CAN LACTOBACILLUS REUTERI PREVENT ALLERGIC DISEASE IN EARLY CHILDHOOD?

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To my family
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ORIGINAL PUBLICATIONS

This thesis is based on the following four papers, which will be referred to in the text by their roman numerals.

I Probiotics in prevention of IgE-associated eczema: A double-blind, randomized, placebo-controlled trial.

Abrahamsson TR, Jakobsson T, Fagerås-Böttcher M, Fredrikson M, Jenmalm MC, Björkstén B, Oldaeus G.
Journal of Allergy and Clinical Immunology 2007, vol 119, p 1174-1180

II Probiotic Lactobacilli in breast milk and infant stool in relation to oral intake during the first year of life.

Abrahamsson TR, Sinkiewicz G, Jakobsson T, Fredrikson M, Björkstén B.
Journal of Pediatric Gastroenterology and Nutrition, in press.

III Low breast milk TGF-β2 is induced by *Lactobacillus reuteri* supplementation and associates with reduced risk of sensitization during infancy.

Fagerås-Böttcher M, Abrahamsson TR, Fredriksson M, Jakobsson T, Björkstén B.
Pediatric Allergy and Immunology 2008, vol 19, p 497-504.

IV A Th1/Th2-associated chemokine imbalance preceding allergic disease is influenced by birth size, breastfeeding, day-care and probiotics.

Abrahamsson TR, Sandberg M, Forsberg A, Björkstén B, Jenmalm MC.
Submitted.
Abstract

Background: An altered microbial exposure may be partly responsible for the increase of allergic diseases in populations with a western lifestyle. Activation of the immune system by microbes early in life is probably required for an accurate maturation of the immune system. Probiotics, live bacteria which are considered to confer health when ingested, have been suggested to prevent eczema and sensitisation infants.

Aim: The general aim of this thesis was to assess the effect of oral supplementation with the probiotic bacterium Lactobacillus reuteri (L. reuteri) in infancy on the development of allergic disease and sensitisation during the first 2 years of life and to examine mechanisms possibly underlying eventual effects on allergic manifestations.

Subjects: The thesis is based on results obtained from a prospective double-blind placebo-controlled multicenter trial, comprising 232 families with allergic disease, of whom 188 completed the study.

Methods: The families were recruited at the antenatal clinic, and the mothers received L. reuteri ATCC 55730 (1 x 10⁸ colony forming units) or placebo daily from gestational week 36 until delivery. Their babies then continued with the same study product from birth until 12 months of age and were followed up for another year. The primary outcomes were allergic disease, with or without positive skin prick test or circulating IgE to food allergens. Bacterial counts and prevalence were assessed in maternal breast milk and faeces and infant faeces, employing conventional cultivation methods. Cytokines and IgA antibodies were analysed in colostrum and mature milk from the mothers with ELISA, and Na/K- ratio in breast milk with ion selective electrodes. Circulating Th1/Th2-associated chemokines were analysed in cord and peripheral blood in the infants with Luminex or ELISA technique.

Results: The cumulative incidence of eczema was similar, 36% in the treated versus 34% in the placebo group. The L. reuteri group had a lower cumulative incidence of IgE-associated allergic disease, 20% versus 35% (p=0.04), and less IgE-associated eczema during the second year, 8% versus 20% (p=0.02). The prevalence of L. reuteri was higher during the first year of life in stool samples from infants, as well as in colostrum, in the active as compared to the placebo treated group. Colostrum from L. reuteri supplemented mothers had lower levels of TGF-β2, and low levels of this cytokine were associated with less sensitisation. Low Th1- and high Th2-associated chemokine levels preceded allergic disease. The presence of L. reuteri in stool was associated with lower levels of the Th2-associated chemokines CCL17 and CCL22 and higher levels of the Th1-associated CXCL11.

