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Review article

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

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, as 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 of the association between the gut microbiota and atopic diseases are discussed. Although most studies indicated an association between the gut microbiota composition and atopic sensitization 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 examining the gut microbiota, the definition of atopic outcomes, confounding and reverse causation. In conclusion, the microbiota hypothesis in atopic diseases is promising and deserves further attention. To gain more insight into the role of the gut microbiota in the etiology of atopy, large-scale prospective birth cohort studies using molecular methods to study the gut microbiota are needed.

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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 rekindled the interest in intestinal microbial ecology. The gut microbiota has been linked to the risk of gastrointestinal diseases such as 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 of 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 colonization of the intestines by a variety of microorganisms begins (10).

Gastrointestinal colonization 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 their location in the gut. The concentration of bacteria ranges from 10^3 colony-forming units per millilitre (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 (Fig. 1) (13). The colonic microbiota is dominated by obligatory anaerobes such as *Bacteroides* spp., *Clostridium* spp., bifidobacteria, eubacteria, and fusobacteria. Facultative anaerobes occur in 100- to 1000-fold lower numbers and include lactobacilli, enterococci,

streptococci and Enterobacteriaceae (Table 1) (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, mucosal 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).

Factors influencing the intestinal microbiota composition can be divided into host factors (such as pH, transit time, bile acids, pancreatic enzymes and 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 confer important health benefits upon the human host (18). Probably the most important function of the gut microbiota is the so-called colonization resistance. By not only competing for nutrients and adhesion sites, but also by the production of antibacterial substances (bacteriocins), the indigenous gut microbiota makes it difficult for 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 of 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 among others decreased Peyer’s patch size, decreased number of IgA-producing lymphocytes in the lamina propria, decreased number of intra-epithelial T cells and a delayed immune response after antigenic challenge compared with conventional animals (12, 19–23).

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.

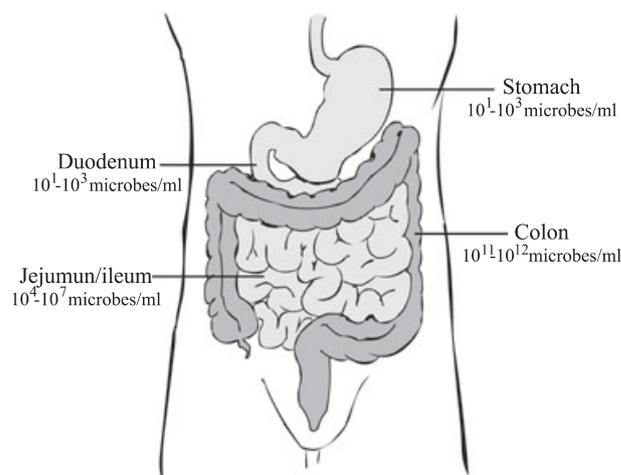


Figure 1. Bacterial densities increase throughout the gut, with highest concentrations found in the colon.

From culture to genotype analysis

Research into the intestinal microbiota composition has relied almost exclusively on the quantitative culture of microbes from fecal 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 cultured), 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 culturable, 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-scale epidemiological studies is that samples require immediate processing (25). The relative inexpensiveness and wide availability on the other hand make these classical techniques the most applied.

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 are 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 the 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), polymerase chain reaction (PCR) combined with denaturing- or temperature-gradient gel electrophoreses (PCR-DGGE/PCR-TGGE) and real-time PCR are molecular techniques which have found application for studying 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, bypassing the cell wall. Gram-positive bacterial cells such as lactobacilli, for example, are more difficult to permeabilize 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-TGGE/-DGGE. In this method of analysis, bacterial DNA is extracted from the fecal sample and fragments of the 16S rRNA gene are amplified by PCR; subsequently the 16S molecular species within the resulting mixture are separated by TGGE/DGGE. The double-stranded 16S fragments migrate through the polyacrylamide gel until each kind of fragment is partially denatured 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 as 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, as DNA/RNA may not be extracted with equal efficiency from all bacteria (13).

