile* assay was done by comparing the results of the assay with the results of culturing on a *C. difficile* selective plate. All 20 samples which were negative in the TaqMan assay were also negative by culture, whereas 19 of the 20 samples which were positive in the TaqMan assay were also culture positive. The *Bifidobacterium spp.* assay was not compared with traditional culturing since there are no reliable selective media for bifidobacteria [8].

DNA extraction and PCR inhibition control

The DNA extraction and amplification procedures were validated by the addition of a ‘spike’ consisting of RCMV particles, before DNA isolation. The C_t values of the RCMV-specific TaqMan assay were similar for all stool samples and bacterial cultures as well as the RCMV-positive control (C_t range 32.4-33.7; data not shown).

Microbial analysis of the faecal samples

The real-time PCR assays were used to analyze faecal samples of 50 exclusively breastfed and 50 exclusively formula-fed one-month-old infants participating in the KOALA-study. Prevalences of colonisation and counts (log_{10} CFU (g wet weight faeces)$^{-1}$) as determined by real-time PCR are shown in table 3. All breastfed as well as formula-fed infants were positive for *Bifidobacterium spp.* Although the number of bifidobacteria in faeces appeared slightly higher in breastfed infants than in formula-fed infants, this difference was not statistically significant.

The prevalence of colonisation with *C. difficile* was more than two times lower in breastfed infants (7/50) than in formula-fed infants (15/50), and in those infants colonised counts were also significantly lower in breastfed compared to formula-fed infants (median values 3.28 and 7.43 log_{10} CFU g^{-1} respectively; p = 0.03).

Ten breastfed infants were not colonised with *E. coli*, whereas only three formula-fed infants were not colonised by this species. Furthermore, the median value of *E. coli* numbers in colonised breastfed infants was almost threefold lower compared to the median value of *E. coli* numbers in the colonised formula-fed infants (p = 0.004).

TABLE 3

Prevalence and counts of *Bifidobacterium spp.*, *E. coli*, and *C. difficile* (log_{10} CFU (g wet weight faeces)$^{-1}$) in faeces determined at one month of age in either breast-fed or formula-fed infants

	Breastfed (n = 50)	Formula-fed (n = 50)	P-value*
Bifidobacterium spp.			
Prevalence	50/50	50/50	
Counts[a]	10.56 (5.52-11.34)	10.24 (6.15-11.37)	0.14
C. difficile			
Prevalence	7/50	15/50	
Counts[a]	3.28 (2.92-6.91)	7.43 (2.71-9.57)	0.03
E. coli			
Prevalence	40/50	47/50	
Counts[a]	9.11 (5.73-10.43)	9.57 (5.14-10.46)	0.004

[a]Median values (ranges) calculated from positive samples only (log_{10} CFU (g wet weight faeces)$^{-1}$)
*P value of Mann-Whitney rank sum test calculated from all samples

Discussion

In order to monitor the influence of either exclusive breastfeeding or formula feeding on composition as well as quantity of gut bacteria in infants, real-time PCR assays for the detection of *Bifidobacterium spp.* and *C. difficile* were developed. In addition, we also employed a previously described real-time PCR assay for the detection and quantification of *E. coli*. Each of the three real-time PCR assays was found to be highly specific, since they did not generate positive signals with closely related bacterial species. The assays were also shown to be very sensitive, detecting as little as 10, 2 and 4 CFU of *Bifidobacterium spp.*, *C. difficile* and *E. coli*, respectively. Spiking of faecal samples with RCMV resulted in similar values for all faecal samples as well as for the RCMV-positive control. This indicated that the assays are not significantly influenced by potentially inhibitory components from the stool samples.

We found the prevalence and counts of *C. difficile* as well as *E. coli* to be significantly lower in the gut microbiota of breastfed infants than in that of formula-fed infants. In contrast, the prevalence and counts of *Bifidobacterium spp.* was similar among both groups of infants.

Throughout the last decades numerous studies have been conducted to compare gut microbiota composition between breast- and formula-fed infants, all based upon traditional culture techniques. Although some studies demonstrated significantly higher counts of bifidobacteria in breastfed infants than in formula-fed infants [22, 23], other, more recent studies did not reveal such differences [6, 24-26]. Since especially the more recent studies did not show significant differences in bifidobacteria between breast- and formula-fed infants, it has been suggested that this might be the result of modifications of the infant formulas. For instance, the protein and phosphorus content of modern formulas are much lower, giving a lower buffering capacity similar to that of human milk [6]. On the other hand, since most previous studies were performed using traditional culturing methods, discrepant results between these studies may also be due to differences in selectivity and specificity of the media used for counting bifidobacteria [6]. In some studies an extremely low prevalence of bifidobacterial colonisation was found in infants aged 4 weeks [27, 28], whereas in other studies (almost) all infants were reported to be colonised with bifidobacteria at the same age [25, 26, 29].

We found *E. coli* to be significantly less prevalent, and in lower numbers, in faecal samples of breastfed infants than in those of formula-fed infants. This finding is in agreement with data from previous studies in which lower counts of enterobacteria were found in breastfed infants than in formula-fed infants around the age of one month [24, 26, 29].

The prevalence of *C. difficile* in our study was also lower in breastfed infants than in formula-fed infants (14% and 30%, respectively). Furthermore, the breastfed infants that were colonised with *C. difficile* harbored lower numbers in their faeces than did the formula-fed infants. Other studies on the prevalence of *C. difficile* in infants showed prevalences ranging from 15 to 70% [30-35]. A study among 343 infants born in a Swedish hospital showed carriage rates comparable to our findings: 21% of breastfed infants and 47% exclusively formula-fed infants carried *C. difficile* at the age of 6 weeks [32].

Several other real-time PCR assays targeting the 16S rRNA gene for the detection of gut bacteria have recently been described. Ott and colleagues described several TaqMan assays for the detection of gut bacteria using universal primers and specific probes [18]. The sensitivity of the assays was similar to the sensitivity of our assays detecting as little as 10 CFU per reaction, the detection of bifidobacteria was not included in this study.

Matsuki and colleagues described several real-time PCR assays for the detection of bifidobacteria not only to the genus-level, but also to the species-level [16, 17]. The detection limit of their assays, however, was only 10^6 CFU bifidobacteria per gram wet weight faeces, whereas our assay could detect as little as $5*10^3$ CFU *Bifidobacterium* spp. per gram/faeces, which corresponds to 10 CFU per reaction. Guiemonde and colleagues described an assay for the detection of bifidobacteria in faecal samples with a detection limit of $5*10^4$ CFU per gram of faeces [19].

The molecular 16S rDNA-based real-time PCR assays evaluated in this study provide more accurate data on intestinal microbiota composition than traditional culture techniques. Furthermore, these real-time PCR assays do not require anaerobic conditions and they can be applied to high-throughput analyses using both fresh and frozen samples, thereby making this technique very suitable for large-scale epidemiological studies on gut microbiota composition.

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Chapter 4

Factors influencing the composition of the intestinal microbiota in early infancy

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Chapter 4

Abstract

The aim of this study was to examine the contribution of a broad range of external influences on the gut microbiota composition in early infancy.

Faecal samples of 1032 infants aged one month, recruited from the KOALA Birth Cohort Study in the Netherlands, were subjected to quantitative real-time PCR assays for the enumeration of bifidobacteria, *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis*-group, lactobacilli and total bacterial counts. Information on potential determinants of the gut microbiota composition was retrieved from repeated questionnaires. The association between these factors and the selected gut bacteria were analyzed with univariate and multivariate analyses.

Infants born through caesarean section had lower numbers of bifidobacteria and bacteroides, whereas they were more often colonised with *C. difficile,* compared with vaginally born infants. Exclusively formula-fed infants were more often colonised with *E. coli*, *C. difficile*, bacteroides and lactobacilli, compared with breastfed infants. Hospitalisation and prematurity were associated with a higher prevalence and counts of *C. difficile*. Infant's antibiotic use was associated with decreased numbers of bifidobacteria and bacteroides. Infants having older siblings had slightly higher numbers of bifidobacteria, compared with infants without siblings.

In conclusion, the most important determinants of the gut microbiota composition in infants are the mode of delivery, type of infant feeding, gestational age, infant hospitalisation, and antibiotic use by the infant. Vaginally home-born term infants who are exclusively breastfed seem to have the most "beneficial" gut microbiota (highest numbers of bifidobacteria and lowest numbers of *C. difficile* and *E. coli*).

Introduction

The gut microbiota plays an important role in human health by providing a barrier for colonisation of pathogens, by exerting important metabolic functions (fermentation of non-digestible fibers, salvage of energy as short-chain fatty acids, production of vitamin K) and by stimulating the development of the immune system [1]. Bifidobacteria and lactobacilli are considered the most important health beneficial bacteria for the human host, whereas bacteria such as staphylococci and clostridia are potentially pathogenic [2]. Because the gut microbiota is involved in many aspects of human health, it is important to understand how the composition of this microbial ecology is established.

During early life, there are major changes in the intestinal microbiota composition. At birth, the intestines are sterile. Within a few hours, bacteria start to appear in the faeces. Because the intestinal environment of neonates shows a positive oxidation reduction potential at birth, the gastrointestinal tract is colonised first by facultative aerobes. Gradually the consumption of oxygen by these bacteria changes the intestinal environment into a more reduced one, permitting the subsequent growth of strict anaerobes [3].

The bacteria colonizing the infant gut during the first days of life originate mainly from the mother and the environment. In early life, one of the first major determinants of the gut microbiota is the mode of delivery. Vaginally born infants are colonised at first by faecal and vaginal bacteria of the mother, whereas infants born through caesarean section are exposed initially to bacteria originating from the hospital environment and health care workers [3, 4]. Other factors that can influence the composition of the intestinal microbiota in neonates are the environment during birth, prematurity, hygiene measures and the type of infant feeding [5]. We showed previously that breastfed infants had less *Clostridium difficile* and *Escherichia coli* in their faeces at the age of one month compared to formula-fed infants, whereas counts of bifidobacteria were comparable in the two groups [6].

Although determinants of gut microbiota composition were investigated previously in several studies, those studies focused on only one or a few determinants at a time and generally involved a limited number of infants [7-11]. Many potential determinants of the intestinal microbiota are clustered. For example, infants born through caesarean section need to stay in the hospital more often and receive antibiotics more frequently than do infants born vaginally. Consequently, the effects of individual determinants on the composition of the intestinal microbiota can be distinguished only if a large population is studied.

The aim of the present study was to examine the influence of a broad range of potential determinants on the gut microbiota composition in a prospective cohort study in the Netherlands. To this purpose, faecal samples of over a

thousand one-month-old infants were analysed using real-time PCR to quantitatively detect several bacterial groups and species.

Materials and methods

Subjects and study design

The KOALA Birth Cohort Study is a prospective birth cohort in the Netherlands. The design of this study was described in detail elsewhere [12]. Briefly, from October 2000 until December 2002, we recruited participants with diverse lifestyles at 34 weeks of gestation. Pregnant women with a conventional lifestyle (n=2343) were recruited from an ongoing prospective cohort study on Pregnancy-related Pelvic Girdle pain in the Netherlands [13]. Additionally, pregnant women with an alternative lifestyle (n=491) were recruited through organic food shops, anthroposophic doctors and midwifes, Steiner Schools, and magazines.

Beginning halfway during recruitment of the cohort (subjects recruited from January 2002 onward), faecal samples of infants (n = 1176) at the age of one month were collected. Subjects received a faeces tube with spoon (Sarstedt, Nümbrecht, Germany), together with a sanitary napkin, an instruction form and a brief questionnaire (faeces questionnaire). Parents collected a faecal sample by placing a sanitary napkin in the diaper (to prevent absorption of the faeces by the diaper) and collecting the faeces out of the napkin into the collection tube, and they sent the sample as soon as possible (the same day) to the Department of Medical Microbiology at the University Hospital of Maastricht by mail. Exclusion criteria were: insufficient amount of faeces (< 1 gram), faeces collected before the age of three weeks or after the age of six weeks, and a missing faeces questionnaire.

Information on potential determinants

During pregnancy and during the first months of the infant ś life, information on perinatal determinants of the child ś health as well as on hygiene, infections, nutrition, child rearing, and other lifestyle characteristics was collected for all members of the cohort with repeated questionnaires. Based on these questionnaires, the following variables were selected as potential determinants of gut microbiota composition: maternal education (lower education, vocational, higher general secondary/pre university, or higher vocational/academic); maternal diet, defined as (1) regular diet (> 50% of meat, eggs, vegetables, fruit and milk of regular origin), (2) vegetarian diet (no meat whereas other products have mainly a regular origin), (3) organic/biodynamic diet (over 50% of meat, eggs, vegetables, fruit and milk of organic or biodynamic origin), or (4) organic/biodynamic vegetarian diet;

maternal probiotic use during pregnancy (frequency of consumption of dairy products with additional lactic acid-producing bacteria); maternal antibiotic use during last month of pregnancy (yes or no); prolonged rupture of membranes (< 24h or > 24h before delivery); place and mode of delivery (vaginal delivery at home, vaginal delivery in hospital, artificial delivery in hospital, or caesarean section in hospital); hospitalisation of the infant after birth (days of hospitalisation immediately following birth); infant gender (male or female); gestational age (< 37 wk, 37-41 wk, or > 41 wk); birth weight (< 2500 g, 2500-4500 g, or > 4500 g); birth season (winter, spring, summer, or fall); type of infant feeding during first month of life (exclusively breastfed, exclusively formula-fed, or combination); brand of infant formula (exclusive consumption of Brand A, Brand B with locust bean gum, Brand C, or Brand D with oligosaccharides during the first month of life); antibiotic/antifungal use by infant during first month of life (yes or no); fever of infant in first month of life (yes or no); number of siblings (0, 1, or $\geq$ 2); living on a farm (yes or no); and having furry pets (none, cat, dog, other, or combination).

DNA purification from faeces

At the laboratory, faecal samples were tenfold diluted in peptone water (Oxoid CM0009) containing 20% (vol/vol) glycerol (Merck, Damstadt, Germany) and were stored at –20°C until analysis. For DNA isolation, 0.2 mL of the diluted faeces was added to a 2-mL vial containing approximately 300 mg of glass beads (diameter 0.1 mm) and 1.4 mL of ASL-buffer from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), then the samples were disrupted in a mini-bead beater (Biospec Products, Bartlesville, Okla.) at 5000 rpm for 3 min. Subsequently, the bacterial DNA was isolated from the samples with the QIAamp DNA Stool Mini Kit, according to the instructions of the manufacturer. The DNA was eluted in a final volume of 200 µL.

Microbial analysis using real-time PCR assays

DNA from all faecal samples was subjected to real-time PCR assays for bifidobacteria*, E. coli, C. difficile, Bacteroides fragilis*-group, lactobacilli and total bacteria based on 16S rDNA gene sequences (primers and probes are listed in Table 1). Development and validation of the real-time PCRs were described in detail elsewhere [6, 14-17]. The 5´-nuclease technique was used for the detection of bifidobacteria*, E. coli., C. difficile* and members of the *B.fragilis*-group. For the detection of bifidobacteria, amplifications were conducted in a total volume of 50 µL, containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems [ABI], Foster City, CA), 300 nM of both primers, 150 nM of TaqMan probe, and 20 µL of purified target DNA (see above). For *E. coli, C. difficile* and *B. fragilis*-group, amplifications were conducted in a total volume of 25 µL, containing 1x TaqMan Universal PCR

Master Mix (ABI), 900 nM of both primers, 200 nM of TaqMan probe, and 10 µl of purified target DNA. The amplification (2 min at 50°C, 10 min at 95°C, followed by 42 cycles of 15 s at 95°C and 1 min at 60°C) and detection were conducted with ABI Prism 7000 sequence detection system (ABI).

For the quantification of lactobacilli and total bacterial load, real-time detection of PCR products was conducted with SYBR Green I. The lactobacilli PCR was conducted in a total volume of 25 µL, containing 1 x iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc, Hercules, CA), 500 nM of both primers, and 5 µL purified target DNA. The amplification was conducted as follows: 5 min at 95°C, followed by 35 cycles consisting of 15 s at 95°C, 20 s at 58°C, 45 s at 72°C, with a final extension step at 72°C for 5 min. Following amplification, melt curve analysis was performed from 60-95 °C using increments of 0.5 °C per 10 s. For quantification of the total bacterial load, amplifications were conducted in a total volume of 25 µL, containing 1 x iQ™ SYBR® Green Supermix (Bio-Rad), 300 nM of both primers, and 5 µL purified target DNA. The amplification consisted of 4 min at 95°C, 30 s at 60°C, followed by 35 cycles of 30 s at 60°C, 15 s at 95°C and 30 s at 60°C. Finally melt curve analysis was performed from 60-95 °C using increments of 0.5 °C per 10 s. Amplification, melting curve analysis and detection were conducted with the MyiQ Single-Color real-time PCR Detection System (Bio-Rad).

TABLE 1

Primers and probes used in this study

Target organisms (amplicon size)	Primer/Probe	Sequence (5´-3´)	T_m (°C)	Ref.
Bifidobacterium spp	forward primer	GCGTGCTTAACACATGCAAGTC	59	[6]
(126 bp)	reverse primer	CACCCGTTTCCAGGAGCTATT	59	[6]
	probe	TCACGCATTACTCACCCGTTCGCC	70	[6]
E. coli	forward primer	CATGCCGCGTGTATGAAGAA	59	[14]
(96 bp)	reverse primer	CGGGTAACGTCAATGAGCAAA	59	[14]
	probe	TATTAACTTTACTCCCTTCCTCCCCGCTGAA	68	[14]
C. difficile	forward primer	TTGAGCGATTTACTTCGGTAAAGA	58	[6]
(114 bp)	reverse primer	TGTACTGGCTCACCTTTGATATTCA	59	[6]
	probe	CCACGCGTTACTCACCCGTCCG	69	[6]
B. fragilis-group	forward primer	CGGAGGATCCGAGCGTTA	58	this study
(92 bp)	reverse primer	CCGCAAACTTTCACAACTGACTTA	59	[43]
	Probe	CGCTCCCTTTAAACCCAATAAATCCGG	68	this study
Lactobacillus spp.	forward primer	AGCAGTAGGGAATCTTCCA	59	[16, 44]
(341 bp)	reverse primer	CACCGCTACACATGGAG	59	[16, 45]
Total count.	forward primer	TCCTACGGGAGGCAGCAGT	59	[17]
(467 bp)	reverse primer	GGACTACCAGGGTATCTAATCCTGTT	58	[17]

Statistical analyses

The log_{10} colony forming units (CFU) per gram of the bacterial groups and species were calculated for each stool sample from the threshold cycle (C_t)-values using the constructed standard curves. The prevalence of colonisation was expressed as the percentage of infants colonised with a specific bacterial group or species. To determine the unadjusted overall effects of the individual determinants on the prevalence and counts of the bacteria simultaneously, the Mann-Whitney rank sum test was used.