Conclusion: Although a preventive effect of probiotics on infant eczema was not confirmed, the L. reuteri treated infants had lower cumulative incidence of IgE-associated allergic disease at two years of age, and therefore possibly run a reduced risk to develop later respiratory allergic disease. The mechanisms underlying this effect require further elucidation.
Sammanfattning

Bakgrund: En ändrad exponering för mikrober anses åtminstone delvis kunna förklara ökningen av allergisk sjukdom i populationer med västerländsk livsstil. Mikrobers aktivering av immunförsvaret tidigt i livet krävs troligen för en normal utmognad av immunsystemet. Probiotika, levande bakterier som anses främja hälsa vid intag, har föreslagits förebygga allergisk sjukdom hos spädbarn.

Syfte: Syftet med avhandlingen var att utvärdera effekten av oral tillförsel av den probiotiska bakterien *Lactobacillus reuteri* (*L. reuteri*) i spädbarnsålder på utvecklingen av allergisk sjukdom och sensibilisering, och att undersöka mekanismer som skulle kunna liga bakom en eventuell effekt på allergiska manifestationer.

Studiepopulation: Denna avhandling baseras på resultat som erhållits från en prospektiv dubbel-blind placebo-kontrollerad multicenterstudie, som ursprungligen inkluderade 232 familjer med allergisk sjukdom, av vilka 188 fullföljde hela studien.

Metoder: Familjerna rekryterades på mödravårdscentraler, och mammorna fick *L. reuteri* ATCC 55730 (1 x 10⁸ kolonibildande enheter) eller placebo dagligen från graviditetsvecka 36 till förlossningen. Deras barn fortsatte sedan med samma studieprodukt från födelsen till 12 månaders ålder och följdes i ytterligare ett år. Primärt utfall var allergisk sjukdom, med eller utan positivt pricktest eller cirkulerande IgE mot födoämnen. För analysen av bakterier i mammans bröstmjölk och avföring samt i spädbarnets avföring användes konventionella odlingsmetoder. Cytokiner och IgA antikroppar analyserades i bröstmjölk från mamman med ELISA, och Na/K kvoten i bröstmjölk med jonselectiva elektroder. Cirkulerande Th1/Th2-associerade kemokiner analyserades i navelsträngsblod och perifert blod hos spädbarnen med Luminex- eller ELISA-teknik.

Resultat: Incidensen av eksem var lika, 36% i den behandlade mot 34% i placebo gruppen. Dock hade *L. reuteri* gruppen lägre kumulativ incidens av IgE-associerad allergisk sjukdom, 20% mot 35% (p=0.04), och färre barn med IgE-associerat eksem det andra levnadsåret, 8% mot 20% (p=0.02). Förekomsten av *L. reuteri* var högre i den aktiva jämfört med placebo gruppen både i avföring från barnens första levnadsår och i kolostrum. Kolostrum från mammor som fått *L. reuteri* hade lägre nivåer av TGF-β₂, och låga nivåer av denna cytokin var associerat med lägre risk för sensibilisering hos barnen. Låga nivåer av Th1- och höga av Th2-associerade kemokiner första levnadsåret innebar en högre risk att utveckla allergisk sjukdom. Förekomst av *L. reuteri* i avföring var associerat med lägre nivåer av de Th2-associerade kemokinerna CCL17 och CCL22 och högre av Th1-associerade kemokinerna CXCL11.

Slutsats: En förebyggande effekt av probiotika på spädbarnseksen kunde inte bekräftas, men de *L. reuteri* behandlade barnen hade en lägre kumulativ incidens av IgE-associerad allergisk sjukdom, vilket kan innebära att de löper en lägre risk att utveckla allergisk astma och rhinokonjunktivit senare under barnaåren. De bakomliggande mekanismerna till denna effekt behöver utredas vidare.
**ABBREVIATIONS**