The microbiota hypothesis in atopy

Very recently it was hypothesized that the gut microbiota may also be involved in the etiology of atopic diseases. 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 (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 the 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 of the association between viral and bacterial infections and allergy were less consistent (38, 39). In 1998 Wold suggested that rather than a decrease in viral or bacterial infections, an altered normal intestinal colonization pattern in infancy, which fails to induce immunological tolerance, could be responsible for the increase in allergies (40). 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 (41); (ii) administration of lipopolysaccharide (LPS; a constituent of the outer membrane of gram-negative bacteria) together with food antigens increases the tolerizing effect of feeding (42); (iii) and bacterial toxins may break oral tolerance (43). Since then, several observational studies of the gut microbiota composition and allergy have been conducted.

Potential immunological mechanisms

The innate immune system may be decisive in determining the type of adaptive immune responses elicited against microbial antigens. Of the innate immune cells, dendritic cells (DC) seem to be pivotal in the earliest bacterial recognition and in shaping T-cell responses (44). Innate immune cells recognize microbial antigens through molecules such as Toll-like receptors (TLRs), and nucleotide-binding oligomerization domain (NODs) molecules, which recognize conserved pathogen-associated molecular patterns, including unmethylated CpG motifs characteristic of bacterial DNA, the bacterial LPS and peptidoglycan (45).

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 (46–48). 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, was also increasing and that chronic parasitic worm (helminth) infections which induce strong Th2 responses and high IgE levels are not associated with an increased risk of allergy (49).

An alternative interpretation conceives anti-inflammatory immune responses to be of fundamental importance in the development of mucosal and systemic tolerance (50). These immunosuppressive mechanisms are orchestrated by regulatory T-cell classes (Treg cells) that control [largely via the production of interleukin (IL)-10 and/or tumor growth factor (TGF)- β] both Th1 and Th2 responses and hence the development of both atopic and autoimmune diseases (50, 51). 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 et al. (52). Furthermore, a study of duodenal biopsies of healthy infants and infants with multiple food allergy showed that the dominant mucosal abnormality was not Th2 deviation but impaired generation of TGF- β -producing Treg cells (53).

Relatively harmless organisms, including not only bifidobacteria and lactobacilli, but also helminths and saprophytic mycobacteria, may skew immune responses toward immunoregulation by inducing Treg cells, rather than eliciting a proinflammatory immune response. For example, *Lactobacillus paracasei* has been reported to inhibit the secretion of both Th1 and Th2 cytokines, while inducing the development of a population of CD4(+) T cells producing TGF- β and IL-10, reminiscent of previously described subsets of regulatory cells implicated in oral tolerance and gut homeostasis (54).

Lactobacillus reuteri and *Lactobacillus casei* have been shown to prime monocyte-derived DCs to drive the development of IL-10-producing Treg cells, through binding of the C-type lectin DC-specific intracellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (55). The *Bifidobacterium* genomic DNA has been reported to induce the secretion of IL-10 by peripheral blood mononuclear cells (PBMCs) from healthy donors *in vitro* (56).

The 'microbiota hypothesis' proposes that the loss of exposure to these harmless microorganisms in the westernized environment might explain the increase in immune dysregulatory disorders (57, 58). 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 (59). Furthermore, the impact of the gut microbiota on the development of IgA antibody responses, which contribute to pathogen and allergen exclusion in the gut lumen, may also be involved (45).

It has been proposed that the effects of the gut microbiota may not only be related to food antigens, but also to aeroallergens and the manifestation of allergic airway symptoms. Noverr et al. developed a mouse model to demonstrate experimentally that antibiotic therapy, leading to bacterial and fungal microbiota changes, could predispose a host to allergic airway disease (60). Furthermore, oral treatment with live *L. reuteri* has recently been

shown to inhibit the allergic airway response in mice (61). These results support the possibility that afferent events in allergic sensitization may occur outside of the lungs and involve host-microbiota communication.