To analyze the effects of the individual determinants, with adjustment for all other determinants, two multivariate approaches were used. First, linear regression analyses were used to examine the effects of the determinants on the bacterial counts. In the linear regression analyses, only infants who were colonised with the bacterial group or species were included. Total bacterial count was added as an additional independent variable to account for differences in consistency of faecal samples. Second, logistic regression analyses were used to examine the effect of the determinants on the prevalence of colonisation (colonised compared to not colonised). Both linear and logistic regression models included all the determinants under study as independent variables and one of the bacterial groups or species as dependent variable at a time. Length of hospitalisation (in days), was included as a continuous variable in the regression models, whereas all the other independent variables were incorporated as categorical variables.

To limit the chance of falsely rejecting the null hypothesis (no association) as a result of multiple testing, we chose to set alpha at 0.01 two-sided instead of the usual 0.05. Accordingly we present 99% confidence intervals for the odds ratios (ORs) from logistic regression analyses.

Ethical considerations

The KOALA Study was approved by the ethical committee of the University Hospital of Maastricht, and all parents signed informed consent for the study.

Results

Faecal samples from a total of 1176 infants were collected. After exclusion of samples that were of insufficient amount (n = 65), samples that were collected before the age of three weeks or after the age of six weeks (n = 54), and samples for which the faeces questionnaire was missing (n = 25), faecal samples of 1032 infants were included for analysis.

TABLE 2

Median counts (log10 CFU/gram faeces) and prevalences (%) of selected gut bacteria in faeces of infants aged one month (n = 1032)

	Bifidobacteria	*E. coli*	*C. difficile*	*B. fragilis*-group	Lactobacilli	Total Counts
Median counts	10.68	9.35	5.32	9.28	8.66	11.12
(range)	(6.84-11.56)	(5.91-10.79)	(2.70-9.57)	(5.74-10.44)	(7.92-10.73)	(9.43-12.14)
Prevalence (%)	98.6	87.7	25.0	81.6	32.4	100

Almost all infants were colonised with bifidobacteria, and these bacteria outnumbered all other bacterial groups and species (Table 2). The majority of infants were also colonised with *E. coli* and members of the *B. fragilis*-group, whereas both the prevalence and counts of lactobacilli and *C. difficile* were much lower.

Table 3 shows the prevalence and counts of the bacterial groups and species for the individual determinants under study. In Table 4, the associations between these determinants and the prevalence and counts of the faecal bacteria under study, as determined in the multivariate analyses, are presented. More detailed information on these associations, including regression coefficients and ORs from the linear and logistic regression analyses, is shown in Table 5.

Maternal education, diet, antibiotic and probiotic use

More than half of the women in this population (n = 572) had a higher vocational or academic degree. Maternal education was not associated, however, with the infant's gut microbiota composition.

Infants whose mothers consumed an organic or biodynamic diet seemed to have slightly lower numbers of *E. coli* in their stools than did infants whose mothers consumed a regular diet (Table 3); however, this association was not observed in the multivariate analysis (data not shown). Mothers who consumed an organic diet more often breastfed their infants exclusively (95%), compared with mothers who consumed a regular diet (59%), which suggests that the type of infant feeding was the underlying cause of the association between maternal diet and infant's microbiota composition in the univariate analysis.

Probiotic use and antibiotic use by the mother during pregnancy had no influence on the infant's gut microbiota composition of the infant.

Delivery and birth characteristics

Amniotic membranes ruptured > 24 hours before delivery for only 36 women. We found no association between prolonged rupture of membranes among women and the gut microbiota composition of their infants.

More than half of the infants were born in the hospital, and approximately ten percent of all infants were born through caesarean section. In comparison with vaginal delivery at home, caesarean section resulted in lower colonisation rates and counts of bifidobacteria and *B. fragilis*-group species, whereas prevalence and counts of *C. difficile* and counts of *E. coli* were higher. The most pronounced differences in colonisation were seen for the *B. fragilis*-group and *C. difficile*; compared with infants born vaginally at home, the median counts of *B. fragilis*-group bacteria and *C. difficile* were approximately one hundred-fold lower and one hundred-fold higher, respectively, for infants born through caesarean section (Table 3). After adjustment for the other determinants caesarean section was still associated with lower counts of bifidobacteria, a lower colonisation rate and counts of *B. fragilis*-group species, and higher counts of *C. difficile*. In contrast, counts and colonisation rate of *E. coli* were no longer associated with caesarean section in the adjusted analyses. This is illustrated in Table 5 by a regression coefficient for counts close to zero and an OR for prevalence close to 1.0. Although vaginal delivery, and artificial delivery in the hospital seemed to be associated with higher counts and prevalences of *E. coli* and *C. difficile,* compared with home birth (Table 3), these associations were only present in the unadjusted analysis.

Hospitalisation of the infant after birth was associated only with a higher *C. difficile* colonisation rate after controlling for other determinants. The prevalence of *C. difficile* increased with about 13% per day of hospitalisation, compared with nonhospitalised infants (OR 1.13; 99%CI 1.01-1.25).

Only eleven infants in this study population were born premature (< 37 weeks). These infants were colonised more often with *C. difficile* (64% as opposed to 23%) and with considerably higher counts (7.12 $\log_{10}$ compared to 5.06 $\log_{10}$ CFU/g faeces) compared with term infants (Table 3). When adjusted for other determinants the counts were still statistically significantly higher (Table 4 and 5). The OR was still consistent with a higher colonisation rate (OR 4.47), but was not statistically significant, probably because of the low power caused by the small number of infants.

No association was found between the gut microbiota and birth weight, birth season, or the gender of the infant.

Infant feeding

Most infants (n = 700) were breastfed exclusively up to the first month of life, whereas 232 infants were formula fed exclusively and 98 infants received a combination of breastfeeding and formula feeding. Exclusively formula-fed

infants were more often colonised with *E. coli, C. difficile, B. fragilis*-group, and lactobacilli than were their exclusively breastfed counterparts, as shown in both the unadjusted (Table 3) and adjusted analyses (Table 4 and 5). The counts of *E. coli, C. difficile, B. fragilis*-group and lactobacilli were also significantly higher for formula-fed infants, compared with breastfed infants in the unadjusted analyses. In the adjusted analyses, only counts of *C. difficile* were still significantly higher for formula-fed infants, whereas counts of both *E. coli* ($p = 0.03$) and *B. fragilis* ($p = 0.027$) still tended to be higher in formula-fed infants (not reaching the level of significance of $p < 0.01$) (Table 5).

In our population, four brands of formula were used frequently. Brand B contained locust bean gum and brand D was enriched with oligosaccharides, whereas the others were not. Infants fed exclusively with one of these four formulas were compared. As shown in Table 3, infants fed with the oligosaccharides enriched formula (brand D) harbored higher numbers of bifidobacteria in their stools. After adjusting for the other determinants under study counts of bifidobacteria (Coef = 0.60; $p = 0.04$) and also counts of lactobacilli (Coef = 0.75; $p = 0.02$) tended to be higher in infants fed with formula D, compared with reference formula A (data not shown).

Antibiotics, antimycotics and fever

Oral use of antibiotics (mainly amoxycillin) by the infant during the first month of life resulted in decreased numbers of bifidobacteria and *B. fragilis*-group species. Lower counts of bifidobacteria were also observed after oral administration of the antimycotic miconazole (Table 3 and 4). Infants who experienced a fever in their first month of life did not have a different gut microbiota composition, compared with infants without a fever.

Home environment

Infants with older siblings had lower total bacterial counts per gram faeces than did infants without siblings, as seen in the unadjusted analysis (Table 3). After adjusting for these differences in total bacterial counts and other potential differences between infants with and without siblings, a greater proportion of bifidobacteria was found for infants with older siblings, compared with infants without siblings (Table 4 and 5).

Infant's gut microbiota was not associated with farm residence or the presence of furry pets in the home, as determined by both the unadjusted (Table 3) and multivariate analyses (data not shown).

TABLE 3

Median counts (log10 CFU/gram faeces), and prevalence (%) of colonisation of selected gut bacteria in faeces of infants aged one month (n = 1032)

Characteristics	N	Bifidobacteria		*E. coli*		*C. difficile*		*B. fragilis*-gr.		Lactobacilli		Total counts
		counts[†]	%	counts[†]	%	counts[†]	%	counts[†]	%	counts[†]	%	counts[†]
Maternal education[‡]												
lower education[¶]	75	10.71	100	9.47	92	4.95	24	9.32	77	8.87	39	11.16
vocational	242	10.73	98	9.43	91	6.37	25	9.41	86	8.65	34	11.18
higher general secondary/pre-university	107	10.68	97	9.41	88	5.49	19	9.29	80	8.66	31	11.06
higher vocational/academic education	572	10.65	99	9.24	86	5.30	25	9.21	80	8.64	30	11.08
Maternal diet[‡§]												
regular[¶]	723	10.67	99	9.49	89	5.40	26	9.39	82	8.70	34	11.15
organic/biodynamic	49	10.56	98	8.91	82*	6.70	16	8.93	75	8.51	33	10.92
vegetarian	47	10.63	98	9.06	85	6.76	17	9.00	81	8.66	32	11.05
organic/biodynamic vegetarian	16	10.61	94	8.66	88	4.17	25	8.16	69	8.48	38	10.91
Maternal probiotic use[‡§]												
never/sporadic[¶]	814	10.67	99	9.30	88	5.06	25	9.24	82	8.64	32	11.10
several times a month	100	10.62	98	9.23	82	6.74	21	9.36	75	8.68	34	11.12
several times a week	65	10.75	100	9.45	95	5.30	39	9.29	83	8.74	39	11.24
daily	23	10.48	100	9.71	70	5.40	13	9.34	83	8.71	35	11.21
Maternal antibiotic use[‡‖]												
no[¶]	972	10.68	99	9.32	88	5.10	25	9.28	82	8.65	32	11.12
yes	38	10.60	92	9.48	95	5.92	16	9.30	74	8.68	50	11.13
Rupture of membranes[‡]												
≤24 hours[¶]	996	10.67	99	9.36	88	5.30	25	9.28	82	8.68	32	11.12
> 24 hours	36	10.61	100	8.93	89	6.97	19	9.23	78	8.50	31	11.05
Place and mode of delivery[‡]												
natural delivery at home[¶]	480	10.67	99	9.09	85	4.20	19	9.21	83	8.58	32	11.02
natural delivery in hospital	346	10.74	99	9.54	91**	5.45	26*	9.40	85	8.65	34	11.18**
artificial delivery in hospital	76	10.80	100	9.73	91**	6.19	34*	9.60	87	8.50	30	11.36**
caesarean section in hospital	108	10.38	96**	9.59	88*	6.36	42**	6.67	63**	8.87	32	11.11

TABLE 3 continued

Hospitalisation after birth‡											
no¶	737	10.69	99	9.24	87	4.38	20	9.27	83	8.58	32	11.10
1 day	91	10.77	100	9.57	90	6.60	30	9.60	81	8.68	33	11.33*
2-3 days	85	10.65	100	9.72	93**	6.39	40**	9.41	79	8.88	37	11.23
4-6 days	73	10.36	96**	9.76	89	6.03	43**	6.56	68**	8.71	41	11.08
≥ 7 days	25	10.25	96*	9.40	88	6.94	40*	8.42	80	9.44	28	10.96
Gender												
male¶	531	10.64	99	9.37	89	5.61	24	9.20	80	8.76	32	11.10
female	501	10.70	98	9.28	86	5.06	25	9.34	83	8.58	33	11.12
Gestational age at birth‡												
< 37 weeks (premature)	11	10.53	91	9.02	73	7.12	64*	8.95	82	8.80	27	10.80
37-41 weeks¶	860	10.68	99	9.30	87	5.06	23	9.27	82	8.61	33	11.12
> 41 weeks (postmature)	37	10.44	100	9.77	84	6.99	35	9.51	78	8.72	32	11.14
Birth weight‡												
< 2500 grams	11	10.45	100	8.67	100	7.12	27	9.31	82	9.12	36	11.28
2500-4500 grams¶	906	10.67	99	9.32	87	5.08	24	9.30	82	8.65	33	11.12
>4500 grams	26	10.61	100	9.59	85	6.07	23	9.30	77	8.60	35	11.03
Birth season‡												
winter¶	285	10.67	99	9.37	85	5.61	22	9.20	81	8.65	32	11.11
spring	241	10.65	96	9.26	88	6.28	25	9.44	82	8.87	37	11.13
summer	286	10.55	99	9.23	92	4.60	23	9.04	82	8.65	33	11.04
fall	216	10.84	99	9.53	86	4.74	30	9.31	81	8.40	26	11.21
Type of infant feeding‡												
exclusively breastfed¶	700	10.67	99	9.06	85	4.53	21	8.99	79	8.54	29	10.98
exclusively formula-fed	232	10.69	97	9.84	94**	7.43	33**	9.76	88**	8.93	41**	11.43**
combination	98	10.78	99	9.76	93**	5.58	35*	9.53	83*	8.71	34	11.36**
Type of infant formula												
Brand A¶	47	10.51	96	9.83	91	7.68	40	9.84	89	8.84	34	11.45
Brand B (with locust bean gum)	19	10.80	95	9.81	89	6.56	21	9.80	95	8.40	47	11.43
Brand C	39	10.81	95	9.82	93	7.28	25	9.70	87	8.68	38	11.28
Brand D (with oligosaccharides)	20	11.19	100**	9.63	95	6.23	30	10.11	75	9.29	55	11.65

Characteristics	N	Bifidobacteria		*E. coli*		*C. difficile*		*B. fragilis*-gr.		Lactobacilli		Total counts
		counts[†]	%	counts[†]	%	counts[†]	%	counts[†]	%	counts[†]	%	counts[†]
Antibiotic/antimycotic use during first month[‡]												
no[¶]	945	10.70	98	9.32	88	5.50	25	9.31	82	8.65	32	11.12
oral antibiotic	28	10.29	100*	9.45	79	7.12	18	6.39	82*	8.62	36	10.92
oral miconazole	22	10.18	100*	9.57	82	4.47	23	9.35	86	8.68	36	11.04
oral nystatine	15	10.77	93	9.67	87	4.81	13	9.33	73	8.64	33	11.00
Fever in first month[‡]												
no[¶]	893	10.67	99	9.31	87	5.07	26	9.25	81	8.61	31	11.11
yes	47	10.58	100	9.37	87	6.89	11	8.95	81	8.90	38	11.08
Siblings[‡]												
no[¶]	414	10.63	98	9.51	86	6.10	27	9.40	80	8.77	34	11.21
1	452	10.70	99	9.27	88	5.06	23	9.21	82	8.64	31	11.07*
$\geq$ 2	165	10.69	100	9.23	90	3.85	25	8.95	82	8.57	33	11.03*
Living on a farm[‡]												
no[¶]	979	10.67	99	9.32	87	5.54	25	9.27	81	8.69	32	11.12
yes	23	10.87	100	9.52	96	4.04	35	9.51	83	8.70	26	11.35
Furry pets												
no[¶]	552	10.70	98	9.30	89	5.40	27	9.23	83	8.63	32	11.12
dog	217	10.74	98	9.28	87	4.71	22	9.27	82	8.72	31	11.08
cat	164	10.62	98	9.57	83	6.57	23	9.39	75	8.73	34	11.15
other	35	10.71	97	9.19	89	4.88	11	9.29	83	8.23	23	10.94
combination	64	10.56	100	9.43	94	5.17	28	9.36	83	8.75	41	11.14

[†]counts are presented as median values ($\log_{10}$ cells (g wet weight faeces)$^{-1}$) calculated from positive samples only, prevalence (%) is presented in parentheses

[‡]may not add up to 1032 because of missing values

[§]consumed during pregnancy

[‖]used somewhere during the last month of pregnancy

[¶]Reference category

* $p < 0.01$, ** $p < 0.001$ as determined by Mann-Whitney rank sum test calculated from all samples (the statistical significance refers to an overall difference incorporating both counts and prevalence)

TABLE 4

Associations between determinants and selected gut bacteria (counts and prevalences) in faeces of infants aged one month, as determined in multivariate analyses*

	Bifidobacteria		***E. coli***		***C. difficile***		***B. fragilis*-gr.**		**Lactobacilli**	
	counts[†]	prevalence[‡]	counts[†]	prevalence[‡]	counts[†]	prevalence[‡]	counts[†]	prevalence[‡]	counts[†]	prevalence[‡]
Caesarean section (compared with vaginal)	--	ND				+	--	--		
Hospitalisation (days)		ND				+				
Prematurity (compared with term infants)		ND			+					
Exclusive formula feeding (compared with excl. breastfeeding)		ND		+	+	++		+		+
Antibiotic use infant (yes/no)	-	ND					--			
Miconazole use infant (yes/no)	-	ND								
Siblings (yes/no)	+	ND								

* Results for determinants without statistically significant results on any of the bacteria are not presented.

[†]Association between determinants and counts examined by linear regression. Models included the following independent variables: maternal education, maternal diet, maternal probiotic use, maternal antibiotic use, rupture of membranes, place and mode delivery, gender, gestational age, birth weight, birth season, hospitalisation following birth, type of infant feeding, antibiotic/antimycotic use infant, fever infant, siblings, farm residence, furry pets and total bacterial count.

[‡]Association between determinants and prevalence of colonisation (colonised compared to not colonised) examined by logistic regression. Models included the following variables: maternal education, maternal diet, maternal probiotic use, maternal antibiotic use, rupture of membranes, place and mode delivery, gender, gestational age, birth weight, birth season, hospitalisation following birth, type of infant feeding, antibiotic/antimycotic use infant, fever infant, siblings, farm residence and furry pets.