ANOVA  Analysis of variance  
APC  antigen presenting cell  
APRIL  a proliferation-inducing ligand  
ARC  allergic rhinoconjunctivitis  
BAL  bronchoalveolar lavage  
BAFF  B cell-activating factor of the TNF family  
CB  cord blood  
CDSA  Clostridium difficile selective agar  
CFU  colony forming units  
CCL5  CC-chemokine ligand 5 [regulated upon activation, normal T cell expressed and secreted (RANTES)]  
CCL11  CC-chemokine ligand 11 (eotaxin-1)  
CCL17  CC-chemokine ligand 17 [thymus and activation-regulated chemokine (TARC)]  
CCL18  CC-chemokine ligand 18 [pulmonary-and activation-regulated chemokine (PARC)]  
CCL22  CC-chemokine ligand 22 [macrophage-derived chemokine (MDC)]  
CXCL9  CXC-chemokine ligand 9 [monokine induced by interferon-γ (MIG)]  
CXCL10  CXC-chemokine ligand 10 [IFN-γ inducible protein 10 (IP-10)]  
CXCL11  CXC-chemokine ligand 11 [IFN-γ inducible T-cell α-chemoattractant (I-TAC)]  
CV  coefficient of variance  
DBPC  double-blind placebo-controlled  
DC  dendritic cell  
DP agar  Colombia agar with dicloxacillin and propionic acid  
ELISA  enzyme-linked immunosorbent assay  
FoxP3  forkhead box P3  
IFN  interferon  
Ig  immunoglobulin
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Descriptive Name</th>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LBS</td>
<td><em>Lactobacillus</em> selection agar</td>
</tr>
<tr>
<td>LCM</td>
<td>lactobacilli coring medium</td>
</tr>
<tr>
<td>LGG</td>
<td><em>Lactobacillus rhamnosus</em> GG</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>MAMP</td>
<td>microbial associated molecular pattern</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MRS</td>
<td>Man-Rogosa-Sharpe agar</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NLR</td>
<td>NOD-like receptor</td>
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<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>OVA</td>
<td>ovalbumin</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>TRFLP</td>
<td>total restriction fragment length polymorphism</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>SCORAD</td>
<td>severity scoring of atopic dermatitis</td>
</tr>
<tr>
<td>SPT</td>
<td>skin prick test</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Tr1</td>
<td>inducible T regulatory cell type 1</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
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**INTRODUCTION**

During the last century, the incidence of allergic diseases has increased dramatically. In the 19th and the beginning of 20th century, allergy was still an uncommon disease and considered to be a problem restricted to the upper class. After an extensive investigation of all the clinics in London 1828, Dr John Bostock, who himself suffered from hay fever, succeeded in finding 27 other patients suffering from the same ailment. Charles Blackley, who in an article from 1873 gave convincing evidence that it was pollen that caused the symptoms of hay fever, also noted that he had never seen a case of "summer catarrh" in the working class. Subsequently, during the 20th century the increase of allergic disease was most prominent in countries with a westernized lifestyle.  

The reason for the increase of allergic disease in affluent societies is not yet clear. Several explanations have been suggested, such as changes in nutrition, timing of allergen exposure and alterations of the exposure to microbes. According to the hygiene hypothesis, a lack of microbial stimulation may affect the maturation of the immune system, resulting in failure of clinical tolerance to harmless antigens, and finally, in the development of allergy. The first to propose this theory were J.W. Gerrard and his colleagues in 1976. They related the lower prevalence of allergic disease in the Metis compared to the white community in Saskatchewan, Canada, to a higher microbial pressure in the Metis community, and proposed: “atopic disease may be the price paid by some members of the white community for their relative freedom from diseases due to viruses, bacteria and helminths”.  

Since then, a wealth of epidemiology studies supporting this theory has been reported. Yet, to confirm the theory, intervention studies are needed. Probiotics, which are live bacteria considered to confer health when ingested, might be appropriate for such interventions.
Review of the literature

General aspects of allergic disease

Allergy is a hypersensitivity reaction initiated by specific immunological mechanisms. There are four types of hypersensitivity reactions. Type I hypersensitivity reactions are mediated by IgE antibodies against soluble antigens, so-called allergens, and induce mast cell activation. Type II and III hypersensitivity reactions involve IgG antibodies against cell surface/matrix associated antigens or soluble antigens, respectively. Type IV hypersensitivity reactions are T-cell mediated. In this thesis, the term allergy is referring to IgE mediated allergy.