The mechanisms by which events in the gut can affect the systemic immune system and local inflammation in remote tissues such as the respiratory tract remain to be determined. However, it has been shown that inhaled particles, fluids and microbes are also swallowed. The gastrointestinal tract will, thus, be exposed to any antigens to which the respiratory tract is also exposed. As ingestion of antigens can induce tolerance to that antigen (oral tolerance), the gastrointestinal (GI) tract may act as a 'sensor' for the development of tolerance to inhaled antigens (11, 62). Induced regulatory T cells may thereafter home in other tissues throughout the body, in particular in other mucosal tissues such as the respiratory tract (63).

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 sensitization) 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, as these studies have to deal with completely different methodological issues. For an overview of studies on the use of probiotics in the prevention and treatment of allergic diseases we refer to an extensive review on this topic (64). The publications were analyzed for population size, design of the study, atopic outcomes, and methods used for analyzing the fecal 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 January 2007. The following keywords and limits were used: [intestines (mesh) OR intestine* OR gut OR gastrointestin* OR enteric] AND (flora OR microbiolog* OR microflora OR bacteria OR bacterial OR colonization OR colonisation OR microbes OR microbial OR microbiota) AND (hypersensitivity OR atopic dermatitis OR allergic OR allergy OR atopy OR atopy OR eczema OR rhinitis OR asthma) AND [English(la)]. Additional reports were found searching the reference lists of pertinent articles.

Results

Using the search criteria as described above, we identified 18 observational studies on the association between the gut microbiota and atopic diseases. Eight studies were exclusively based on traditional bacteriological culture techniques to study the microbial composition of fecal samples, and another seven studies (additionally) used molecular techniques. The remaining three studies were not based on the examination of fecal 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 (65) was not incorporated in Table 2, because it lacked statistical analysis and a reference group.

Culture-dependent studies

Already in the early 1980s, Russian scientists linked food allergy to abnormal intestinal microbiota (65). The authors examined 60 infants with dermatological syndrome, caused by food allergy and reported dysbacteriosis of different degrees 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 analysis and it was not clear how a deficiency of lactic acid bacteria and an increase of Enterobacteriaceae were defined, as data pertaining to a reference group of infants without dermatological symptoms was not mentioned in the paper.

It was not until the late 1990s when Sepp et al. initiated the research into the potential role of the gut microbiota in the etiology of allergic diseases. They studied the gut microbiota of 1-year-old healthy infants in Estonia with a low prevalence and Sweden with a high prevalence of childhood allergy (66). Especially lactobacilli and eubacteria were more prevalent in Estonian infants. Swedish infants harbored higher counts of clostridia, and especially *Clostridium difficile* was more common than in 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 (67). Allergic infants were less often colonized by lactobacilli compared with non-allergic infants in both countries. In contrast, the allergic children harbored higher counts of facultative aerobic microorganisms, especially coliforms in the Estonian and *Staphylococcus aureus* in the Swedish 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 as it was cross-sectional a conclusion on what comes first could not be drawn. Therefore in a follow-up 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 (68). Fecal samples were collected several times throughout the first year of life and infants were clinically followed up for the presence of allergic symptoms and sensitization until the age of 2 years. The prevalence of colonization by bifidobacteria was consistently lower throughout the first year of life in infants who developed

allergy compared with 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 as comparing both prevalence and counts of multiple bacterial groups at several different time points introduces a considerably risk of false-positive findings because of multiple testing. Furthermore numbers were too small for statistical analysis for the two countries separately.

Differences in the bifidobacterial species composition were first investigated in a small Finnish case-control study (69). In this study, faecal samples of seven breast-fed allergic infants and six breast-fed, healthy, age-matched controls were examined. Allergic infants were more often colonized with *Bifidobacterium adolescentis*, whereas colonization with *Bifidobacterium bifidum* was more frequent in their healthy counterparts.