+ (positive association $p < 0.01$); ++ (positive association $p < 0.001$)

- (negative association $p < 0.01$); -- (negative association $p < 0.001$)

ND: not determined (logistic regression on prevalence of bifidobacteria was not performed since 99% of infants were colonised)

TABLE 5

Linear regression coefficients for bacterial counts and odds ratio's for presence of the gut bacteria, depending on determinants in multivariate analyses*

	Bifidobacteria		***E. coli***		***C. difficile***		***B. fragilis*-gr.**		**Lactobacilli**	
	Coef† (p-value)	OR‡ (99% CI)	Coef † (p-value)	OR‡ (99% CI)	Coef † (p-value)	OR‡ (99% CI)	Coef† (p-value)	OR‡ (99% CI)	Coef † (p-value)	OR‡ (99% CI)
Caesarean section (compared with vaginal)	**-0.34 (0.003)**	ND	0.07 (0.677)	1.04 (0.38-2.83)	0.88 (0.24)	**2.07 (1.01-4.25)**	**-1.36 (0.000)**	**0.28 (0.13-0.61)**	0.31 (0.032)	0.84 (0.42-1.70)
Hospitalisation (days)	-0.01 (0.365)	ND	0.04 (0.108)	1.00 (0.86-1.17)	0.06 (0.364)	**1.13 (1.01-1.25)**	0.01 (0.621)	1.02 (0.90-1.16)	0.02 (0.306)	1.02 (0.92-1.12)
Prematurity (compared with term infants)	0.38 (0.282)	ND	-0.81 (0.109)	0.11 (0.01-1.15)	**2.83 (0.007)**	4.47 (0.48-41.85)	0.38 (0.432)	0.96 (0.09-10.38)	-0.23 (0.580)	0.68 (0.09-5.25)
Excl. formula-fed (compared with excl. breastfed)	-0.10 (0.233)	ND	0.24 (0.031)	**2.90 (1.22-6.89)**	**1.03 (0.003)**	**1.88 (1.13-3.11)**	0.25 (0.027)	**2.22 (1.16-4.24)**	0.056 (0.564)	**1.64 (1.03-2.60)**
Antibiotic use infant (yes/no)	**-0.66 (0.001)**	ND	0.06 (0.825)	0.57 (0.12-2.66)	0.94 (0.324)	0.59 (0.13-2.75)	**-1.10 (0.000)**	1.30 (0.27-6.19)	-0.16 (0.470)	1.11 (0.34-3.63)
Miconazole use infant (yes/no)	**-0.59 (0.003)**	ND	0.41 (0.142)	0.60 (0.13-2.90)	0.04 (0.965)	1.01 (0.25-4.09)	0.174 (0.506)	1.49 (0.27-8.20)	0.17 (0.468)	1.27 (0.38-4.25)
Siblings (yes/no)	**0.25 (0.000)**	ND	0.21 (0.025)	1.45 (0.82-2.57)	-0.32 (0.277)	1.01 (0.66-1.56)	0.004 (0.907)	1.09 (0.68-1.74)	0.01 (0.923)	0.88 (0.59-1.29)

* Results for determinants without statistically significant results on any of the bacteria are not presented.

† Regression coefficient of association between determinants and counts by linear regression. Models included the following independent variables: maternal education, maternal diet, maternal probiotic use, maternal antibiotic use, rupture of membranes, place and mode delivery, gender, gestational age, birth weight, birth season, hospitalisation following birth, type of infant feeding, antibiotic/antimycotic use infant, fever infant, siblings, farm residence, furry pets and total bacterial count.

‡ Odds ratios of association between determinants and prevalence of colonisation (colonised compared to not colonised) by logistic regression. Models included the following variables: maternal education, maternal diet, maternal probiotic use, maternal antibiotic use, rupture of membranes, place and mode delivery, gender, gestational age, birth weight, birth season, hospitalisation following birth, type of infant feeding, antibiotic/antimycotic use infant, fever infant, siblings, farm residence and furry pets.

ND: not determined (logistic regression on prevalence of bifidobacteria was not performed since 99% of infants were colonised)

Statistically significant results (at alpha = 0.01 two-sided) are printed in bold.

Discussion

To our knowledge, this is the first large, prospective, epidemiological study on determinants of gut microbiota composition in early infancy. As a consequence of the large number of infants in the KOALA study, we were able to study the potential determinants in a multivariate manner and to distinguish their independent effects. The faecal samples were analysed using real-time quantitative PCR assays. This molecular approach can be applied for high-throughput analyses using frozen samples, which makes this technique very suitable for a large-scale epidemiologic study such as this. Furthermore, real-time quantitative PCR analyses overcome many of the limitations of the traditional bacteriologic culture techniques, such as the low sensitivity, the low level of reproducibility because of the multitude of species to be identified and quantified and the time-consuming aspects of the conventional methods [14]. Molecular techniques based on amplification of 16S rDNA however require that the microbial cells in the sample first be lysed for the extraction of DNA. There is a vast difference in the susceptibility of the cells of different microbial species to lytic procedures. When only one lytic method is used it is unlikely that template DNA for the real-time PCR is extracted with equal success from all species [18], therefore we chose to add an additional mechanic lyses-step in addition to the chemical lyses of the Qiagen Stool Mini Kit.

A drawback of this study is the time between collection of the samples by the parents and processing of the samples in the laboratory, which was approximately one day. Ott and colleagues clearly demonstrated that the total amount of bacterial DNA as well as the diversity of the microbiota significantly decreased over such a time-span. However they also showed that the similarity (determined by DGGE) of faecal samples directly processed and processed after 24 hours remained high. This means that the dominant microbiota appears to be relatively stable. Furthermore the aim of the present population-based study was to examine differences in gut microbiota composition between subjects [19]. It is not likely that the possible change in composition of the samples during transport was influenced by the determinants under study.

It has previously been demonstrated that diet can have an influence on the gut microbiota. For instance, introduction of an extreme vegan diet has been shown to change bacterial fatty acid profiles in the faeces [20]. Also, an organic diet (consumption of foods which are produced without the use of synthetic inputs, such as synthetic fertilizers and pesticides, veterinary drugs, genetically modified seeds and breeds, preservatives, additives and irradiation) may influence the gut microbiota since organically produced foods include spontaneously fermented vegetables containing lactobacilli [21]. We

therefore hypothesized that maternal diet may not only be a determinant of her own gut microbiota, but also influences her infant's gut microbiota composition. Indeed, maternal diet appeared to have some influence on her offspring's microbiota as shown in the univariate analysis: *E. coli* counts were lower and also the prevalence and counts of *B. fragilis*-group species tended to be lower in infants from mothers consuming organic diets. However this association completely disappeared after adjusting for other determinants. This can at least partly be explained by the fact that mothers consuming an organic diet more often breastfeed their infants than do mothers with regular diets. This clearly emphasizes the need for multivariate analysis when studying the effects of determinants of the gut microbiota composition.

We did not find an association between maternal use of probiotics during pregnancy and the intestinal microbiota composition in their offspring at the age of one month. It is however not unlikely that transfer of probiotic bacterial strains from mother to child occurs, especially when the child is exposed to maternal faeces during vaginal delivery. And although probiotic bacteria do not permanently colonize the gut of adults [22, 23], this might be different for neonates which do not yet have an established gut microbiota. Indeed Schultz and colleagues confirmed such a transmission of probiotic strains from mother to child during birth, although the specific probiotic strain was only present in very low numbers in most infants (10^4-10^5 CFU/g faeces), it persisted at least until the age of six months [24]. In the present study, we did not investigate the presence of specific (probiotic) strains, but focussed on the total numbers of bifidobacteria and lactobacilli. Consequently, a putative low contribution of probiotic bacterial strains on the total number of lactobacilli or bifidobacteria, will not be noticed. Furthermore, the pregnant women in our cohort used a wide range of different probiotic products, including different strains. This heterogeneity may also explain why an association between probiotic use of the mothers and gut microbiota composition of the infants was not found. Firm conclusions of the effect of changes of the maternal microbiota itself can however not be made, since we have no data on the impact of both maternal diet and probiotic use on her own microbiota.

Apart from maternal diet and probiotic use, antibiotic use of the mother during the last month of pregnancy was also selected as a potential determinant of her infant's microbiota. This was based on the notion that changes in maternal microbiota could affect the infant's microbiota. However, as for the previous two determinants, maternal antibiotic use was not found to be associated with any of the examined gut bacteria.

Prolonged rupture of (amniotic) membranes before delivery increases the risk of foetal infection, especially with group B streptococci [25]. We found however no association between prolonged rupture of membranes and the commensal gut bacteria under study. However, as the presence of

streptococci was not examined, we cannot draw any conclusions about the association between prolonged rupture of membranes and the presence of this specific group of bacteria in the infant ṡ gut.

The colonisation rate and counts of the *Bacteroides fragilis*-group differed most markedly between vaginally delivered infants and infants born by caesarean section. This is in agreement with two previous studies, which also found members of the *B. fragilis*-group to be greatly reduced as a result of caesarean section [4, 26]. Furthermore we found bifidobacterial counts to be lower, although less pronounced and colonisation rates of *C. difficile* to be higher in infants born by caesarean section than in vaginally home-born infants.

After adjusting for potential confounding by the other determinants, the hospital environment itself had only an effect on the colonisation rate of *C. difficile.* Indeed, it is generally believed that infants are being colonised with this spore-forming anaerobic micro-organism mainly through the (hospital) environment. It was previously reported that vaginal swabs, collected just before delivery, were uniformly negative for this organism [27]. By contrast, *C. difficile* has been isolated from hands and stools of healthy hospital personnel and from a newborn intensive care unit, where spores may persist for months. [28]. Although carriage of *C. difficile* in healthy adults is uncommon [29], the relatively high colonisation rates among asymptomatic infants as found in our study are in accordance with findings from previous studies [30-33]. The highest carriage rate of *C. difficile* in our birth cohort was amongst premature infants: 64% of the infants born before 37 weeks of gestation were colonised. As prematurity is strongly associated with hospitalisation this could (partly) explain the relative high prevalence of *C. difficile* in preterms. However, after controlling for other determinants, the counts of *C. difficile* were still significantly higher in preterms than in term infants. Several factors, which were not included in the present study, may account for the higher numbers of *C. difficile* in preterms, such as the immature gastrointestinal tract and delayed oral feeding.

Like previous studies [11, 34, 35], we found that breastfed infants have a microbiota dominated by bifidobacteria, colonisation rates with *E. coli, C. difficile, B.* fragilis-group species and lactobacilli were significantly lower compared to formula-feds. Infants fed exclusively with a formula supplemented with a mixture of galacto-oligosaccharides and fructo-oligosaccharides (brand D) had higher counts of bifidobacteria and lactobacilli in their stools compared to infants fed with an unsupplemented formula. Although the composition of the formulas differed not only regarding the oligosaccharides supplementation, it is well known that supplementation with oligosaccharides increases counts of lactic acid bacteria [36, 37]. As opposed to the time of this study, most formulas are presently supplemented with oligosaccharides in the Netherlands, therefore differences in gut microbiota

composition as a result of different formulas probably have disappeared by now.

Although the use of antibiotics may have a major effect on the composition of the gut microbiota, the effect differs between antibiotics [38]. Due to the small number of infants in our cohort who received oral antibiotics in their first month, we were not able to distinguish between different antibiotics. Nevertheless, the administration of oral antibiotics had clear-cut effects on the anaerobic microbiota, counts of bifidobacteria and bacteroides being decreased.

In the unadjusted analyses total bacterial counts were lower in infants with siblings than in first children. After adjusting for these differences in total bacterial counts and all the other determinants, the proportion of bifidobacteria was significantly higher in infants with older siblings compared to first children, whereas counts of *E. coli* also tended to be higher in infants with older sibs. Again, this emphasizes the importance of multivariate analyses in studying a range of determinants of the gut microbiota. Studies on the aetiology of allergic diseases have often found a protective effect of older siblings [39]. This sibling-effect has been hypothesized to be a marker of early life infections. However, direct evidence for this hypothesis is lacking [40]. Since allergic diseases have also been linked to aberrant gut microbiota composition [41, 42], it has been suggested that this sibling-effect in allergy could be (partly) due to the commensal gut microbiota composition. To our knowledge, we are the first to demonstrate that having older siblings is indeed associated with the composition of the gut microbiota. It is clear, however, that the difference between children with or without siblings is small and that the (clinical) relevance of this difference remains to be elucidated.

In conclusion, infant feeding has a major effect on the gut microbiota composition in infants at the age of one month. Breastfed infants were less often colonised with bacteria other than bifidobacteria compared to formula-fed infants. The effect of mode of delivery is also of major importance, especially regarding bacteroides. Although passing the birth canal (vaginal delivery) and thus getting in contact with maternal faeces is strongly associated with the infant's gut microbiota, we found no effect on the infant's gut microbiota by determinants, which potentially influence the microbial composition of maternal faeces (maternal diet, antibiotic and probiotic use). Hospitalisation and prematurity are both positively associated with *C. difficile*. Antibiotic use greatly reduces the obligate anaerobes (bifidobacteria and bacteroides), whereas having older siblings is associated with slightly higher bifidobacterial counts. Vaginally home-born term infants who are exclusively breastfed seem to have the most "beneficial" gut microbiota, with the highest numbers of bifidobacteria and lowest numbers of *C. difficile* and *E. coli*.

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Chapter 5

Molecular fingerprinting of the intestinal microbiota of infants in whom atopic eczema was or was not developing

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Chapter 5

Abstract

The rise in atopic diseases has been linked to disturbances in the intestinal microbiota composition. The purpose of this study was to investigate the intestinal microbiota composition in infants in whom atopic (IgE-associated) eczema was or was not developing, using a molecular fingerprinting technique.

Within a prospective birth cohort study, faecal samples have been collected at the infant's age of one month. Within the context of this cohort, we conducted a nested case-control study comparing faecal samples of 26 infants who became sensitised and developed eczema within the first year of life with 52 non-sensitised non-eczematous infants. The composition of the faecal samples was examined using PCR combined with denaturing gradient gel electrophoresis. Using real-time PCR, total bacterial counts and bifidobacterial counts were enumerated.

Neither total bacterial profiles nor the type and proportion of bifidobacteria in the faeces were associated with the development of atopic eczema. The similarity of bacterial profiles was low; mean similarity was approximately 33% in both infants with or without atopic eczema. The prevalence of one specific band in total bacterial profiles was significantly higher in infants with atopic eczema compared with controls (96% vs. 71%, $P = 0.01$). Identification of this band revealed that it represented *Escherichia coli*.

In conclusion, although no association was found between the development of IgE-associated eczema and the dominant gut microbiota as a whole or with the bifdobacterial microbiota, the association with *E. coli* indicates that differences in gut microbiota do precede the development of atopy.

Introduction

Intestinal microorganisms have a major impact on the development and functioning of the intestinal immune system of the host, while the intestinal immune system in turn regulates the colonisation of the gut by these microorganisms. This interplay has been suggested to have a major influence on the host even beyond the intestines [1]. For example, the rise of atopic diseases has been linked to disturbances in the composition of the gut microbiota [2]. Differences in intestinal microbiota have been described between healthy and allergic children [3-5]. Furthermore, in prospective studies, it has been shown that differences in the gut microbiota composition are already present prior to the development of allergic sensitisation and symptoms [6, 7].

Bifidobacteria are generally considered to be health-beneficial and have been associated with a lower risk of atopy [3, 4, 6, 7]. The effect of bifidobacteria seems, however, to be species-dependent. In vitro studies have shown that bifidobacterial species differentially affect the production of cytokines by dendritic cells and macrophages [8, 9]. Furthermore, allergic infants have been reported to harbour a more adult-type *Bifidobacterium* flora containing *Bifidobacterium adolescentis*, whereas healthy infants had high levels of the infant-type *Bifidobacterium bifidum* [10].

Most studies on the gut microbiota and atopy were based on the quantification of selected bacterial groups or species that are commonly present in the intestines. Some of the disadvantages of classical culturing techniques are that samples require immediate processing and that extensive expertise and specialized equipment is needed to isolate strict anaerobes [11]. The introduction of molecular techniques to study the gut microbiota has overcome these problems, and using these techniques now gives us the opportunity to analyze frozen samples collected from large cohorts.

Polymerase Chain Reaction combined with Denaturing Gradient Gel Electrophoresis (PCR-DGGE) is a genetic fingerprinting technique, which provides a pattern or profile of the genetic diversity in a microbial community. In this technique it is not required for the bacteria or their sequences to be previously characterized [12]. In principle, PCR-DGGE can be used to distinguish two (bacterial) DNA molecules that differ from as little as a single-base substitution [13]. Murray and colleagues used this technique to compare the gut microbiota of sensitised wheezy and non-sensitised non-wheezy infants aged 4 years [14]. Although no differences were found between cases and controls, the authors concluded that further studies using molecular techniques are necessary to establish whether differences between faecal microbiota composition truly exist in younger allergic and non-allergic children with different allergic diseases [14].

In the present study, faecal samples collected at the age of one month postpartum, of infants in whom atopic eczema was or was not developing within the first year of life were compared using PCR-DGGE.

Material and methods

Subjects and samples

Within the prospective KOALA Birth Cohort Study on the aetiology of allergic diseases, we conducted a nested case-control study. The design of the cohort has been described previously [15]. Briefly, from October 2000 until December 2002, 2834 pregnant women were recruited at 14-18 weeks of gestation. Information on perinatal determinants of the child's health as well as on hygiene, infections, nutrition, child rearing, other lifestyle characteristics and on atopic manifestations was collected for all members of the cohort by repeated questionnaires at 34 weeks of gestation and 3, 7, 12 months post-partum. In the second part of the cohort (those participants recruited from October 2002 onwards), parents were asked to collect a faecal sample at the infant's age of one month and to consent to collecting of capillary blood for determination of total and specific IgE at the infant's age of 1 year.