Atopy and sensitisation

Atopy is defined as personal and/or familiar tendency to become sensitised and produce IgE antibodies to allergens. The term atopy should not be used until the presence of IgE antibodies has been documented. These antibodies can be demonstrated in vivo with skin prick test or by analysing circulating IgE antibodies. In this aspect, atopy is synonymous with the term sensitisation in allergy research. Allergic disease such as food allergy, infant eczema and asthma are typically caused by IgE-mediated mechanisms. Yet, sensitised individuals can be non-symptomatic, and patients with allergic disease can be non-sensitised, implicating a substantial part of allergic disease is not IgE-mediated. Besides supporting the diagnosis of allergy against a specific allergen, sensitisation is also a reliable predictor in infancy for later development of allergic asthma and rhinoconjuntivitis. Moreover, sensitisation in combination with eczema, i.e. IgE-associated eczema, might enhance the predictive value for subsequent development of respiratory allergic disease. Approximately 60% of sensitised infants with eczema develop asthma in school age compared with 14% of the non-sensitised infants with eczema.
**Eczema, food allergy and asthma**

Eczema, food allergy and asthma are the most common allergic manifestations in the first years of life. The cumulative incidence of infant eczema is approximately 20-30% in an unselected population and 40-50% in infants with a family history of allergic disease. Eczema is defined as pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution. The criteria by Hanifin and Rajka are the most commonly employed in allergy research. Since these criteria are not applicable for infants, several modified criteria have been proposed. The pathogenesis of eczema is multifactorial. During the past years, it has been increasingly well recognised that skin barrier function plays a critical role in the development of eczema. These defects in skin barrier likely result from a combination of factors including deficiency in skin barrier proteins, the lack of certain protease inhibitors and lipid abnormalities. Loss-of-function mutations in the skin structural protein filaggrin are now considered a major risk factor for eczema. Interestingly, filaggrin mutations markedly enhance systemic allergen sensitisation via the skin, supporting the theory that encountering allergen via the skin instead of the mucosa may increase the risk for sensitisation.

Although IgE-mediated sensitisation and Th2 deviation are common features in eczema, recent articles suggest that IgE-mediated reactions are only part of a much more complex immunological picture in eczema. This was supported recently by large epidemiologic study showing only a weak association between sensitisation and eczema. Furthermore, serum levels of IL-31, a pruritogenic Th2 cytokine, correlates with severity of eczema, suggesting a role for T cells in the pathogenesis of pruritus that may be, at least partially, mast cell independent. Thus, prevention measures affecting the development of sensitisation might have a low impact on eczema incidence. On the other hand, prevention strategies may succeed in reducing eczema despite an absent effect on sensitisation and respiratory allergies.

Food hypersensitivity affects about 6-8% of children in affluent countries. A variety of symptoms such as eczema, urticaria, gastrointestinal and respiratory manifestations
are involved. The condition is caused either by IgE-mediated or by non-IgE-mediated mechanisms. Since skin prick test and analyses of circulating IgE often are connected with both false positive and negative results, the gold standard for diagnosis of food allergy is the double-blind placebo-controlled food challenge. The major offending allergens are hen’s egg, cow milk and peanut. Children with other allergic diseases have a higher prevalence of food allergy. About 35% of the children with moderate-to-severe eczema have IgE-mediated food allergy and 6-8% of asthmatic children may have food-induced wheezing. Before the age of five, almost 80% of the children have outgrown their food allergy, i.e. developed tolerance.