In a Japanese case-control study, among minors with atopic dermatitis (AD) 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 AD. Furthermore the prevalence of colonization with staphylococci was higher in the AD group than in the control group (70).

In another Japanese case-control study (71), fecal 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 et al. 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 (72).

Recently a case-control study was published investigating the gut microbiota of infants with AD with or without sensitization to food allergens (73). No differences were found in the intestinal microbiota composition of infants with IgE-associated and non-IgE-associated AD. As this study compares two subgroups of infants with AD and does not include a reference group of healthy infants, it can only be concluded that no differences exist between these subgroups. It may well be that both subgroups of infants have a different gut microbiota composition compared with healthy infants.

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.

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 analysis	Results for atopic compared with non-atopic subjects
67	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 <i>S. aureus</i> (in atopic compared with non-atopic infants from Sweden)
68	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 <i>S. aureus</i> (at 6 months) Lower counts of bacteroides (at 12 months) Lower prevalence of bifidobacteria (during year 1)
75	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, <i>Clostridium difficile</i> , <i>E. coli</i>), although some genera were associated with severity of atopy
69	Ouwehand, 2001	Atopic dermatitis (Hanifin & Rajka) and at least 1 positive SPT	7 allergic and 6 healthy infants (2–7 months)	Cross-sectional case-control	Bacteriological culture and biochemical identification of bifidobacteria to the species level	More often colonized with <i>Bifidobacterium adolescentis</i> and less often colonized with <i>Bifidobacterium bifidum</i>
76	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 colonization (at 3 weeks and 3 months as determined by culture) Lower bifidobacteria : clostridia ratio (at 3 weeks as determined by FISH)
74	Bottcher, 2000	At least one positive SPT at age 12 months	25 allergic 43 non-allergic 13-month-old infants	Cross-sectional, nested case-control	Gas chromatography	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
70	Watanabe, 2003	Atopic dermatitis (Japanese Dermatological Association)	30 allergic and 68 non-allergic children	Cross-sectional, case-control	Bacteriological culture and biochemical identification	Higher prevalence of <i>S. aureus</i> Lower counts of bifidobacteria Significant trend between percentage bifidobacteria of total flora and severity of atopic eczema
71	Matsumoto, 2004	Severe atopic dermatitis	11 allergic and 14 non-allergic adults	Cross-sectional, case-control	Bacteriological culture and measurement of slgA in faeces	Lower total counts and total anaerobes Higher proportion of Enterobacteriaceae Higher content of slgA

Table 2. (Continued)

Ref.	Year	Definition of allergy	Study population	Design	Bacteriological/serological analysis	Results for atopic compared with non-atopic subjects
77	Mah, 2006	Atopic dermatitis (Hanifin & Rajka)	21 allergic and 28 non-allergic 3-year-old children	Cross-sectional	Bacteriological culture and biochemical identification (for aerobes) and FISH (for anaerobes)	Higher counts of LAB (especially enterococci) Lower counts of bifidobacteria and clostridia
79	Woodcock, 2002	Recurrent wheeze (≥ 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 <i>C. difficile</i>	Higher levels of <i>C. difficile</i> -specific IgG levels
78	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 colonization with lactobacilli and bifidobacteria. No differences in bifidobacterial counts (lactobacilli not enumerated), or in bifidobacterial species composition
80	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 <i>C. difficile</i>
81	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 <i>B. vulgatus</i> in subjects with 2 or more symptoms
72	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
82	Penders, 2006	Eczema (based on ISAAC-questionnaire) specific, serum IgE or recurrent wheeze (≥ 3 episodes)	957 newborns	Prospective birth cohort (until age 2 years)	Quantitative real-time PCR at age 1 month	Higher prevalence and counts of <i>E. coli</i> in infants who subsequently developed eczema Higher prevalence of <i>C. difficile</i> in infants who subsequently developed eczema, recurrent wheeze and/or became sensitized
83	Penders, 2006	Eczema (based on ISAAC-questionnaire) and specific serum IgE to at least 1 allergen	26 allergic and 52 non-allergic 1-year old children	Prospective nested case-control	PCR-DGGE and quantitative real-time PCR at age 1 month	<i>E. coli</i> more prevalent in infants who subsequently developed IgE-associated eczema No differences in total bacterial profiles No difference in bifidobacterial counts or in bifidobacterial species composition.
73	Kendler, 2006	Atopic dermatitis and specific serum IgE to at least 1 food allergen	93 children (<7 years) with AD (25 children without and 68 with specific IgE)	Cross-sectional case-control	Bacteriological culture	No differences in the fecal microbiota of infants with AD and specific IgE compared to infants with AD without specific IgE