For this nested case-control study the selection criteria were the availability of infant's faeces and capillary blood, full term birth and no history of antibiotic use within the first month of life. From those infants who met these criteria, we selected all infants who subsequently developed atopic eczema, and randomly selected twice as many control infants. Cases had to meet the following criteria: 1) having an itchy relapsing rash during the first year of life (parentally reported), excluding cases with only diaper rash, rash around the eyes and/or scalp scaling; and 2) being IgE-sensitised (> 1 IU/ml) for cow's milk, hen's egg or peanut at age 1 year. Control subjects were randomly selected out of the infants without an itchy relapsing rash during the first year of life and without detectable specific IgE levels (< 0.36 IU/ml). Determination of specific IgE from capillary blood has been performed as described previously [16, 17], with adjustments made for testing in capillary blood [18].

Collection of infant's faeces

To collect faeces at the infant's age of one month, parents received a faeces tube with spoon attached to the lid (Sarstedt, Nümbrecht, Germany), together with a sanitary napkin, an instruction form and a brief questionnaire (faeces questionnaire). Parents collected a faecal sample by placing the sanitary napkin in the diaper (to prevent absorption of the faeces by the

diaper) and collect the faeces out of the napkin into the collection tube and sent it, together with the questionnaire, immediately to our laboratory by post. At the laboratory, faecal samples were tenfold diluted in peptone-water (Oxoid CM0009) containing 20% v/v glycerol (Merck, Damstadt, Germany) and stored at –20°C until analysis.

DNA purification from faeces

0.2 ml of the diluted faeces was added to a 2-ml vial containing approximately 300 mg glass beads (diameter 0.1 mm) and 1.4 ml of ASL-buffer from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The bacterial samples were disrupted in a mini-bead beater (Biospec Products, Bartlesville, Okla.) at 5,000 rpm for 3 min. Subsequently, the bacterial DNA was isolated from the samples using the QIAamp DNA Stool Mini Kit, according to the instructions of the manufacturer. The DNA was eluted in a final volume of 200 µl.

Denaturing Gradient Gel Electrophoresis

All primers used in the study are listed in Table 1. Primers 968-GC-f and 1401-r were used to amplify the V6 to V8 regions of bacterial 16S rRNA. *Bifidobacterium* genus-specific PCR was performed using 16S rDNA-targeted primers Bif-164-f and Bif-662-GC-r. A 40-bp GC-clamp was attached to the 5 ' end of primer 968-GC-f and Bif-662-GC-r (Table 1) in order to facilitate the analysis of the PCR products by DGGE.

PCR reactions were performed as described previously [19, 20], using the HotStarTaq polymerase kit from Qiagen (Hilden, Germany). The reaction mixtures contained 1x PCR buffer (Qiagen), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.25 U Taq polymerase and 5 µl of appropriately diluted template DNA in a final volume of 50 µl. The PCR thermocycling program with primers 968-GC-f and 1401-r was the following: 94 °C for 5 min; 35 cycles of 94 °C for 30s, 53 °C for 20 s, 68 °C for 40 s; and 68 °C for 7 min. The reactions were subsequently cooled to 5 °C. The annealing temperature was changed from 53 °C to 62 °C for primers Bif-164-f and Bif-662-GC-r.

Analysis of PCR amplicons by DGGE was performed essentially as described previously [19, 21], using the DCode detection system (Bio-rad, Hercules, Calif.). Polyacrylamide gels (8% w/v, acrylamide:bisacrylamide-37.5:1) in 0.5x TAE with a denaturing gradient were prepared with a gradient mixer and pump. The following denaturing gradients were used: 45-65% for bacterial and 45-55% for bifidobacterial PCR products. A 100% denaturant corresponds to 7 M urea and 40% (v/v) formamide. PCR amplicons were separated by electrophoresis at a constant voltage of 85 V and a temperature of 60°C for 16 h. The DNA fragments were visualised by $AgNO_3$ staining, as described previously [22]. Identification of bacterial species represented in

the bifidobacterial DGGE profiles was based upon comparison with an identification ladder consisting of control strains of *B. breve, B. bifidum, B. infantis, B. longum* and *B. adolescentis*. Identification of bands of interest in total bacterial profiles was done by elution of DNA fragments from the gels followed by sequencing [23]. Selected DGGE bands were cut out of the gel with a sterile scalpel followed by elution of the DNA by mechanically disrupting the gel slices and resuspension in 50 µl H_2O. Two microliter of the eluted DNA of each DGGE band was reamplified by using the conditions described above (using primers 968-GC-f and 1401-r) after which PCR products were analysed by DGGE, together with the amplicons of the original samples in order to verify that the correct bands were isolated. Upon confirmation, PCR products were purified and sequenced using primers 968-GC-f and 1401-r.

Quantification of bifidobacteria in faecal samples

For the enumeration of bifidobacteria and total bacterial counts, samples were subjected to a quantitative real-time PCR, as described previously [24]. Briefly, the 5 ' nuclease chemistry was used for the quantification of bifidobacteria with primers Bif-f and Bif-r and probe Bif-pr. The SYBR-Green chemistry was used for the determination of total bacterial counts using primers Total-f and Total-r.

TABLE 1

Primers and probes used in this study

Primer/probe	Sequence (5 '– 3)	Reference
968-GC-f	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-AACGCGAAGAACCTTA	[19]
1401-r	CGGTGTGTACAAGACCC	[19]
Bif-164-f	GGGTGGTAATGCCGGATG	[19, 20]
Bif-662-GC-r	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-CCACCGTTACACCGGGAA	[19, 20]
Bif-f	GCGTGCTTAACACATGCAAGTC	[24]
Bif-r	CACCCGTTTCCAGGAGCTATT	[24]
Bif-pr	TCACGCATTACTCACCCGTTCGCC	[24]
Total-f	TCCTACGGGAGGCAGCAGT	[35, 36]
Total-r	GGACTACCAGGGTATCTAATCCTGTT	[35, 36]

Statistical analysis

Total bacterial profiles were compared using the GelCompar II software (Applied Maths, St-Martens-Latem, Belgium). With this software Dice's similarity coefficients were calculated, following the equation:

$$S = [2a/(b+c)] \times 100\%$$

where b is the number of PCR-DGGE bands in subject 1, c is the number of PCR-DGGE bands in subject 2, and a is the number of matching PCR-DGGE bands [25]. Comparing the bacterial profile of each case with every other case created a matrix of similarity coefficients; therefore a mean similarity coefficient for cases could be calculated. This was also done for controls.

All the other statistical analyses were performed using SPSS for Windows, version 12.01. Demographic characteristics of cases and controls were compared using χ^2-tests.

The number of bands for each individual in the total bacterial profile (band richness) was calculated as an indicator of diversity of the total microbiota. The mean number of bands (band richness) in the total bacterial profiles was compared between cases and controls using the Student's t-test. Furthermore, bands (in total bacterial profiles) present in twenty or more infants were compared for their prevalence in cases and controls using the χ^2-test.

The Mann-Whitney rank sum-test was used to compare bifidobacterial counts (expressed as proportion of total bacterial counts) between cases and controls. To test for differences in the prevalence of specific bifidobacterial strains, as shown in the bifidobacterial profiles, the χ^2-test was used.

Results

Of the 646 infants who met the selection criteria (presence of faeces, capillary blood, full term birth and no history of antibiotic use in first month), 26 infants had both developed eczema in the first year of life along with specific IgE antibodies against peanut, hen's egg and/or cow's milk (> 1 IU/ml). All these infants were included as cases in the present study. Of the 375 infants who had not developed eczema in the first year and had no detectable specific IgE antibodies in their blood, 52 were randomly selected as controls.

There were no statistically significant differences between cases and controls in gender, infant feeding, birth season and mode of delivery (Table 2). However, the cases more often had an allergic mother (self-reported doctor's diagnosed eczema, hay fever, asthma, pet and/or house dust mite allergy)

compared to healthy infants (54% compared with 14%, *P* = 0.00) and also, although not statistically significant, tended to have an allergic father more often (46% compared to 27%, *P* = 0.09). To account for a potential confounding effect of parental atopic status, all following analyses were performed both unstratified and stratified for parental atopic status (at least one parent atopic vs. none parents atopic).

The number of bands in total bacterial profiles or band richness represents the number of bacterial species present in a faecal sample and thus the diversity of the microbiota. The band richness was similar in cases and controls (mean; 7.54 vs. 7.63 bands, *P* = 0.92). Also after stratification on parental atopic status (data not shown), no difference in mean band richness between cases and controls was found.

Comparison of total bacterial profiles showed that similarities between individuals were low, with mean similarity coefficients of 32.5% for cases and 33.7% for controls.

Five bands in total bacterial profiles were present in twenty or more infants; these bands were tested for difference in prevalence between cases and controls. One of these bands was present in all except one of the cases and in only 37 of 52 controls (96% vs. 71%, *P* = 0.01). This association persisted after adjusting for parental atopic status. This particular band had a similar position on gel as did the *Escherichia coli* control band from the identification ladder (Fig. 1). Excising and sequencing of this band from several lanes confirmed that it represented a fragment that was derived from the genome of *Escherichia coli* (Fig. 1). The prevalence of the other four bands was similar in cases and controls.

Bifidobacteria were detected in all infants and the proportion that bifidobacteria comprised of the total bacterial counts was not significantly different between cases (median 37.6%, range 0.07- >99%) and controls (median 56.9%, range 0.001- >99%, *P* = 0.66). The proportion of bifidobacteria of total bacterial counts remained similar between cases and controls in stratified analyses on parental atopic status.

The prevalence of the different bifidobacterial species, as determined by *bifidobacterium*-specific DGGE, is shown in Table 3. *B. breve* was most frequently detected (36/78 infants), followed by *B. bifidum* (32/78 infants). The prevalence of the different bifidobacterial species was similar between cases and controls (Table 3), and adjusting for parental atopic status did not change these results (data not shown).

TABLE 2

Characteristics of study population

	Cases	Controls	P-value
Boys	17/26 (65%)	24/52 (46%)	0.11
excl breast-fed	21/26 (81%)	37/52 (71%)	0.36
birth season	-	-	0.33
vaginal delivery	20/26 (77%)	44/52 (85%)	0.40
mother allergic	14/26 (54%)	7/52 (14%)	0.00
father allergic	12/26 (46%)	14/52 (27%)	0.09

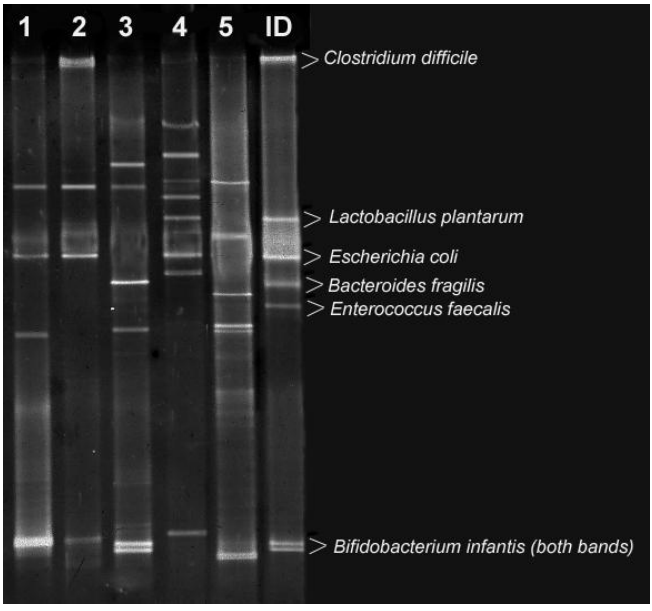
TABLE 3

The composition of the *Bifidobacterium* population in cases and controls

Bifidobacterium species	Cases	Controls	P-value
B. breve	15 (58%)	21 (40%)	0.15*
B. bifidum	9 (35%)	23 (44%)	0.42*
B. infantis/B. longum	3 (12%)	10 (19%)	0.53 †
B. adolescentis	0 (0%)	3 (6%)	0.55 †

*χ^2 test. †Fisher exact test.

FIGURE 1



An example of total bacterial profiles of faecal samples of one-month-old infants (1-5) and of an identification ladder (ID), as determined by denaturing gradient gel electrophoreses.

Samples 1, 2 and 4 have a band corresponding to *E. coli*.

Discussion

In the present study, PCR-DGGE was used to examine total bacterial profiles and the type and proportion of bifidobacteria in faecal samples of one-month-old infants in whom IgE-associated eczema was or was not going to develop within the first year of life. Neither total bacterial profiles nor the type and proportion of bifidobacteria in the samples were associated with the development of IgE-associated eczema. *E. coli* was however significantly more often detected in infants who were going to develop atopic eczema compared to healthy controls.

Matching may increase efficiency if matching on strong risk factors is employed. Valid estimates can, however, also be obtained by controlling for important confounders in the analyses. Instead of matching, we therefore chose to perform stratified analysis on parental atopic status, the only population characteristic under study which differed between cases and controls. We found that this stratification did not change the results.

It has been suggested that if differences in gut microbiota composition truly exist in early life between children who go on to develop atopic sensitisation compared with those who do not, it is possible that these differences are genetically determined [14]. As the association between *E. coli* and IgE-associated eczema in our study persisted after adjusting for parental atopic status, this explanation seems unlikely. Furthermore, as faecal sampling was conducted before the manifestation of atopic symptoms, we can conclude that differences in *E. coli* colonisation precede the development of IgE-associated eczema.

Kirjavainen and colleagues reported the number of *E. coli* in faeces to be positively correlated with total serum IgE levels in infants with atopic dermatitis [26]. Conversely, oral introduction of a non-enteropathogenic *E. coli* strain after birth has been shown to reduce the risk of developing allergic diseases in another study [27]. It seems therefore likely that the effects of *E. coli* are strain-dependent. Interestingly, administration of probiotic *E. coli* strains in the intestine has been shown to decrease the presence of pathogenic bacterial strains [28], and to enhance the barrier defense against some pathogenic *E. coli* strains [29].

It has been argued that the impact of the indigenous microbiota on the development of the infant's immune system may not be dependent on specific microbial strains, but that continuing stimulation by the dynamic microbiota as a whole might override the importance of any individual strain or infection [30]. For instance, a high turnover of appropriate bacteria may provide such a continuous immune stimulation necessary to prevent atopy and atopic diseases [31]. A lower diversity of the bacterial microbiota could be related to a limited bacterial turnover, suggesting to reduce the

stimulation of the immune system [32, 33]. This would imply that atopic infants have a less diverse microbiota compared with healthy infants. However, we did not find that cases had a lower diversity (fewer bands) than controls. Total bacterial profiles of both atopic and healthy infants under study comprised on average seven to eight different bands indicating a relatively low diversity of the dominant intestinal microbiota. Indeed, it is known that infants at this young age have a relatively simple microbiota [34].

Another way to examine if the intestinal microbiota as a whole is involved in the development of atopic diseases is by looking at the total bacterial profiles. If infants in whom IgE-associated eczema is going to develop would have a predisposing microbial make-up in common early in life, this would be expressed in a high similarity index. Mean similarity coefficients in both atopic and healthy infants were low (approximately 33% in both atopic and healthy infants) meaning that already at this age each individual has a fairly unique bacterial profile, and that we found no common bacterial profile among infants becoming atopic.

Although we did not find that the dominant microbiota as a whole (diversity and similarity) was associated with the development of IgE-associated eczema, it should be noted that using PCR-DGGE only dominant species (> 1% of total microbiota) can be visualized. Therefore, it cannot be excluded that differences exist in the number and profiles of minor species between infants in whom IgE-associated eczema was or was not going to develop.

Bifidobacteria have been associated with a lower risk of developing atopic diseases [3, 4, 6, 7]; furthermore different bifidobacterial species have been shown to exert different effects on the immune system [8, 9], and were differently associated with atopic eczema [10], but not with IgE-associated wheeze [14]. We found no association between the proportion bifidobacteria of total counts or specific bifidobacterial strains and IgE-associated eczema. The infant-type bifidobacteria (*B. breve* and *B. bifidum*) were most frequently detected, whereas the adult-type *B. adolescentis*, which has previously been linked to allergy [10], was only detected in three healthy infants in our study.

In conclusion, we did not find an association between the dominant gut microbiota as a whole and IgE-associated eczema. Already at this young age each infant appeared to have a unique, although still simple, bacterial profile. Also, no association was found between the number of bifidobacteria or the type of bifidobacterial species and the development of atopy. The development of IgE-associated eczema was however associated with colonisation by *E. coli*, indicating that differences in gut microbiota do precede the development of atopy. Larger differences may be found for those species not part of the dominant microbiota.

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Chapter 6

Gut microbiota composition and development of atopic manifestations in infancy: the KOALA Birth Cohort Study

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Chapter 6

Abstract

Perturbations in the intestinal microbiota composition, as a result of changed lifestyles, may be involved in the development of atopic diseases. We examined the gut microbiota composition in early infancy and the subsequent development of atopic manifestations and sensitisation.

Faeces of 957 one-month-old infants, participating in the KOALA Birth Cohort Study, were analyzed using quantitative real-time PCR. Information on the manifestation of atopic symptoms (eczema, wheeze) and potential confounders was retrieved through repeated questionnaires. Total and specific IgE were measured in venous blood samples collected during home visits at the infant's age of 2 years. During these home visits also a clinical diagnosis of atopic dermatitis was made according to the UK Working- Party criteria.

The presence of *E. coli* was associated with a higher risk of developing eczema (ORadjusted = 1.87; 95%CI 1.15-3.04), this risk being increased with increasing numbers of *E. coli* ($P_{for\ trend}$ = 0.016). Infants colonised with *C. difficile* were at higher risk of developing eczema (ORadjusted = 1.40; 95%CI 1.02-1.91), recurrent wheeze (ORadjusted = 1.73; 95%CI 1.08-2.77) and allergic sensitisation (ORadjusted = 1.54; 95%CI 1.02-2.31). Furthermore, the presence of *C. difficile* was also associated with a higher risk of a diagnosis of atopic dermatitis during the home visit (ORadjusted = 1.73; 95%CI 1.08-2.78).

In conclusion, this study demonstrates that differences in the gut microbiota composition precede the manifestation of atopy. Since *E. coli* was only associated with eczema, whereas *C. difficile* was associated with all atopic outcomes, the underlying mechanims explaining these association may be different.