Asthma is a chronic inflammatory disease of the airways in which many cell types play a role, in particular mast cells, eosinophils and T lymphocytes. In susceptible individuals the inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung. The diagnosis of asthma in early childhood is challenging, since wheezing and cough is very common even in children who do not have asthma. Most obstructive episodes are triggered by infections in infancy. Whether this recurrent wheezing is mainly a non-allergic condition with a good prognosis or an early onset of allergic asthma is debated. Hospitalisation because of wheeze in infancy and Respiratory Syncytial Virus bronchiolitis (RSV) seems to be risk factor for later asthma in school age and early adulthood. On the other hand, other epidemiological studies indicate that non-sensitised infants without family history of asthma do not run an increased risk of asthma in school age. Thus, wheezing triggered by infection may indicate an inherent vulnerability in the child.

Atopic march
The phenomenon that manifestations of allergic diseases tends to vary with age within the same individual is called the “atopic march.” According to this, eczema and food allergy are typically outgrown and replaced in school age by allergic asthma and rhinoconjuntivitis to inhalant allergens, e.g. dust mite, cat and birch. However, as pointed out above, the pathogenesis of the different allergic diseases is heterogeneous.
For example, a substantial part of food allergy is non-IgE-mediated. Moreover, non-sensitised infants with eczema do not run an increased risk for allergic asthma and rhinoconjunctivitis $^{6-8}$.

In conclusion, the pathogeneses underlying different allergic manifestations are heterogeneous. Hence, although a prevention strategy does have effect on one allergic manifestation, it may fail to reduce the development of another. Sensitisation and IgE-associated eczema in infancy seem to be the most reliable predictors for subsequent respiratory allergic disease in school age.

**Immunological mechanisms**

**Mucosa**

The gastrointestinal tract is the largest immunologic organ in the body and the gut mucosa has a surface of approximately 400 m$^2$. The surface epithelium of the mucosa is constantly exposed to myriads of microbes and dietary constituents.

There are several components in the mucosal barrier (Figure 1). A key component is the production of mucus from goblet cells, creating a thick barrier covering the epithelial cells. Within the mucus layer, there are non-specific, *e.g.* mucins and defensins, and specific secretory components, *i.e.* secretory IgA (sIgA) antibodies, preventing attachment to the underlying epithelium $^{35,36}$. The epithelial cell line also constitutes an effective barrier, as cells are joined together by tight junctions, only allowing ions to pass. Peristalsis, low pH in the stomach, proteolytic enzymes and the normal gut microbiota are also important for the defence against harmful bacteria and breakdown of polypeptides to less immunogenic peptides. Yet, the gut must pursue a delicate balance between an effective barrier against pathogens and foreign structures and its absorptive function of nutrients. There are several routes for the uptake of antigens and microbial components in the gut: *e.g.* via M cells covering organised lymphoid tissue such as Peyer’s patches, via dendritic cells (DC) interspersing the
epithelial cells, or via epithelial cells themselves (Figure 1). Increased antigen uptake is also observed when the permeability of the gut is increased, e.g. during inflammation.

In conclusion, interventions affecting the immune system via the gastrointestinal tract have to be adapted to the mucosal barrier and immune system.
Innate immune system
The innate immune system provides the first line of defence in the body, including the physical barriers of the mucosa as well as the cells responding immediately with phagocytosis of microorganisms, extinction of infected cells and cooperation with adaptive immunity (Figure 1). The innate immune system oversees the gateway to immunity with its microbial sensors. Pattern recognition receptors (PRR) recognise so-called pathogen associated molecular patterns (PAMPs), evolutionary conserved structures from bacteria, viruses, parasites and fungi. The PRRs are expressed on various cells of the immune system such as monocytes, macrophages, DC, NK cells as well as mucosal epithelial and endothelial cells. Examples of PRRs are NOD-like receptor (NLR), RIG-1-like receptor (RLR) and Toll-like receptors (TLR), mannose receptors, β-glucan receptors and other C-type lectins. Examples of TLR ligands are lipoteichoic acid (LTA) on Gram positive bacteria and lipopolysaccharide (LPS) on Gram negative bacteria, binding to the extracellular TLR2 and TLR4, respectively, and CpG, bacterial DNA, that binds to the intracellular TLR9.