SPT, skin-prick test; FISH, fluorescence *in situ* hybridization; GLC, gas-liquid chromatography.

Prevalence, as percentage of infants colonized with a certain bacterial group or species.

Counts, as mean or median log₁₀ colony-forming units per gram of feces.

Proportion, as mean or median percentage a certain bacterial group or species contributes to the total bacterial counts.

Culture-independent studies

In a study of microflora-associated characteristics it was shown that sensitized infants had higher levels of i-caproic acid in their stools compared with non-sensitized infants.

This rather uncommon short-chain fatty acid was suggested to indicate the presence of *C. difficile* (74).

Molecular techniques to quantify intestinal bacteria were first used in two studies conducted in Finland (75, 76). In a case-control study using FISH to characterize

the gut microbiota, no differences in concentrations of specific genera were found between healthy infants and infants suffering from AD. Higher bacteroides counts and lower counts of bifidobacteria were however associated with more severe manifestations of dermatitis (75). In a cohort study prospectively following up 76 high-risk infants during their first year of life (76), bacterial fatty acid profiles in fecal samples collected at age 3 weeks significantly differed between infants in whom atopy (positive skin-prick test) was or was not developing. As bacterial culture 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 infants in Singapore, the numbers of clostridia, as determined by FISH, were lower in infants with eczema compared with those in healthy controls (77). 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 with healthy controls.

Two case-control studies nested within the prospective National Asthma Campaign Manchester Asthma and Allergy Study (^{NAC}MAAS) investigated the association between the intestinal microbiota composition and recurrent wheeze. The use of PCR combined with DGGE showed no differences in the dominant fecal microbiota and the bifidobacterial and lactobacilli composition between sensitized wheezy and non-sensitized non-wheezy 4-year olds (78). In a second study within the ^{NAC}MAAS cohort, 1-year-old sensitized wheezy infants had significantly higher serum IgG levels against *C. difficile* compared with non-sensitized non-wheezy infants (79).

The use of IgG serology to determine exposure to intestinal microbes was also used in two large-scale population-based cross-sectional studies (80, 81). In one of these studies conducted in Copenhagen, both sensitized subjects and subjects suffering from allergic rhinitis were more often IgG-seropositive to intestinal bacterial pathogens, especially *C. difficile*, compared with subjects without sensitization and allergic rhinitis respectively (80). 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 (81). Infants with two or more allergic symptoms (asthma, rhinitis, eczema and/or food allergy) had significantly higher IgG titers to *B. vulgatus*.

The KOALA Birth Cohort Study conducted in the Netherlands was the first large-scale study in which the association between the gut microbiota and atopic

manifestations has been investigated prospectively. Using real-time quantitative PCR, fecal samples of 957 infants have been examined. Subsequently infants were followed up for the development of atopic symptoms (82). In this study, colonization with *E. coli* was associated with an increased risk of developing eczema in a concentration-dependent manner. Furthermore colonization with *C. difficile* was associated with an increased risk of developing eczema, recurrent wheeze and becoming sensitized.

A case-control study nested within this same cohort examined the intestinal microbiota of 26 infants with IgE-associated eczema compared with 52 healthy controls using PCR-DGGE (83). No differences were found in the bifidobacterial counts and species composition. However one band corresponding to *E. coli* was more prevalent in the total bacterial profiles of cases compared with that of controls.