Introduction

The prevalence of atopic manifestations has been increasing worldwide in the past decades, predominantly in the Western world and particularly amongst children.[1] An enhanced T helper 2 (Th2) immune response and the elaboration of cytokines such as interleukin (IL)-4, IL-13 and IL-5 contribute to the induction of atopic diseases.[2] Although genetic susceptibility plays an important role in atopy, changes in the prevalence of these diseases have been much faster than the genetic constitution of any population can possibly shift.[3] Therefore environmental changes associated with "western" lifestyles are thought to be involved in the atopic epidemic. In 1989 Strachan introduced the "hygiene hypothesis", which states that reduced exposure to infections during childhood results in aberrant immune responses to innocuous antigens later in life.[4, 5] An alternative interpretation of this hypothesis is however, that perturbations in the gastrointestinal microbiota composition as a result of changed lifestyles (antibiotic use, diet) in ´westernized countries´ have disrupted mechanisms that are involved in the development of immunological tolerance.[6] Regulatory antigen presenting cells (APCreg) and regulatory T cells (Treg) play a crucial role in the development of immunological tolerance. Maturation of these cells might be hampered as a result of reduced exposure to certain microbes ("old friends"); consequently a person may develop a T helper 1 (Th1), but also a Th2 mediated inflammatory disorder.[7]

Indeed differences in intestinal microbiota composition have been shown between infants in countries with high (Sweden) and low (Estonia) prevalences of allergy and also between allergic and healthy infants.[8-11] The majority of studies were based on small populations and although in most observational studies conducted so far an association between gut microbiota and allergy was found, no protective or potentially harmful bacteria can yet be identified.[12]

Further support for the role of the gut microbiota comes from several clinical trials using probiotics in the treatment [13-15] and prevention [16, 17] of atopic eczema, although not all studies have shown to be effective.[18, 19]

In a large prospective birth cohort study in the Netherlands, we examined the composition of the intestinal microbiota of nearly one thousand one-month-old infants and the subsequent development of atopic manifestation and/or sensitisation within the first two years of life.

Material and methods

Subjects and study design

The KOALA Birth Cohort Study is a prospective birth cohort in the Netherlands, aimed at identifying factors influencing atopic diseases. The design of the KOALA study has been described, in detail, elsewhere.[20] Briefly, from October 2000 until December 2002 we recruited pregnant women with diverse lifestyles at 34 weeks of gestation. Pregnant women with a conventional lifestyle (n=2343) were recruited from an ongoing prospective cohort study on Pregnancy-related Pelvic Girdle pain in the Netherlands.[21] Additionally, pregnant women with 'alternative lifestyles '(n=491) with regard to child rearing practices, dietary habits (organic, vegetarian), vaccination schemes and/or (restricted) use of medication, were recruited through organic food shops, anthroposophic doctors and midwifes, Steiner Schools, and magazines.

During pregnancy and early childhood data on perinatal determinants of the child's health as well as on hygiene, infections, nutrition, child rearing, other lifestyle characteristics and on atopic manifestations was collected for all members of the cohort by repeated questionnaires at 34 weeks of gestation and 3,7,12 and 24 months post-partum.

Participants recruited from January 2002 onwards (both conventional and alternative) were asked to consent to sample infant's faeces at the age of 1 month post-partum (n=1176). Subjects received a faeces tube with spoon attached to the lid (Sarstedt, Nümbrecht, Germany), together with a sanitary napkin, an instruction form and a brief questionnaire (faeces questionnaire). Parents collected a faecal sample by placing a sanitary napkin in the diaper (to prevent absorption of the faeces by the diaper) and collected the faeces out of the napkin into the collection tube and sent it immediately to our laboratory by post.

After exclusion of premature infants, infants who received antimicrobial agents during their first month of life, infants of whom insufficient amount of faeces (<1 gram) was collected, infants of whom faeces was not collected between the age of 3-6 weeks postpartum and infants for which the faeces questionnaire was missing, the study cohort comprised 957 infants.

Home-visits were made at the infant's age of 2 years (n = 607) by trained nurses. During these visits a clinical diagnosis of atopic dermatitis was determined using the UK-Working Party (UK-WP) criteria.[22-24] Furthermore venous blood of the infants was collected in order to determine total (n = 590) and specific (n = 583) serum immunoglobulin (Ig)E.

The KOALA-study was approved by the Ethical Committee of the University Hospital of Maastricht and all parents signed informed consent for the study.

DNA purification from faeces

At the laboratory faecal samples were tenfold diluted in peptone-water (Oxoid CM0009) containing 20% v/v glycerol (Merck, Damstadt, Germany) and stored at –20°C until analysis. For the DNA isolation, 0.2 ml of the diluted faeces was added to a 2-ml vial containing approximately 300 mg glass beads (diameter 0.1 mm) and 1.4 ml of ASL-buffer from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), then the samples were disrupted in a mini-bead beater (Biospec Products, Bartlesville, Okla.) at 5,000 rpm for 3 min. Subsequently, the bacterial DNA was isolated from the samples using the QIAamp DNA Stool Mini Kit, according to the instructions of the manufacturer. The DNA was eluted in a final volume of 200 µl.

Microbial analysis of faecal samples

Faecal samples were subjected to real-time PCRs for the enumeration of *Bifidobacterium spp.*, *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis*-group, *Lactobacillus spp.*, and total bacterial counts as described previously [25] (primers and probes are listed in Table 1).

The $\log_{10}$ colony forming units (CFU) per gram of the bacterial groups and species were calculated for each stool sample from the threshold cycle (C_t)-values using the constructed standard curves. The prevalence of colonisation was expressed as the percentage of infants colonised with a specific bacterial group or species.

Table 1

Primers and probes used in this study

Target organisms (amplicon size)	Primer/Probe	Sequence (5´-3´)	T_m (°C)	Ref.
Bifidobacterium spp	forward primer	GCGTGCTTAACACATGCAAGTC	59	[26]
(126 bp)	reverse primer	CACCCGTTTCCAGGAGCTATT	59	[26]
	probe	TCACGCATTACTCACCCGTTCGCC	70	[26]
E. coli	forward primer	CATGCCGCGTGTATGAAGAA	59	[27]
(96 bp)	reverse primer	CGGGTAACGTCAATGAGCAAA	59	[27]
	probe	TATTAACTTTACTCCCTTCCTCCCCGCTGAA	68	[27]
C. difficile	forward primer	TTGAGCGATTTACTTCGGTAAAGA	58	[26]
(114 bp)	reverse primer	TGTACTGGCTCACCTTTGATATTCA	59	[26]
	probe	CCACGCGTTACTCACCCGTCCG	69	[26]
B. fragilis-group	forward primer	CGGAGGATCCGAGCGTTA	58	[25]
(92 bp)	reverse primer	CCGCAAACTTTCACAACTGACTTA	59	[28]
	probe	CGCTCCCTTTAAACCCAATAAATCCGG	68	[25]
Lactobacillus spp.	forward primer	AGCAGTAGGGAATCTTCCA	59	[29, 30]
(341 bp)	reverse primer	CACCGCTACACATGGAG	59	[30, 31]
Total count.	forward primer	TCCTACGGGAGGCAGCAGT	59	[32]
(467 bp)	reverse primer	GGACTACCAGGGTATCTAATCCTGTT	58	[32]

Determination of total and specific IgE

Blood samples, collected during home visits at infant's age of two years, were analysed for total and specific serum IgE by Sanquin Research (Amsterdam). For total IgE levels, samples were analysed as described earlier.[33, 34] For values < 150 IU/ml a sandwich RIA was used,[34] for values >150 IU/ml a competitive RIA.[33] The detection limit for total serum IgE was <0.5 IU/ml. Blood samples were analysed for specific IgE against hen's egg, cow's milk, peanuts, birch, grass pollen, cat, dog and house dust mite using RAST as described previously.[33] Calculation was performed by means of a standard curve that was obtained by RAST with dilution series of a chimeric monoclonal IgE antibody against the major house dust mite allergen Der-p2 and Sepharose-coupled recombinant Der-p2.[35] A RAST value of >0.3 IU/ml was regarded as positive.

Definition of infant's atopic manifestations and sensitisation

Our definitions of eczema and wheeze were based on questions adapted from the ISAAC (International Study of Asthma and Allergies in childhood) questionnaires.[36] In the 7,12 and 24 months postpartum questionnaires, parents were asked: 'has your child ever had an itchy rash that was coming and going in the past months? 'If this question was answered affirmatively, infants were defined as having developed eczema in the first two years of life. Infants for whom only diaper rash, rash around the eyes and/or scalp scaling was reported, were not regarded as having developed eczema.

'Recurrent wheezing 'was defined as reported presence of wheezing with at least 4 attacks between 0-7 months, mentioned in the 7 months postpartum questionnaire and/or between 7-12 months of life, mentioned in the 12 months postpartum questionnaire and/or between 13-24 months, mentioned in the 24 months postpartum questionnaire.

Besides the parentally reported eczema, a diagnosis of atopic dermatitis could be made using the UK-WP criteria for those infants who were visited at home.[22-24] Briefly, the probability of the presence of atopic dermatitis was derived from the presence of four clinical symptoms: presence of itchy rash, (ii) history of flexural dermatitis, (iii) visible flexural dermatitis, and (iv) onset before age of 2 years. In this study, infants with a probability of AD > 0.9 were considered to have atopic dermatitis.

Infants with specific serum IgE-levels >0.3 IU/ml against one or more of the tested food or inhalant allergens were considered to be sensitised.

Definition of potential confounders

The following variables were included as potential confounders: subcohort (conventional; alternative), parental atopic history, defined as self-reported doctor's diagnosed eczema, hay fever, asthma, pet and/or house dust mite allergy (none; at least one parent), siblings atopic history, defined as

parental reported doctor's diagnosed food allergy, eczema, hay fever, asthma, pet and/or house dust mite allergy (no siblings; > 1 siblings, all none-atopic; or > 1 siblings, at least one atopic), age at collection of faecal sample (age in days), infant's gender (boy; girl). Maternal probiotic use during the last month of pregnancy (never/sporadic; several times a month; several times a week; daily), place and mode of delivery (vaginal delivery at home; vaginal delivery in hospital; artificial delivery in hospital; or caesarean section in hospital), type of infant feeding during first month (exclusively breastfed; exclusively formula-fed or combination) were included as a separate set of confounders. These latter variables may be more distal determinants in the association between the gut microbiota and atopic manifestations in stead of true confounders, and adjusted analyses were therefore performed with and without adjustment for these variables.

Statistical analyses

Logistic regression analyses were used to test for (unadjusted) associations between colonisation with the gut bacteria (colonised or uncolonised) under study and the development of atopic outcomes (eczema (parentally reported), atopic dermatitis (UK-WP criteria), recurrent wheeze and/or atopic sensitisation). Adjusted associations were tested by incorporating the potential confounders into the logistic regression models. Two different sets of confounders were used, the first set included the variables subcohort, parental and sibling's atopic history, age at collection of the faecal sample and infant's gender and the second set included these variables as well as maternal probiotic use during pregnancy, place and mode of delivery and type of infant feeding. The association between colonisation with bifidobacteria and atopic outcomes was analysed as 'low' (< 10.68 log10 CFU/g) vs. 'high' (> 10.68 log10 CFU/g) bifidobacterial counts since almost all infants were colonised; the few uncolonised infants (n = 12) were added to the low counts group. The cut-off point of 10.68 log10 CFU/g was chosen to create two equal groups.

Logistic regression analyses were also used to test for associations between the concentration (counts) of the gut bacteria and the atopic outcomes. Here we additionally adjusted for total bacterial counts to account for differences in the consistency of faecal samples in the analyses on bacterial numbers, besides the confounders as described above. To test for trend, bacterial counts were categorised (uncolonised infants were used as the reference category and the remaining colonised infants were accommodated in two (*C. difficile*, lactobacilli) or three equal groups (bifidobacteria, *E. coli*, *B. fragilis*-group)).

Linear regression analyses were used to test for associations between the gut bacteria under study and total serum IgE levels, controlling for the same confounders as mentioned above. Since separate analyses of the

conventional and alternative subcohort showed that the key findings were similar within these two subcohorts, in the final analyses we combined the two groups adjusting for 'subcohort'.

To examine the possibility of selection bias in those infants visited at home, we performed non-response analyses. Using logistic regression analyses, infants visited at home (n = 607) were compared with infants not visited at home (n = 305) regarding the gut microbiota composition and the prevalence of eczema and recurrent wheeze.

Results

Of the 957 infants participating in this study, almost all infants were colonised with bifidobacteria (98.7%) at the age of one month (Table 2). The majority of the infants were also colonised with *E. coli* (88.6%) and members of the *B. fragilis*-group (81.6%), whereas colonisation with lactobacilli (32.2%) and *C. difficile* (25.1%) was less common. Bifidobacteria were detected in highest numbers, followed by *E. coli* and *B. fragilis*-group species. More than half of the infants had at least one parent with a positive history of doctor's diagnosed atopic manifestations (Table 2). Almost one third of the infants who had older siblings, had an atopic sibling. Over thirty percent of the infants had developed eczema at the age of two years, whereas recurrent wheeze was reported in approximately ten percent of the infants. Over one fourth of the infants had circulating IgE antibodies (>0.3 IU/ml) in their blood against one or more of the food and/or inhalant allergens and thus were regarded to be sensitised.

Non-response analyses showed that there were neither differences in the gut microbiota composition nor in the prevalence of eczema and recurrent wheeze between infants who were visited at home and those who were not (data not shown).

Table 3 shows the adjusted association between colonisation with the gut bacteria under study at the age of one month and the development of atopic manifestations within the first two years of life. The risk of eczema was significantly higher in infants colonised with *E. coli* (ORadjusted=1.87; 95%CI 1.15-3.04) compared to infants not colonised with this bacterium in their intestines at the age of one month. For those infants visited at home we were also able to define atopic dermatitis according to the UK-WP criteria. However a higher risk for infants colonised with *E. coli* was not found when atopic dermatitis was defined according to these criteria (ORadjusted=1.02; 95%CI 0.49-2.10).

Table 2
Characteristics of the participants in this study

	Conventional subcohort N = 652[§]	Alternative subcohort N = 305[§]
Prevalence of colonisation with intestinal bacteria	%	%
Bifidobacteria	98.3	99.7
Escherichia coli	89.5	86.6
Clostridium difficile	24.7	25.9
Bacteroides fragilis-group	83.0	78.7
Lactobacilli	33.1	30.2
Counts of intestinal bacteria (log_{10} CFU/g)	Median (range)	Median (range)
Bifidobacteria	10.71 (6.84-11.56)	10.68 (6.85-11.49)
Escherichia coli	9.45 (5.91-10.79)	9.12 (5.92-10.62)
Clostridium difficile	5.12 (2.70-8.41)	5.70 (2.85-8.81)
Bacteroides fragilis-group	9.40 (5.74-10.36)	9.07 (5.79-10.33)
Lactobacilli	8.70 (7.92-10.73)	8.58 (7.95-10.33)
Total counts	11.15 (9.43-12.14)	11.08 (9.58-11.98)
Age at collection faecal sample (days)	Mean (sd) 31.60 (3.28)	Mean (sd) 31.75 (3.31)
Parental history of atopic manifestations	%	%
At least one parent with atopy	54.8	51.8
Sibling history of atopic manifestations	%	%
No siblings	43.7	32.8
$\geq$ 1 siblings, all non-atopic	40.5	46.2
$\geq$ 1 siblings, at least one atopic	15.6	21.0
Sex of infant (percentage boys)	49.4	53.4
Maternal probiotic use[‖]	%	%
Never/Sporadic	78.5	79.9
Several times a month	9.5	9.8
Several times a week	6.7	6.2
Daily	1.4	3.0
Place and mode of delivery	%	%
Natural delivery at home	44.0	53.8
Natural delivery in hospital	34.8	29.2
Artificial delivery in hospital[¶]	7.5	6.9
Caesarean section in hospital	10.7	9.5
Type of infant feeding	%	%
Exclusively breast-fed	58.3	87.9
Exclusively formula-fed	29.4	7.2
Combination	12.0	4.9
Infants atopic outcome at age 2 yr.	%	%
Eczema	32.8	31.7
Recurrent wheeze	12.8	6.3
Sensitisation[*]	29.9	25.0
Total IgE[**]	Median (range) 10.0 ($\leq$ 0.5-5300.0)	Median (range) 16.5 ($\leq$ 0.5-3700.0)

[§]Overall numbers are not always 652 for the conventional subcohort and 305 for the alternative subcohort due to missings in bacterial count data or outcome data.
[‖]consumed during the last month of pregnancy
[¶]forcipale or vacuum extraction
[*]only available for those infants visited at home, total numbers are 391 for conventional subcohort and 192 for alternative subcohort
[**]only available for those infants visited at home, total numbers are 396 for conventional subcohort and 194 for alternative subcohort

TABLE 3

Adjusted association between colonisation with gut bacteria at 1 month of age and atopic sensitisation and atopic disease manifestation at 2 years of age (N = 957)

		Eczema[†]		**Recurrent wheeze**[†]		**Atopic dermatitis (UK-WP)**[‡]		**Sensitisation**[§]	
		Prevalence % (n/N[‖])	OR_{adj} (95%CI)[¶]	Prevalence % (n/N[‖])	OR_{adj} (95%CI)[¶]	Prevalence % (n/N[‖])	OR_{adj} (95%CI)[¶]	Prevalence % (n/N[‖])	OR_{adj} (95%CI)[¶]
Intestinal bacteria									
Bifidobacteria[††]	Low	32.4% (148/457)	1.0	9.4% (41/434)	1.0	17.9% (54/302)	1.0	26.5% (76/287)	1.0
	High	32.5% (156/480)	1.02 (0.77-1.35)	11.8% (55/466)	1.32 (0.85-2.06)	14.4% (44/305)	0.79 (0.51-1.23)	30.1% (89/296)	1.23 (0.85-1.77)
E. coli	No	22.2% (24/108)	1.0	5.8% (6/104)	1.0	15.9% (10/63)	1.0	30.6% (19/62)	1.0
	Yes	33.8% (279/826)	1.87* (1.15-3.04)	11.1% (88/793)	1.92 (0.80-4.59)	16.2% (88/542)	1.02 (0.49-2.10)	28.1% (146/519)	0.86 (0.48-1.54)
C. difficile	No	30.3 % (213/702)	1.0	9.4% (63/671)	1.0	14.1% (64/454)	1.0	26.1% (115/440)	1.0
	Yes	38.7% (91/235)	1.40* (1.02-1.91)	14.4% (33/229)	1.75* (1.09-2.80)	22.2% (34/153)	1.73* (1.08-2.78)	35.0% (50/143)	1.54* (1.02-2.31)
B. fragilis-group	No	32.0% (55/172)	1.0	9.0% (15/166)	1.0	12.5% (14/112)	1.0	29.9% (32/107)	1.0
	Yes	32.5% (249/765)	1.02 (0.71-1.47)	11.0% (81/734)	1.20 (0.66-2.18)	17.0% (84/495)	1.41 (0.76-2.60)	27.9% (133/476)	0.90 (0.57-1.43)
Lactobacilli	No	31.2% (199/637)	1.0	10.3% (63/613)	1.0	15.7% (64/408)	1.0	28.0% (110/393)	1.0
	Yes	35.0% (105/300)	1.23 (0.91-1.65)	11.5% (33/287)	1.22 (0.77-1.93)	17.1% (34/199)	1.14 (0.72-1.81)	28.9% (55/190)	1.04 (0.71-1.53)

[†]Based on parental reports in 7, 12 and/or 24 months questionnaires

[‡]As determined by trained nurses during home-visits

[§]specific IgE antibodies to at least one allergen (cow's milk, henn's egg, peanut, birch, gras pollen, cat, dog, house dust mite)

[‖]Numbers in table do not always add up to 957 due to missing bacterial count data or outcome data.