Antigen presenting cells (APC), such as DC, are obligatory for activation of naïve T helper cells and their differentiation into different subtypes such as Th1, Th2, Th17 and Treg cells, defined by their cytokine secretion, as described in the next section. Dendritic cells take up antigen, become activated and migrate to the lymphatic tissue and present the peptides to T cells. Microbial stimulation of the DC leads to secretion of cytokines such as the anti-inflammatory IL-10 and Th1-inducing IL-12 and up regulation of co-stimulatory molecules such as CD40, CD80 and CD86. The DC stimulation pattern by bacteria varies profoundly, and bacterial strains differ in their subsequent modulation of T cells via DC priming. For example, two different lactobacilli, *Lactobacillus reuteri* and *Lactobacillus casei*, but not a strain of *Lactobacillus plantarum*, primed monocyte-derived DCs to drive the development of Treg cells. These Treg cells produced increased levels of IL-10 and inhibited the proliferation of bystander T cells in an IL-10–dependent fashion. Furthermore, DC can attract immune cells via the secretion of chemokines. For example, the secretion of
the Th2-associated chemokines CCL17 and CCL18 by DC is important for the recruitment of Th2-cells to the lung during asthma exacerbations 42.

Monocytes are bone marrow derived leukocytes with high CD14 expression, which circulate in the blood. They mature and differentiate into macrophages as they enter the tissue. Monocytes are differentially activated by Gram positive and Gram negative bacteria, via the PRRs TLR2 and TLR4, respectively 43, 44. Macrophages can be divided into M1 and M2 subtypes. In response to bacterial moieties and IFN-γ, M1-macrophages typically produce reactive nitrogen and oxygen intermediates and Th1-associated cytokines and chemokines such as IL-12, CXCL9, CXCL10 and CXCL11 45. The M2-activation, originally discovered as a response to IL-4, typically leads to secretion of the Th2-associated chemokines CCL17 and CCL22 45. However, recent results indicate that subpopulations of M2-polarized macrophages also produce the anti-inflammatory cytokine IL-10 upon stimulation by microbial components, inducing differentiation of regulatory T cells 46.

Macrophages and DC also produce proinflammatory cytokines such as TNF and IL-6 upon PAMP stimulation 47. Besides inducing inflammatory response such as C-reactive protein (CRP) and fever 48, IL-6 has several other important features such as directing the transition from innate to acquired immunity, including Th17 cell differentiation 47,49,50, inducing IgA synthesis in Peyer’s patches 51, and regulating the epidermal barrier after tissue injury 52. In addition, IL-6 might also have anti-inflammatory effects, since it induces the release of IL-10, IL-1 receptor antagonist, and plasma cortisol in humans 53. Interestingly, infants developing allergic disease in infancy have been reported to have lower levels of the IL-6 induced CRP in peripheral blood the first year of life, indicating the importance of innate immune activation in the maturation of the immune system 54.

Although they are not innate immune cells, epithelial cells also contribute to the innate response upon stimulation by PAMPs. Besides production of cytokines, human intestinal epithelium also produces the Th1-associated chemokines CXCL9, CXCL10
and CXCL11 after exposure to gut microbiota, favouring recruitment of Th1 cells to the gut mucosa.

*In conclusion, the innate immune system has a crucial role in the modulation of the adaptive immune response to antigens, and this modulation is influenced by the nature of the initial microbial stimulation.*

**T cells, cytokines and chemokines in allergic disease**

The adaptive immune system is governed by the T helper cells. Upon stimulation, naïve T helper cells differentiate along the Th1, Th2 or Th17 pathway, which differ in cytokine pattern and function. The Th1 lineage, typically producing IFN-γ, is important for the host defence against intracellular pathogens. The Th2 lineage produces IgE-inducing IL-4 and IL-13 and eosinophilia-inducing IL-5 for protection against parasites, while the Th17 lineage, producing IL-17, contributes to the host defence to extracellular bacteria and fungi. On the other hand, in contrast to these protective functions, inappropriate Th2 cell responses give rise to IgE-mediated allergic disease, whereas autoimmune diseases result from inappropriate Th1 and Th17 responses.