Discussion

The microbiota hypothesis in atopic diseases is promising. Fourteen of the 17 observational studies conducted so far indicate an association between the gut microbiota composition and atopic sensitization or symptoms. No specific harmful or protective microbes could however be identified yet.

Lactobacilli are microorganisms that stimulate immunoregulation by triggering regulatory T-cell responses (84). However current observational studies do not endorse a protective role of lactobacilli; only previous studies of Swedish and Estonian infants reported a higher colonization by lactobacilli in non-allergic infants in both countries (67). It should however be noted that different species of lactobacilli induce distinct and even opposing DC responses with regard to their Th1/Th2/Treg-driving capacity (85). As 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 (86). Of the 11 studies which included the quantification of total 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, as especially the culture of these bacteria is subject to bias (87). Similar to 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 (88). Furthermore allergic infants have been reported to harbor different *Bifidobacterium* species compared with healthy infants (69, 89). This difference was however not confirmed in the two nested case-

control studies using *Bifidobacterium*-specific PCR-DGGE to compare the fecal microbiota of healthy control infants with that of IgE-associated wheezy infants or IgE-associated eczematous infants (78, 83).

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, clostridia, Enterobacteriaceae and staphylococci are potential candidates as 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 emphasize 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, 45, 90). 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 (91). Van der Velden et al. observed a significantly increased production of IL-4 at 6 months of age in children developing atopy compared with 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 clinical symptoms characteristic of an atopic disease (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 sensitization (92).

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 (45). Indeed the importance of timing for the effect of the gut microbiota

was suggested in a number of studies of 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 (41). In another study it was shown that colonization 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 (93).

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 etiology 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 those quantifying the microbial composition in early life.

Timing and number of samples

Although the exact window of opportunity is not known, the ideal timing for studying the intestinal microbiota composition seems to be somewhere during the first year of life (94). Collecting several samples per infant during this first year of life can give more information than when only one sample is collected. Furthermore, this reduces the risk that the sample is collected outside of the window of opportunity. However, as mentioned before, analyzing several bacterial groups and species in several samples per infant and relating this to several atopic outcomes result in an increased risk of false-positive findings because of multiple testing. The number of samples collected per infant and the age at which these samples are collected are thus of major importance.

An important aspect to take into account when choosing the age at which samples are collected is the developmental process of the gut microbiota composition. This process can be divided into four separate phases (95). Phase 1 is the initial acquisition phase over the first 1–2 weeks of life. Several studies show that the microbial composition fluctuates markedly from day to day (96, 97) and even from hour to hour (98) within each infant during this perinatal period. Phase 2 starts about the end of the second week until the introduction of supplementary feedings of any kind. In this phase the microbiota composition of breast-fed and formula-fed infants is quite different. Fluctuations within each infant are far less marked than in phase 1, except when the feeding regime is switched from exclusively breastfeeding to a combination with formula-feeding (99). Phase 3 is the introduction of solids. During this phase the microbiota of breast-fed infants diversifies and differences between breast-fed and formula-fed infants are lost. Phase 4

begins when weaning is completed. This phase represents the period of conversion to adult microbiota patterns.

Phases 1 and 3 are thus the phases of greatest time-to-time fluctuations in gut microbiota composition within each infant. Furthermore, the introduction of solids starts for most infants between 3 and 6 months of life. The start of phase 3 is thus different for every infant. Altogether, the most optimal timing to analyze the gut microbiota seems to be during phase 2 (2 weeks to 3 months), and thereafter during phase 4.