[¶]From logistic regression analysis: adjusted for subcohort, parental history of atopy, siblings history of atopy, age at collection faecal sample, and infant's gender.

Infants colonised with *C. difficile* were also at higher risk of eczema (ORadjusted=1.40; 95%CI 1.02-1.91) compared to uncolonised infants. This association was even stronger for atopic dermatitis according to the UK-WP criteria (ORadjusted=1.73; 95%CI 1.08-2.78). Colonisation with *C. difficile* was furthermore associated with a higher risk of developing recurrent wheeze (ORadjusted=1.75; 95%CI 1.09-2.80) and atopic sensitisation (ORadjusted=1.54; 95%CI 1.02-2.31).

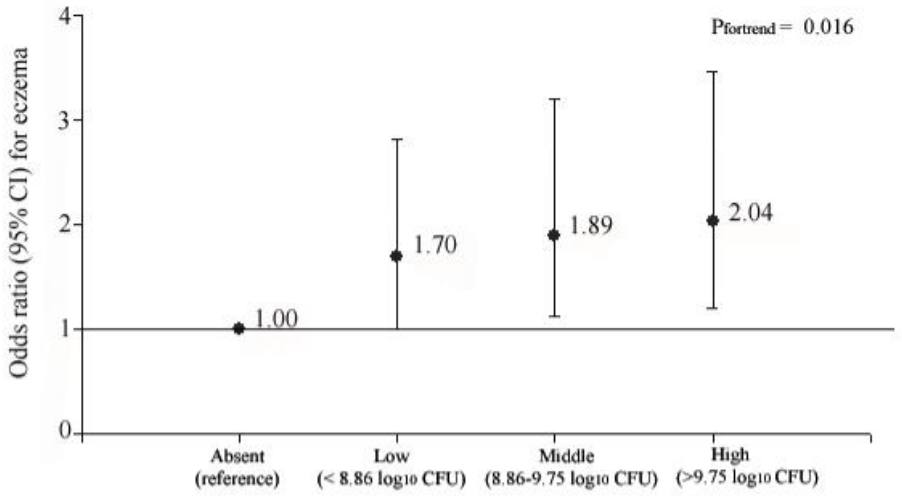
Colonisation with bifidobacteria, *B. fragilis*-group species and lactobacilli was not associated with any of the atopic outcomes. Results of the unadjusted analyses (data not shown) were comparable with the adjusted analyses. Also the results of the analyses in which we additionally adjusted for maternal probiotic use during pregnancy, place and mode of delivery and type of infant feeding (data not shown) were comparable with the results as presented in Table 3.

We subsequently analysed the association between the counts of the bacteria under study and the atopic outcomes. Both unadjusted (data not shown) and adjusted analysis revealed that the risk of developing eczema increased with increasing numbers of *E. coli* in the faecal samples ($P_{for\ trend}$ = 0.016) (Figure 1).

Infants with high *E. coli* counts in their stools had a twofold higher risk of developing eczema compared with uncolonised infants. Again this association was not found when atopic dermatis was defined according to the UK-WP criteria. The association between *C. difficile* colonisation and eczema, atopic dermatitis, recurrent wheeze and atopic sensitisation was not influenced by the concentration of this bacterium in the faecal samples. Furthermore counts of bifidobacteria, *B. fragilis*-group species and lactobacilli were neither associated with atopic disease manifestation nor with sensitisation.

Total serum IgE level at the age of two years was neither associated with colonisation rates nor with counts of gut bacteria.

Figure 1



Adjusted association between numbers of *Escherichia coli* (log_{10} CFU / g faeces) in faecal samples of one-month-old infants and the development of eczema in the first two years of life. Adjusted for subcohort, parental history of atopy, siblings history of atopy, age at collection faecal sample, and infant's gender and total bacterial count. Dots represent the Odds ratios, bars represent the 95% Confidence Intervals.

Discussion

This prospective study demonstrates that differences in the gut microbiota composition in early infancy are associated with the subsequent development of atopic disease manifestation and sensitisation. The presence of *E. coli* was associated with a higher risk of developing eczema, this risk being increased with increasing numbers of *E. coli.* This association was however not found when atopic dermatitis was defined according to the UK-WP criteria. The colonisation with *C. difficile* in early infancy was associated with an increased risk of all atopic outcomes (eczema, atopic dermatitis, recurrent wheeze and atopic sensitisation), independent of the concentration of this bacterium.

This is the first large-scale prospective study on the gut microbiota composition in relation to atopic manifestations. Although previous epidemiological studies were the instigators of a potential role of the gut microbiota in the aetiology of atopic diseases and have gained a lot of insight,

these studies were often based on small populations, not able to adjust for potential confounders. Furthermore only two previous studies [9, 11] were prospective, thereby proficient to determine if differences in the gut microbiota precede the development of atopic symptoms. Another strength of our study is the molecular techniques used, overcoming many of the problems associated with traditional bacteriological culture. Analyses of the gut microbiota using bacteriological culture are biased, since many selective culture media are not absolutely selective. Furthermore, these media do not equally support the growth of different species comprising a population, and far from all bacteria are cultivatable.[37, 38] The real-time PCR assays used in the present study are a quantitative culture-independent approach suitable for high-throughput analyses of both fresh and frozen samples.

At present faeces is the only realistic sample in large non-invasive epidemiological studies on the gut microbiota. However a limitation of using faecal samples to study the gut microbiota is that the bacterial composition in the lumen does not reflect the composition of bacteria adhering to the mucosa, and the composition of bacteria differs furthermore throughout the intestinal tract.[39] Nevertheless it can be assumed that while the proportions and activities of the microbiota change with passage through the intestinal tract, most viable and nonviable intestinal bacteria will still be detectable in the faeces by molecular methods.[40] A drawback of this study is the time between collection of the samples by the parents and processing of the samples in the laboratory, which was approximately one day. Ott and colleagues [41] demonstrated that the total amount of bacterial DNA as well as the diversity of the microbiota significantly decreased over such a time-span. However they also showed that the similarity (determined by DGGE) of faecal samples directly processed and processed after 24 hours remained high. This means that the dominant microbiota appears to be relatively stable. Furthermore the aim of the present population-based study was to examine differences in gut microbiota composition between subjects. It is not likely that the possible change in composition of the samples during transport was different for infants who developed atopic manifestations later on in life and infants who did not.

In the present study, only one faecal sample per infant was collected at an age of one month postpartum. There are several reasons why we chose to collect faeces at this age. First of all the gut microbiota is thought to drive the postnatal development of the immune system,[12] therefore the first months of life seem to be of major importance. Second, at the age of one month colonisation is complete and although the composition may fluctuate, large shifts in the composition do not occur until weaning.[42] Finally, we wanted to exclude the chance of reverse causation, therefore the gut microbiota composition had to be analysed prior to the manifestation of atopic symptoms.

Since manifestation of atopic symptoms and sensitisation to allergens do not always go together (sensitised infants do not always show symptoms and infants with symptoms are not always sensitised),[43] we chose to report the manifestation of atopic symptoms and sensitisation as separate outcome parameters. As a consequence we decided to use the term eczema instead of atopic eczema or atopic dermatitis. We however did use the term atopic dermatitis for those infants visited at home and fulfilling the UK-WP criteria, since Williams and colleagues when introducing these criteria originally used the term atopic dermatitis.[22]

The positive association we found between *E. coli* and eczema is difficult to compare with previous studies, since several previous studies did not determine *E. coli* [9] or measured this bacterium as part of total coliforms,[8] enterobacteriaceaea [10] or gram-negative rods.[11]

In contrast to the association between *E. coli* and eczema (based on parental reports in questionnaires), we did not find an association between *E. coli* and atopic dermatitis based on the UK-WP criteria. Selection bias could not explain this difference since non-response analyses showed that the gut microbiota and the prevalence of eczema and recurrent wheeze was similar for those infants who were visited at home and those who were not. The percentage of infants with atopic dermatitis according to the UK-WP criteria is much lower than the percentage of infants with parentally reported eczema. This can be explained by the fact that parentally reported eczema is based upon the presence of this condition somewhere during the infants first two years of life, whereas a large number of these infants are probably in remission or have already outgrown this condition at time of the home-visit. Therefore it is possible that *Escherichia coli* is only associated with a milder eczematous condition that has already disappeared at the time of the home-visit. Another explanation for these discrepant findings is that the UK-WP criteria included specific predilection sites of atopic dermatitis (flexural involvement),[22-24] whereas the questionnaire data were based on the presence of an itchy rash anywhere (except diaper rash, rash around eyes and scalp scaling). The increased risk of eczema in infants colonised with *E. coli* may therefore also be limited to eczema other than the specific predilection sites for atopic eczema/dermatitis and thus may be non-atopic. This idea is supported by the fact that we also found no association between *E. coli* and sensitisation.

By contrast, *C. difficile* appears to be associated with a higher risk of atopic eczema since a positive association was also found for atopic dermatitis according to UK-WP and for sensitisation. Our findings of an association between *C. difficile* and atopy are in agreement with several previous studies. In a study on microflora-associated characteristics, allergic infants had higher levels of i-caproic acid in their stools compared to non-allergic infants. This short chain fatty acid is suggested to indicate the presence of *C. difficile.*[44]

Two studies used IgG serology against *C. difficile.* Woodcock and colleagues found increased specific IgG against *C. difficile* in sensitised wheezy infants compared to non-sensitised non-wheezy infants.[45] Linneberg and colleagues found a positive association between IgG seropositivity against *C. diffcile* and both allergic rhinitis and sensitisation.[46]

Furthermore several studies found an association between high numbers of the genus Clostridium and atopic dermatitis and/or sensitisation,[9, 11, 47] whereas others did not find such an association.[10, 48] The genus clostridium is a very heterogeneous group comprising several different clusters.[49] It seems therefore unlikely that the members of such a phyologenetically diverse genus like clostridium all have the same effect on the human host. It is more likely that certain species (such as *C. difficile*) or a certain cluster of species within this genus is responsible for the increased risk of developing atopic manifestations.

In contrast to several other studies [8-11, 47] we did not find a negative association between allergies and bifidobacteria. A possible explanation is the lack of contrast in our study with respect to bifidobacterial counts, because almost all infants were colonised with high numbers of bifidobacteria. This is probably the consequence of the very young age of our population at which bifidobacteria are known to dominate the gut microbiota.[50]

Altogether our results support a role of the gut microbiota in the aetiology of atopic diseases. There are several hypotheses by which the associations we found between *C. difficile* and *E. coli* and atopic manifestations could be explained. First of all it should be noted that many of the bacteria in the gut are still unknown; differences in *E. coli* and *C. difficile* colonisation as found in our study could therefore also reflect differences in other unknown bacteria.[9]

The presence of *E. coli* and *C. difficile* could be associated with a decrease of other (unknown) health benificial bacteria. This could result in reduced induction of Treg cells by these health beneficial bacteria leading to immune dysregulation. In the absence of optimal levels of immune regulation, an individual may develop an Th1 (such as Crohn ŝ disease or autoimmunity) or Th2 (such as atopic diseases) mediated inflammatory disorder depending on his/her own Th1/Th2 bias, immunological history and genetic background.[7] Secondly, *E. coli* and *C. difficile* could have a direct effect on the production of cytokines by antigen-presenting cells, thereby affecting the differentiation of T cells [51] Another hypothesis is that *E. coli* and/or *C. difficile* increase the intestinal permeability (for instance by the production of toxins). This increased permeability of the intestinal barrier could facilitate the penetration of innocuous antigens and subsequent sensitisation.[46] Indeed it has been shown that *C. difficile* toxins A and B compromise the intestinal cell barrier.[52, 53] Furthermore, increased intestinal permeability has been described in patients with food allergies, eczema and asthma compared to

healthy subjects.[54-57] Finally, it has also been suggested that infants susceptible for the development of allergies are also susceptible for an aberrant colonisation pattern of the gut. However, this explanations seems less likely since we have controlled for familial history of atopy. Additionally, the fact that differences in the gut microbiota are already present at such a young age preceding atopic symptoms makes this hypothesis less likely.

The consistent findings of a positive association between *C. difficile* and all atopic symptoms as well as sensitisation strengthen the probability of a causal relationship between the gut microbiota and atopy, and are supportive for the potential role of probiotics in the prevention and treatment of these diseases. Perturbations in the gut microbiota may as well be related to other atopic outcomes which manifest at older ages such as asthma, rhinoconjunctivitis and persistent food allergies, long term follow up of cohort studies is necessary to examine if these perturbations are also related to these outcomes.

In conclusion, we demonstrated that differences in the gut microbiota composition precede the manifestation of atopic symptoms and atopic sensitisation. Especially *C. difficile* was associated with all atopic symptoms and sensitisation, whereas *E. coli* appeared to be only associated with (non-atopic) eczema. Different immunological mechanisms may underlie the effects of *E. coli* and *C. difficile*. This calls for further research on the mechanisms by which intestinal microbes interfere with our (gastrointestinal) immune system.

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Chapter 7

General discussion

Chapter 7

Introduction

The causes for the increasing prevalence of allergic diseases over the last few decades in the Westernized world remain largely unknown. It is generally believed that the causes of this increase have to be sought in changes in the environment. Plausible causative factors should be able to influence the immune system (biological plausibility) and should be distributed in such a way that the reported differences can explain the differences in the occurrence of atopic diseases between populations [1].

As outlined in Chapter 2, the gut microbiota drives the maturation of the immune system and perturbations in the composition of the gut microbiota are therefore a biological plausible explanation.

There is little direct information about changes in the composition of the gut microbiota during the last decades. Differences in methodology hinder direct comparisons between recent studies and studies from previous decades.

There is however at least one study, in which alterations in the infant gut microbiota have been examined over time [2]. In this German study, concerning alterations in the infant microbiota between 1958 and 1978, a decrease in numbers of bifidobacteria and an increase in numbers of *E. coli* were reported, while numbers of bacteroides remained unaffected.

Further, indirect evidence comes from several studies showing differences in the gut microbiota composition between countries with a different degree of westernization [3-5].

As we have shown in Chapter 4, among the most important environmental determinants of the gut microbiota composition in infants were the mode of delivery, type of infant feeding, and antibiotic use by the infant. Vaginally home-born term infants, who were exclusively breastfed and did not receive antibiotics, seem to have the most "beneficial" gut microbiota (highest numbers of bifidobacteria and lowest numbers of *C. difficile* and *E. coli*). If changes in these factors have occurred during the past decades, this will

most likely have also resulted in changes in the gut microbiota composition of infants. Of these determinants changes in the mode and place of delivery is the most striking. In the Netherlands, the number of infants born by caesarean section has tripled in the last 20 years from 4.5% to 14%, while the number of infants born at home has decreased (Statistics Netherlands). Furthermore, increased hygienic procedures during delivery have limited the faecal contamination of the vaginally born baby. Also the presence of faecal bacteria in the environment appears to have diminished today [6]. It thus seems likely that changes in the gut microbiota composition have occurred along with changes in lifestyle and hygiene.

Methodological considerations

Validity of measurements of the gut microbiota

The use of molecular techniques in the analysis of the microbial composition of environmental samples has rapidly evolved since the end of the previous century and led to a whole new specialism, known as molecular microbial ecology [7]. At the start of our study quantitative real-time PCR was only applied for the detection and quantification of pathogens, meanwhile several papers have been published describing the application of this technique to quantify commensal gut bacteria [8-20]. The use of molecular techniques to examine the gut microbiota is one of the major strengths of our studies. In fact, this is the first large-scale prospective birth cohort study in which molecular approaches has been used to examine the microbial composition of a considerable number of faecal samples. In Chapter 3 it is shown that the real-time PCR assays had a high sensitivity and specificity, were reproducible and not susceptible to inhibition. Furthermore the ability to perform high-throughput analyses proved their suitability in epidemiological studies. The field of molecular microbial ecology is, however, in its early youth and there are still uncertainties, which have to be considered. Furthermore, it is still a matter of debate whether the microbial composition of faeces is representative for the composition of the gut microbiota.

Using faecal samples to study the gut microbiota

Due to relative ease of stool collection most of the intestinal microbiota studies, including our study, have been performed under the assumption that faeces contain a representative sample of the prevalent intestinal microbiota. Although some studies have shown clear differences between the faecal microbiota composition and the kinds of bacteria that are present at other anatomical sites, including bacteria in the cecum [21] and those associated with the mucosa [22], differences in the human microbiota at various

anatomical sites are not well documented. It can be assumed that while the proportions and activities of the microbiota change with passage through the intestinal tract, most viable as well as nonviable commensal intestinal bacteria will still be detectable in faeces with molecular methods. However it cannot unequivocally be established that analyzing microbiota composition in faeces is representative of important intestinal parameters. Therefore using faeces can be a limitation of studies on the gut microbiota. Yet at this point faeces remain the only realistic sample in large non-invasive studies [23].