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 (100). It is generally known that not all children with a clinical phenotype of (atopic) eczema are actually sensitized against allergens (101–103). This has led to the idea that there may exist at least two variants of eczema, an atopic and a non-atopic variant (103, 104). 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, as non-IgE mediated rhinitis, wheeze and asthma have also been reported (105, 106). Furthermore although eczema, asthma and allergic rhinitis tend to cluster in the same individuals and families, the exact relationship over time is far from clear. The question whether eczema is a risk factor for asthma in a progressive atopic march or that a co-manifestation of two phenotypes at an early age exists, remains to be resolved (101, 107).

The studies conducted so far on the association between the gut microbiota and atopic outcomes have focused on a variety of outcomes: 10 studies analyzed both allergic sensitization and clinical symptoms (of which five on AD/eczema, two on recurrent wheeze, one on allergic rhinitis and two on several atopic symptoms); 2 studies investigated allergic sensitization only; and five studies clinical symptoms only (four AD/eczema and one 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 examine the association of the gut microbiota composition with both allergic sensitization and different atopic phenotypes, this could provide further insight into the role of the gut microbiota in the etiology of childhood atopy.

Confounding effects

To prevent a potential confounding effect, some studies chose to exclude certain subgroups: e.g. the exclusion of infants born by cesarean section or those treated with

antibiotics to prevent a confounding effect of mode of delivery and antibiotic use (68, 75, 82). Other studies verified an association between certain potential confounders and (markers of) the gut microbiota (74) or the disease status (73, 76), or just reported that both allergic and non-allergic infants were comparable regarding dietary or other potential confounding factors. Matching on potential confounding variables was performed in at least one study (78). Overall most studies only took one or few potential confounders into account and only one study, so far, performed multivariate analysis (82). Because of the limited population sizes, most studies lack the power to perform multivariate analysis including several confounders. However, although adjusting for confounders is important it can be argued whether factors such as mode of delivery, type of infant feeding and antibiotic use are true confounders. These factors are not independently associated with the gut microbiota composition, but determine it (partly) (108–110). Therefore these factors are more likely to be distal determinants in the relationship between the gut microbiota and atopy, rather than confounders. As a consequence, it is unlikely that the studies not adjusting to these determinants are biased.

Recent observational studies on the gut microbiota and allergy may, however, be susceptible to a new type of confounding, namely confounding by indication with respect to probiotic or prebiotic use. The increasing attention in the media on the potential health effects of probiotics and prebiotics may introduce this kind of confounding. Allergic mothers may be more aware of the potential beneficial effects of probiotics and prebiotics and may therefore more often consume these products themselves during pregnancy and/or feed them to their children, in order to prevent the development of allergy in their offspring. Consequently the effect of the gut microbiota on the development of allergy will be confounded by a family history of allergy (confounding by indication). Confounding by indication because of a family history of allergic manifestations can best be controlled for by considering allergy in parents and siblings as separate confounding variables (111). Another important reason to adjust for a parental history of allergy is to limit the chance of reverse causation, as will be discussed in the subsequent section.

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 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 toward atopy, rather than the cause of disease (78). It is thus possible that differences in the gut microbiota and differences in the development of immunity are consequences of one or more other unknown factors, associated with the atopic genotype (68). 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 into the underlying mechanisms. However these studies can give no information as to whether perturbations in the indigenous microbiota themselves 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 three 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 (76), thereby indirectly taking the possible effect of a positive family history into account. In one of the other prospective studies, the KOALA study, multivariate analyses were used to account among others for parental and sibling allergy.

In two cross-sectional studies, the potential effect of a positive family history was taken into account by matching for this factor (78, 79).

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 sensitization. The introduction of molecular techniques has simplified the analyses of the gut microbiota in large-scale studies as fecal samples can be frozen prior to the analyses. Furthermore these molecular techniques provide more accurate and sensitive results compared with traditional culture. In such large-scale prospective studies, different atopic outcomes and sensitization can be studied separately, thereby leading to more insight into the etiology of atopy and atopic disorders. Currently several prospective studies such as the IMPACT Study (112) and the Allergyflora project, are examining the association between intestinal colonization in infancy and development of atopic diseases. Results of these studies are expected to be available soon.

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