Collection and processing of the samples

The faecal samples were collected by the parents and sent to the laboratory by mail. The parents received a detailed instruction on how to collect the faecal samples in order to minimize bias and transport time.

The collection procedure and sample handling has far less impact on the composition of the samples when studied by molecular methods compared to the use of traditional techniques, since non-viable bacteria will also be detected. Yet degrading of bacterial DNA, as well as the potential overgrowth of some bacterial species and suppression of others, during transport could have biased the results [24]. Ott and colleagues showed that keeping faeces at room temperature resulted in loss of microbial diversity, especially due to the loss of minor species. Furthermore storage increased the similarity of the microbial composition between individuals, and thus decreased the inter-individual contrast. Such a loss in contrast between individuals tends to produce estimates closer to the no-effect value than the actual effect and does not exaggerate the effect of the gut microbiota.

The extraction of DNA can also introduce bias as different bacteria are more or less susceptible to lysis, with gram-positive bacteria being particularly resistant [25]. We have, however, minimized the potential of bias due to DNA extraction, by using both a mechanical and chemical lysis-step successively.

Age of the infants at the time of faecal collection

In the KOALA Study we collected faecal samples at the infants 'age of one month. The choice for this age was based upon the assumption that the influence of the gut microbiota on the maturation of the immune system is most prominent in early infancy [26]. Furthermore at the age of one month the composition is more stable than during the first weeks of life, therefore stool-to-stool variation is less of a concern [27]. Finally, we wanted to exclude the chance of reverse causation, therefore the gut microbiota composition had to be sampled prior to the manifestation of atopic symptoms. However much is still unknown about the exact critical window period in which the gut microbiota may play a role in the aetiology of atopic diseases. The collection of faecal samples at only one age, as in our study,

introduces the risk that a potential health effect of the gut microbiota is missed simply because the wrong age-period is studied.

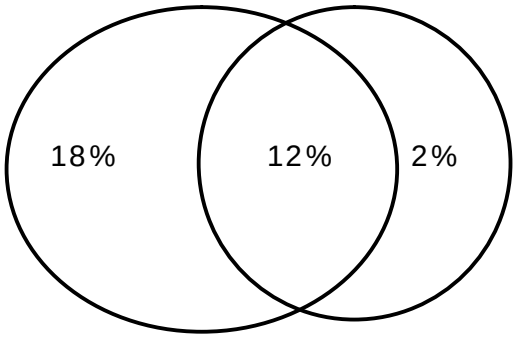
Validity of measurements of atopic manifestations

Reliable diagnostic criteria for eczema and wheeze are essential in order to identify possible disease risk factors. The definition of eczema in the KOALA Study was based on questions adapted from ISAAC questionnaires and included generally accepted characteristics, like a chronically relapsing course and an itchy rash. The definition of recurrent wheeze was also based on the ISAAC questionnaires. Atopic outcomes like infant eczema and wheeze may be fairly unspecific for infants aged 2 years, and therefore, especially when parentally reported, subject to misclassification. In Chapter 6, we used the U.K. Working Party criteria for the definition of `atopic dermatitis´, along with the questionnaire-based diagnosis of eczema. The strength of the U.K. criteria is that visual flexural dermatitis, based upon the clinical examination of a trained nurse, is incorporated (higher specificity). A drawback of these criteria is, however, that infants who have outgrown their disease or who are in remission may not get diagnosed, because of the absence of flexural dermatitis at the time of the clinical examination (lower sensitivity). Both the higher specificity and lower sensitivity of the U.K. criteria are responsible for the lower prevalence of AD compared to eczema as reported by parents (Figure 1).

Thus, the questionnaire-based definition of eczema has probably resulted in an overestimation of cases, whereas the U.K. criteria might have underestimated the number of cases of infants 'eczema in the first two years of life.

It can be assumed that the misclassification of the outcome status is independent of the gut microbiota composition and thus non-differential. Non-differential misclassification is far less problematic than differential misclassification, as it almost always tends to underestimate the actual effect and does not overestimate the effect of the studied exposure.

Figure 1



Venn diagram showing the proportion of infants with eczema (parentally reported in questionnaires), AD (according to U.K. criteria) or both in the KOALA study.
Subset areas presented above are not proportional to the actual relative subset size.

Of those infants with a diagnosis of atopic dermatitis (AD) according to the U.K. criteria, 44% had specific IgE levels to at least on of the tested allergens at the age of two years, compared to 25% of the infants without AD (Odds ratio (OR) 2.33 95% Confidence interval (CI) 1.54.3.53). 40% of infants with parentally reported eczema and 22% of infants without eczema were sensitised (OR 2.44 95% CI 1.76-3.38). By consequence, both definitions of childhood eczema had almost a similar strength of association with sensitisation. These associations were furthermore consistent with correlations between eczema and sensitisation as reported in other population-based studies [28].

Study design and potential sources of bias

The prospective study design enabled us to examine whether differences in the gut microbiota actually precede the development of atopic manifestations. Most studies so far were cross-sectional of design, so that they could not exclude the possibility of reverse causation. Our study has the advantage of being population-based, with relatively short intervals between subsequent follow-up questionnaires, which decreases the possibility of recall bias.

Follow-up rates were very high: of those infants of whom faeces was collected less then 5% were lost to follow-up by the age of two years, which limited differential bias with regard to baseline characteristics or outcomes.
As in most studies on the aetiology of atopy, families with allergic complaints are over-represented in the KOALA Study. This phenomenon is probably due to self-selection, because people with a positive family history of allergic disease will tend to be more interested in participating in a study on health-related topics [29]. However, it seems unlikely that the association between the gut microbiota composition and atopic diseases would be different in those who participated and those who did not.

Determinants of the infant gut microbiota

The most important determinants of the gut microbiota composition in infants were found to be the mode of delivery, type of infant feeding, gestational age, hospitalisation and antibiotic use of the infant. These determinants can serve as keystones to modify the gut microbiota when necessary.
Infants born through caesarean section miss the natural colonisation by their mother's vaginal and faecal bacteria. In this situation the (hospital) environment becomes the most crucial source of colonizing bacteria [27]. This results in a delay in faecal colonisation with low numbers of bifidobacteria and bacteroides and high colonisation rates of *C. difficile*. One may speculate that the best way to accelerate the faecal colonisation of the gut of infants born through caesarean section is to somehow expose them to their mother's vaginal and faecal microbiota.
Besides all other beneficial effects of breastfeeding, it is also the best type of infant feeding with respect to the composition of the gut microbiota. During the last two decades, many attempts have been made to mimic the intestinal microbiota of breastfed infants in formula fed infants. As a result differences in the gut microbiota composition between these two groups seem to be less dramatic as in former times [30]. Especially the supplementation of formulas with oligosaccharides (prebiotic fibres) has proven to be successful in increasing the numbers of lactic acid bacteria in formula fed infants [31, 32] and this supplementation is currently implemented by many manufacturers. It is however still unclear, whether the prevalence and counts of potential pathogens, such as *C. difficile* or *Staphylococcus aureus* are completely reduced to a level comparable to that in breastfed infants [33]. This is of particular interest, since the higher number of potential pathogens in the gut microbiota of formula-fed infants may be partly responsible for the higher risk of enteral infections in these infants [34]. It is thus obvious that one of the goals for improvement of current infant formulas is to achieve an

intestinal microbiota with formula feeding that is identical with that in breastfed infants [35].
The disruption of the gut microbiota caused by antibiotic therapy may be especially unfavourable for young children in which the gut microbiota is still evolving. Probiotics may assist in re-establishing the disrupted intestinal microbiota caused by antibiotic treatment. Indeed several studies have shown that supplementation with probiotic bacteria during and post antibiotic therapy reduces the extent of disruption to the intestinal microbiota [36, 37] and that probiotics show promise as effective therapies for antibiotic associated diarrhoea (AAD) [38-40].

The gut microbiota and atopy

In this thesis it has been shown that the composition of the gut microbiota is associated with atopic manifestations and sensitisation. We report a positive association between *E. coli* and eczema and a positive association between *C. difficile* and eczema, recurrent wheeze, sensitisation and atopic dermatitis (according to the U.K. Working Party). By conducting a longitudinal study, we were able to show that differences in the gut microbiota precede the development of atopic manifestations. This correct temporal relationship strengthens the evidence for a causal relationship [41]. The dose-response relationship between *E. coli* and eczema, as reported in Chapter 6, also strengthens the evidence for a causal relationship. Furthermore an association between *E. coli* and eczema was found in both the study using DGGE to quantify the gut microbiota of selected cases and controls (Chapter 5), and the study using real-time PCR to quantify the gut microbiota of the complete study population (Chapter 6).
With respect to *C. difficile*, the consistent findings of a postive association between this bacterium and all atopic manifestations, including sensitisation, strengthen the evidence for a causal relationship. (Chapter 6). Since *C. difficile* is only present in relatively low numbers in the faecal microbiota of colonised infants, whereas only the dominant microbiota is visualized by DGGE analysis, it is not surprising that an association between this bacterium and atopic eczema was not found when using DGGE (Chapter 5).
The biological mechanisms that can explain these associations are diverse: a marker effect for other bacteria associated with atopy [42], a direct effect on the cytokine production [43], or an increased intestinal permeability [44] all seem plausible explanations.
In contrast to several other studies [42, 45-48] we did not find a negative association between atopic diseases and bifidobacteria, nor did we find differences in the bifidobacterial species detected in the faeces of healthy and

atopic infants. A possible explanation is the lack of contrast in our study with respect to bifidobacterial counts, because almost all infants were colonised with high numbers of bifidobacteria. This is probably the consequence of the very young age of our population at which bifidobacteria are known to dominate the gut microbiota. [27]. Furtermore the adult-type *B. adolescentis*, which has previously been linked to allergy [49], was rarely detected in our study.

In conclusion, the gut microbiota is a biological plausible etiological factor in the aetiology of atopic diseases, giving its ability to drive the development of the immune system. Furthermore it seems likely that the infant gut microbiota composition has changed during recent decades. Our data, along with data from most of the previous studies support a role of the gut microbiota in the development of atopic diseases. Inconsistencies however exist between studies regarding which bacteria are relevant. A potential protective effect of (certain species of) bifidobacteria and a risk promoting effect of clostridia (especially *C. difficile*) are the most consistent findings between studies.

Would this mean that perturbations in the gut microbiota composition are able to account for the existing epidemiological data?

The answer probably has to be no. The aetiology of atopic diseases is likely to be multifactorial [50] and perturbations in the gut microbiota alone will thus not be able to explain the increased prevalence. Yet the gut microbiota is an interesting and feasible target for intervention. Clinical trials using priobiotcs in the treatment and prevention of atopic diseases have already shown promising results, but are far from conclusive at this moment. At this time our understanding of the immunological and biological mechanisms of probiotic actions is still limited [51]. If we get a better comprehension on how bacteria and their products educate our immune system perhaps we could learn to mimic its action without giving up our hygienic lifestyle. This concept of safe immune-education by the use of microbial products, aimed at facilitating harmonious development and maintainance of the immune system, may one day become normal practice not only for protection against infectious diseases, but also for prevention of immune-mediated diseases, including atopic diseases [52].

Recommendations for future research

More large-scale longitudinal studies, in which the role of the gut microbiota in the aetiology of atopic diseases is studies, are needed. The use of molecular techniques is inevitable in such studies, one of the reasons being that frozen samples can be used. Since the exact window of opportunity in which the gut microbiota may play a role in the development of atopic

diseases is unknown, the collection of faecal samples at different ages can give more insight in this issue.
Long-term follow-up of studies like ours is furthermore necessary to relate the gut microbiota composition to other, more specific, atopic manifestations like allergic asthma or rhinoconjunctivitis.
The promising findings on the role of the gut microbiota in the aetiology of atopic diseases, also ask for more studies on the therapeutic and preventive effect of probiotics. The selection of probiotic strains is fundamental for the potential success of these studies. If upcoming observational studies confirm our findings of a positive associations between *C. difficile* and/or *E. coli* and atopic diseases, probiotic studies should also consider the inhibiting effect on these bacteria and not only the induction of anti-inflammatory cytokines when selecting probiotic strains.
As mentioned in Chapter 1, environmental factors are thought to be responsible for the rise in the prevalence of atopic diseases. However, the development of atopic diseases depends not only on environmental but also on genetic factors and it is likely to be an interaction of these, particularly in early life, which determines the atopic status of a person [53, 54] . The effects of certain microbes on the development of atopy may therefore differ according to the genetic susceptibility of an individual. Gene-environment interaction studies are therefore the next step in studying the aetiology of atopic diseases. Candidate genes are those genes encoding for pattern recognition receptors on cells of the innate immune system. Examples of such receptors are toll-like receptor (TLR) 4 and CD14 involved in Lipopolysaccharide signalling, TLR2 involved in the signalling complex to a wide range of microbial components, and the caspase recruitment domain protein (CARD) 4 interacting with muropeptides [55, 56]. For example, in two independent populations, a polymorphism in the toll-like receptor (TLR) 4 gene that disrupts TLR4-mediated LPS signaling was associated with bronchial hyperresponsiveness and allergies, respectively, but only in those subjects heavily exposed to endotoxin [56, 57]. In a recent study it was shown that polymorphisms in the gene encoding the caspase recruitment domain protein (CARD)4, modify the protective effect of the farm environment [55]. So far, no gene-environment studies have been performed on the interaction between candidate genes and gut bacteria.
Up to now studies on the gut microbiota were based upon relatively small study populations, whereas large study populations are needed to study gene-environment interactions. The availability of molecular techniques makes large-scale studies on the gut microbiota and thereby studies on gene-gut microbiota interactions feasible.

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Summary

Summary

Eczema, food allergy, hay fever (and other airway allergies) and allergic asthma are collectively known as 'atopic disorders', chronic inflammatory disorders associated with an aberrant T helper 2 (Th2)-type immune response and IgE mediated hypersensitivity against common 'innocuous' environmental antigens (allergens). Examples of such allergens are antigens from (grass) pollen, house dust mite, cat, dog, hen's egg or cow's milk. A largely unexplained increase in the prevalence of these diseases was noted during the past decades, prominently in westernized countries. Many now consider atopic disorders a result of an immature immune system, possibly caused by insufficient microbial stimulation of the immune system as a result of westernization.
The human gut microbiota consists of approximately 10^{14} (100 billion) bacteria, which can be subdivided in over 500 different species. The gut microbiota is thereby the quantitatively most important source of microbial stimulation and may provide a primary signal for driving the postnatal maturation of the immune system and the induction of a balanced immunity. The microbiota hypothesis postulates that an altered normal intestinal colonisation pattern in western infants is (partly) responsible for the increase in atopic disorders.

The aims of the studies described in this thesis were to investigate the gut microbiota composition of infants in order to subsequently examine the external factors influencing the composition of this gut microbiota and to examine the role of the gut microbiota in the development of atopic diseases.

The studies described in this thesis were carried out within the framework of the KOALA Birth Cohort Study. This prospective cohort study on the aetiology of atopic diseases was initiated in October 2000 by the recruitment of pregnant women (n = 2834). Data was collected during pregnancy and early childhood by repeated questionnaires.
A subgroup of participants was asked to consent to sampling of maternal blood (n = 1355), breast milk (n = 317) and faeces of the infant at 1 month post-partum (n = 1176), capillary blood at age 1 year (n = 956), venous blood at the age of 2 years (n = 829), and buccal swabs for DNA isolation from child–parent trios. Furthermore parents were asked to consent to a physical examination of their child on manifestation of atopic dermatitis at the age of two years (n = 826).

In chapter 2, we review the observational studies conducted thus far on the association between the gut microbiota and atopic diseases. Eleven out of the 13 observational studies indicated an association between the gut microbiota composition and atopic sensitisation or symptoms. Although the microbiota hypothesis thus seems a promising hypothesis, no specific harmful or protective microbes were however identified yet. The available studies on the gut microbiota and atopy appeared to be heterogeneous and therefore difficult to compare. Several methodological issues that have to be considered when interpreting these studies are: the microbiological methods used (traditional culture vs. molecular techniques); the timing of examining the gut microbiota; the definition of atopic outcomes and the possibility of reverse causation. The best way to gain more insight into the association between the gut microbiota and atopic disorders is to conduct large-scale prospective birth cohort studies, in which the gut microbiota can be studied in early infancy and infants can be followed up for the subsequent development of atopic symptoms and sensitisation.

Such a study is now possible by culture-independent molecular techniques to investigate ecological microbial communities. This has provided the microbiologist with a vast array of new techniques to study the human intestinal microbiota. With these techniques, unculturable species are detectable, anaerobic handling and expertise is not required and samples can be kept frozen for later analysis.
Chapter 3 describes the development and validation of 16S rDNA-based real-time PCR assays for the quantification of *Bifidobacterium spp., Clostridium difficile* and *Escherichia coli,* and the application of these assays on faecal samples of 50 exclusively breastfed and 50 exclusively formula-fed infants. The evaluated assays were found to be highly specific and sensitive and provided more accurate data on intestinal microbiota composition than traditional culture techniques.
The prevalence and counts of *C. difficile* as well as *E. coli* were significantly lower in the gut microbiota of breastfed infants than formula-fed infants, whereas the prevalence and counts of *Bifidobacterium spp.* was similar among both groups.

Major changes in the intestinal microbial composition occur especially early in life. The establishment of the gut microbiota commences immediately after birth. The sterile newborn's intestinal tract becomes colonised with bacteria originating from the mother and the environment. In chapter 4, the contribution of a broad range of external influences on the gut microbiota composition in early infancy is investigated.
Infants born through caesarean section had lower numbers of bifidobacteria and bacteroides, whereas they were more often colonised with *C. difficile,*

compared with vaginally born infants. Exclusively formula-fed infants were more often colonised with *E. coli*, *C. difficile*, bacteroides and lactobacilli, compared with breastfed infants. Hospitalisation and prematurity were associated with a higher prevalence and counts of *C. difficile*. Infants' antibiotic use was associated with decreased numbers of bifidobacteria and bacteroides. Infants having older siblings had slightly higher numbers of bifidobacteria, compared with infants without siblings. Altogether vaginally home-born term infants who are exclusively breastfed seemed to have the most "beneficial" gut microbiota (highest numbers of bifidobacteria and lowest numbers of *C. difficile* and *E. coli*).

We performed two studies using different molecular approaches (PCR-DGGE and real-time PCRs respectively) to examine the potential role of the gut microbiota composition on the development of atopic manifestations (Chapter 5 and 6). In chapter 5 a case-control study nested within the KOALA cohort is presented, comparing the gut microbiota of 26 infants who became sensitised and developed eczema within the first year of life with that of 52 non-sensitised non-eczematous infants. A profile of the microbial genetic diversity in the faecal samples was provided by subjecting the samples to PCR combined with DGGE. No association was found between the development of IgE-associated eczema and the dominant gut microbiota as a whole (total bacterial profiles) or with the species composition and proportion of bifidobacteria. The prevalence of one specific band in total bacterial profiles was, however, significantly higher in infants with atopic eczema compared with controls. Identification of this band revealed that it represented *E. coli*. This association with *E. coli* indicates that differences in gut microbiota do precede the development of atopy.

Chapter 6 presents a study on the gut microbiota composition of nearly one thousand one-month-old infants participating in the KOALA study and the subsequent development of atopic manifestations and sensitisation within the first two years of life.

Bifidobacterium spp., Lactobacillus spp., and members of the *Bacteroides fragilis*-group were not associated with the development of atopic manifestations. The presence of *E. coli* was associated with a higher risk of developing eczema, this risk being increased with increasing numbers of *E. coli.* Infants colonised with *C. difficile* were at higher risk of developing eczema, recurrent wheeze and allergic sensitisation. Furthermore, the presence of *C. difficile* was also associated with a higher risk of a clinical diagnosis of atopic dermatitis by a trained nurse.

This study again demonstrates that differences in the gut microbiota composition precede the atopic manifestations. Since *E. coli* was only associated with eczema, whereas *C. difficile* was associated with all atopic outcomes, the underlying mechanims explaining these association may be different.

In chapter 7 the main findings from the studies described in this thesis are summarised and discussed in the light of methodological considerations.
The use of molecular techniques to examine the gut microbiota was one of the major strengths of our studies. In fact, this is the first large-scale prospective birth cohort study in which molecular approaches have been used to examine the microbial composition of a considerable number of faecal samples. The use of faecal samples can, however, be a limitation of studies on the gut microbiota composition, since it cannot unequivocally be established that analyzing microbiota composition in faeces is representative of important intestinal parameters.
By conducting a longitudinal study and analyzing the gut microbiota in early infancy, we were able to show that differences in the gut microbiota precede the development of atopic manifestations. Thereby we ruled out the possibility of reverse causation.
When atopic outcome data are based upon parentally reported symptoms, it is inevitable that misclassification occurs. However, it can be assumed that the misclassification of the outcome status is independent of the gut microbiota composition and thus non-differential. Due to the relatively short intervals between subsequent follow-up questionnaires and the high follow-up rates, it seems unlikely that the results were subject to extensive recall and differential bias.
In this thesis, we found support for the hypothesis that the gut microbiota is involved in the development of atopic manifestations. However, the aetiology of atopic diseases is likely to be multifactorial and perturbations in the gut microbiota alone will probably not be able to explain all of the increased prevalence in atopic diseases. Yet the gut microbiota is an interesting and feasible target for intervention. If we better understand how bacteria and their products educate our immune system perhaps we could learn to mimic its action and hence could target interventions to prevent atopic diseases.
The development of atopic diseases depends not only on environmental but also on genetic factors and it is likely to be an interaction of these, particularly in early life, which determines the atopic status of a person. The effects of certain microbes on the development of atopy may therefore differ according to the genetic susceptibility of an individual. Gene-environment interaction studies are therefore the next step in studying the aetiology of atopic diseases.

Samenvatting

Samenvatting

Eczeem, voedselallergie, hooikoorts (en andere luchtwegallergieën) en allergisch astma behoren tot de atopische aandoeningen. Dit zijn chronische ontstekingsziekten die veroorzaakt worden door een verstoorde T helper 2 (Th2)-type immuunrespons tegen alledaagse onschadelijke antigenen (allergenen). Voorbeelden van dergelijke allergenen, zijn (gras)pollen, huisstofmijt, kat, hond, kippenei en koemelk. In de afgelopen decennia is het voorkomen van atopische aandoeningen, vooral in westerse landen, sterk gestegen. Er is (nog) geen duidelijke verklaring voor deze stijging, maar veel onderzoekers beschouwen atopische aandoeningen als het gevolg van een onderontwikkeld immuunsysteem. Een mogelijke verklaring hiervoor is dat het immuunsysteem van mensen uit westerse ontwikkelde landen onvoldoende geprikkeld wordt door bacteriën en virussen, waardoor het immuunsysteem zich niet goed ontwikkeld.
De humane darmflora bestaat uit ongeveer 10^{14} (100 biljoen) bacteriën, die onderverdeeld kunnen worden in meer dan 500 verschillende soorten. De darmflora is kwantitatief gezien de belangrijkste bron van microbiële stimulatie van het immuunsysteem en draagt mogelijk bij aan de postnatale ontwikkeling van dit immuunsysteem.
De darmflora-hypothese of 'old friends' hypothese oppert dan ook dat een verandering in de samenstelling van de darmflora van westerse kinderen (mede) verantwoordelijk is voor de stijging van atopische aandoeningen.

De onderzoeken die in dit proefschrift worden beschreven hadden als doel de samenstelling van de darmflora van kinderen in kaart te brengen, om vervolgens te bestuderen welke externe factoren de darmflora beïnvloeden en wat de rol van de darmflora is bij het ontstaan van atopische aandoeningen.

Alle onderzoeken in dit proefschrift waren ingebed in het KOALA geboortecohort onderzoek, een longitudinale cohortstudie naar het ontstaan van atopische aandoeningen. Het KOALA onderzoek ging in oktober 2000 van start met de werving van 2834 zwangere vrouwen. De verzameling van gegevens vond plaats gedurende de zwangerschap en de eerste twee levensjaren van het kind door middel van herhaalde vragenlijsten. Aan een subgroep van deelnemers werd toestemming gevraagd om lichaamsmateriaal af te nemen: bloed van de moeder (n = 1355), moedermelk (n = 317) en een ontlastingmonster van het kind op de leeftijd van 1 maand (n = 1176), capilair bloed van het kind op de leeftijd van 1 jaar (n = 956), veneus bloed op de leeftijd van 2 jaar (n = 829) en wangslijmvlies van zowel de ouders als het kind voor de isolatie van DNA. Verder werd toestemming gevraagd om

het kind op 2-jarige leeftijd te onderzoeken op de aanwezigheid van atopisch eczeem (n = 826).

In hoofdstuk 2 wordt een literatuuroverzicht gegeven van de observationele onderzoeken naar de associatie tussen de darmflora en atopische ziekten. Elf van de dertien onderzoeken rapporteerden een associatie tussen de samenstelling van de darmflora en atopische sensitisatie of symptomen. Hoewel de darmflora-hypothese dus een veelbelovende hypothese lijkt, heeft men tot op heden nog niet weten te achterhalen welke specifieke bacteriën mogelijk een rol spelen. De heterogeniteit van de verschillende onderzoeken bemoeilijkt de onderlinge vergelijking van de studieresultaten. Verschillende methodologische aspecten zijn van belang bij de interpretatie van de onderzoeken, onder andere: de gebruikte microbiologische methoden om de darmflora te karakteriseren (traditionele kweekmethoden versus moleculaire technieken), de leeftijd waarop de samenstelling van de darmflora wordt bepaald, de definiëring van de atopische uitkomstmaat en de mogelijkheid van "reverse causation" (omgekeerde oorzakelijkheid).
Grootschalige longitudinale geboortecohortonderzoeken zijn waarschijnlijk de beste manier om meer inzicht te krijgen in de associatie tussen de darmflora en atopische aandoeningen. In dit soort onderzoeken kan de darmflora op jonge leeftijd worden gekarakteriseerd en kunnen kinderen in de tijd gevolgd worden om te kijken of ze atopische sensitisatie of symptomen ontwikkelen.

De ontwikkeling van kweek-onafhankelijke moleculaire technieken biedt de microbioloog een breed scala aan nieuwe technieken om de darmflora te bestuderen. Met deze technieken kunnen ook niet-kweekbare bacteriën worden gedetecteerd. Bovendien is het niet nodig om de ontlastingmonsters anaeroob te verzamelen en verwerken en kunnen de monsters worden ingevroren alvorens ze worden geanalyseerd. Hoofdstuk 3 beschrijft de ontwikkeling en validatie van 16S rDNA-gebaseerde real-time PCR's voor de kwantitatieve bepaling van *Bifidobacterium spp., Clostridium difficile* and *Escherichia coli.* De real-time PCR's werden vervolgens toegepast op ontlastingmonsters van 50 exclusief borstgevoede en 50 exclusief flesgevoede kinderen. De geëvalueerde PCR's bleken zeer specifiek en gevoelig en leverden betrouwbaardere resultaten over de darmflora samenstelling op dan de traditionele kweekmethoden.
C. difficile en *E. coli* kwamen minder vaak en in lagere aantallen voor in de darmflora van borstgevoede kinderen ten opzichte van flesgevoede kinderen. Bifidobacteriën kwamen even vaak en in vergelijkbare aantallen voor in de darmflora van borst- en flesgevoede kinderen.

Grote veranderingen in de samenstelling van de darmflora vinden voornamelijk in de eerste levensfase plaats. De totstandkoming van de

darmflora begint meteen na de geboorte, wanneer de darmen van het kind gekoloniseerd worden door bacteriën afkomstig van de moeder en de omgeving. In hoofdstuk 4 wordt de invloed van een breed scala aan externe factoren op de samenstelling van de darmflora van jonge kinderen onderzocht.
Kinderen geboren via een keizersnede hadden lagere aantallen bifidobacteriën en bacteroides, terwijl ze vaker gekoloniseerd waren met *C. difficile* dan kinderen die op de natuurlijke wijze (vaginaal) werden geboren.
Kinderen die uitsluitend flesvoeding kregen, waren in vergelijking met borstgevoede kinderen vaker gekoloniseerd met *E. coli*, *C. difficile*, bacteroides en lactobacillen. De ontlastingmonsters van kinderen die waren opgenomen in het ziekenhuis en van te vroeg geboren kinderen bevatten vaker en hogere aantallen *C. difficile.* Antibioticagebruik door het kind was geassocieerd met lagere aantallen bifidobacteriën en bacteroides. Kinderen met oudere broertjes of zusjes hadden iets hogere aantallen bifidobacteriën in hun ontlasting.
Samenvattend bleken voldragen kinderen die op natuurlijke wijze ter wereld waren gekomen en vervolgens uitsluitend borstvoeding kregen, de meest gunstige darmflorasamenstelling te hebben (hoogste aantallen bifidobacteriën en laagste aantallen *E. coli en C. difficile*)

We hebben twee studies uitgevoerd, met verschillende moleculaire technieken (respectievelijk PCR-DGGE en real-time PCR), om de rol van de darmflora op de ontwikkeling van atopie te onderzoeken (Hoofdstuk 5 en 6). In hoofdstuk 5 worden de resultaten van een patiënt-controle onderzoek genest in het KOALA cohort gepresenteerd. In dit onderzoek werd de samenstelling van de darmflora op de leeftijd van 1 maand vergeleken van 26 kinderen die tijdens hun eerste levensjaar werden gesensitiseerd en bovendien eczeem ontwikkelden (IgE-geassocieerd of atopisch eczeem) met de darmflora van 52 kinderen die niet werden gesensitiseerd en ook geen eczeem ontwikkelden.
Door de ontlastingmonsters te analyseren met behulp van PCR-DGGE, werd een bandenpatroon van de microbiële genetische diversiteit verkregen. Er werd geen associatie gevonden tussen de ontwikkeling van IgE-geassocieerd eczeem en de darmflora-samenstelling in zijn geheel (totale bacteriële bandenpratronen). Ook werd er geen associatie gevonden tussen atopisch eczeem en de verschillende soorten en proportie van bifidobacteriën. Één specifiek bandje bleek echter vaker voor te komen bij kinderen met atopisch eczeem dan bij de kinderen zonder atopisch eczeem. Na identificatie bleek dat dit bandje *E. coli* representeerde. Deze associatie tussen *E. coli* en atopisch eczeem duidt erop dat verschillen in de darmflora voorafgaan aan de ontwikkeling van atopie.

Hoofdstuk 6 beschrijft een onderzoek naar de samenstelling van de darmflora van bijna duizend kinderen, die deelnamen aan het KOALA onderzoek, en de ontwikkeling van atopische symptomen en sensitisatie in de eerste twee levensjaren.
Bifidobacterium spp., Lactobacillus spp., en bacteriën behorende tot de *Bacteroides fragilis*-groep waren niet geassocieerd met de ontwikkeling van atopie. De aanwezigheid van *E. coli* in de darmflora was geassocieerd met een hoger risico op het ontwikkelen van eczeem. Dit risico nam toe naarmate de aantallen *E.* coli in de ontlasting hoger waren. Kinderen die waren gekoloniseerd met *C. difficile* hadden een verhoogd risico op het ontwikkelen van eczeem, piepen op de borst en allergische sensitisatie. Verder bleek de aanwezigheid van *C. difficile* in de darmflora ook geassocieerd met een hoger risico op atopisch eczeem, wanneer dit werd gediagnostiseerd door een speciaal opgeleide verpleegkundige.
Ook deze studie toont weer aan dat verschillen in de samenstelling van de darmflora voorafgaan aan de manifestatie van atopie. Aangezien *E. coli* alleen met eczeem was geassocieerd, terwijl *C. difficile* met alle atopische uitkomsten was geassocieerd, zijn de mechanismen die aan deze associaties ten grondslag liggen mogelijk verschillend.

In hoofdstuk 7 worden de belangrijkste resultaten van de onderzoeken die beschreven zijn in dit proefschrift samengevat en bediscussieerd in het licht van methodologische afwegingen. Het gebruik van moleculaire technieken om de samenstelling van de darmflora te karakteriseren was een van de voornaamste sterke punten van onze studies. Het KOALA onderzoek is zelfs het eerste prospectieve geboortecohort waarin moleculaire methoden zijn toegepast om de microbiële samenstelling van een groot aantal ontlastingmonsters te bepalen. Het gebruik van ontlastingmonsters kan echter een beperking in het darmflora-onderzoek zijn, aangezien het niet onomstotelijk is bewezen dat de microbiële samenstelling van ontlasting representatief is voor belangrijke intestinale parameters.
Door het uitvoeren van een longitudinale studie en het analyseren van de darmflora op zeer jonge leeftijd, waren we in staat om aan te tonen dat verschillen in de samenstelling van de darmflora vooraf gingen aan de ontwikkeling van atopische manifestaties. Hierdoor waren we in staat om de mogelijkheid van "reverse causation" uit te sluiten.
Wanneer de atopische uitkomsten van kinderen gebaseerd zijn op hetgeen de ouders rapporteren, dan is het onvermijdelijk dat er misclassificatie optreedt. Het is echter aannemelijk dat het hier non-differentiële misclassificatie betreft, aangezien de misclassificatie van uitkomsten onafhankelijk is van de samenstelling van de darmflora. Door de korte intervallen tussen de opeenvolgende vragenlijsten en de hoge follow-up percentages, lijkt het

onwaarschijnlijk dat de resulatetn van onze onderzoeken sterk vertekend zijn door recall bias (vertekening door fouten in herinnering) en differentiële bias.
De bevindingen in dit proefschrift ondersteunen de hypotheses dat de darmflora een rol speelt bij de ontwikkeling van atopische aandoeningen. De etiologie van atopische aandoeningen lijkt echter multifactorieel en veranderingen in de samenstelling van de darmflora is waarschijnlijk slechts een van de factoren die een rol speelt bij de toename van atopische aandoeningen. Toch is de darmflora een interessant en geschikt doelwit voor interventie. Wanneer we meer inzicht weten te verschaffen in de manier waarop bacteriën en hun producten ons immuunsysteem helpen te ontwikkelen, zouden we dit wellicht na kunnen bootsen zonder dat we onze hygiënische westere levensstijl hoeven op te geven.
De ontwikkeling van atopische aandoeningen is niet alleen van omgevingsfactoren (zoals de darmflora), maar ook van genetische factoren afhankelijk. Waarschijnlijk is het een wisselwerking tussen genetische en omgevingsfactoren vroeg in het leven, die de atopische status van iemand bepaalt. Afhankelijk van de genetische gevoeligheid van een persoon kan het dus zijn dat het effect van darmbacteriën op de ontwikkeling van atopie verschilt. Gen-omgevingsinteracties zijn dan ook de volgende stap in het onderzoek naar de rol van de darmflora bij het ontstaan van atopische aandoeningen.

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About the author

John Penders was born on Februari 12 1978 in Elsloo, the Netherlands. After completing secondary school (VWO) at the Scholengemeenschap Groenewald in Stein in 1996, he studied Environmental Health Sciences at the Faculty of Health Sciences of Maastricht University. During his study, he fulfilled two internships. The first internship was carried out at the department of Epidemiology, Maastricht University, in which he studied the association between hormonal factors and the risk of colorectal cancer in postmenopausal women participating in the Netherlands Cohort Study on diet and cancer. The second internship was performed at the department of Medical Microbiology, University Hospital of Maastricht, where he examined the prevalence of antibiotic resistance in Dutch fish farms. He graduated in Environmental Health Sciences in 2002. From 2002 till 2006, he worked as a PhD student at the department of Epidemiology at Maastricht University, on the KOALA Birth Cohort Study examining the gut microbiota composition and the development of atopic manifestations in infancy. This project resulted in the present thesis. In December 2006 he was awarded with the "Talent voor de toekomst" grant, which funds an extension of his work on the gut microbiota and atopy as a researcher at the same department.