Atopy is characterised by Th2 deviated cytokine response to allergens, with high levels of IL-4, IL-5, IL-9 and IL-13, while the Th1 cytokines, e.g. IFN-γ and IL-12, usually are observed at equal or lower levels. Moreover, children developing allergic disease have been reported to have a delayed maturation of the immune system with a decreased allergen induced IFN-γ production at birth and a prolonged postnatal Th2-deviation in childhood. Yet, it is still somewhat controversial whether Th2-deviation increases the risk for allergic disease or not. For example, children in developing countries with a strong Th2-deviation, due to chronic parasite infection, do not run an increased risk for allergic disease. Also, both Th1-associated autoimmune diseases, such as Mb Crohn and diabetes mellitus, and allergic disease have increased in affluent countries.
Albeit a very useful one, the Th1/Th2 paradigm in allergic disease is obviously an oversimplification. There are other subtypes of T cells that are involved in the allergic process. The T regulatory (Treg) cells assemble T cell types that may regulate immune responses via cell to cell interactions and/or production of anti-inflammatory cytokines. There are many different types of Treg cells. Tr1 and Th3 cells are induced in the periphery in an antigen-dependent manner and mediate their immune-suppressive effect mainly via IL-10 and TGF-β dependent mechanisms. T helper 3 cells release TGF-β, while Tr1 cells are defined by their production of IL-10 and TGF-β. In contrast, naturally occurring CD4+CD25+ regulatory cells, are derived from the thymus, and mediate their suppressive effect through ligation of the T cell receptor and cell to cell contact, possibly via IL-35.

T regulatory cells have been proposed to be involved in the suppression of allergen-specific response in several ways, e.g. suppression of APC, Th1 and Th2 effector cells, regulation of B cells resulting in reduced IgE and increased IgG4 and IgA synthesis, and suppression of mast cells, basophils and eosinophils. During specific immunotherapy with allergens, the reduction of allergic symptoms is accompanied with induction of Tr1 cells, suppressing the allergen specific Th1 and Th2 responses via e.g. IL-10 and TGF-β.

Although the increase of allergic disease obviously depends on environmental factors, it has been difficult to link laboratory markers of Th2-deviation to such factors in humans in vivo. This might be a methodology issue. Circulating Th1 and Th2 cytokine levels are very low and close to detection limits, making them less appropriate for discriminating between factors possibly influencing allergy development.

Chemokines, on the other hand, are easily detected in peripheral blood. They comprise a large protein family responsible for the trafficking of leukocytes to the site of inflammation and the regulation of leukocyte maturation. They are produced by several cell types but macrophages are considered to be the most important source.
Their receptors are expressed on the surface of several cell types involved in the allergic inflammation (Figure 2): e.g. CC receptor 4 (CCR4) on Th2 lymphocytes and CXC receptor 3 (CXCR3) on Th1 lymphocytes and natural killer cells. Accordingly, atopic dermatitis has been associated with high circulating levels of the Th2-cytokine induced CCR4 ligands CCL17 and CCL22, as well as CCL18 (unknown receptor), in children and adults. Furthermore, increased levels of CCL17, CCL18 and CCL22 in bronchoalveolar lavage (BAL) fluid have been reported in asthmatics and after allergen challenge.

In contrast, the IFN-γ induced CXCR3 ligands CXCL10 and CXCL11 are associated with Th1-like diseases, such as sarcoidosis, tuberculosis and Crohn’s disease. The CXCR3 ligands have been reported to be stimulated by microbial stimulation. CXCL10 is induced by LPS via TLR4 and are elevated in bacterial sepsis in infants, and enteroinvasive bacteria enhance the production of CXCL9, CXCL10 and CXCL11 from human intestinal epithelium. The role of the CXCR3 ligands in allergic disease is still not clear. Although CXCL10 favoured Th1-like response in lymph nodes in a mouse model, it attracted Th2-cells and eosinophils locally in lung at a late stage of airway inflammation. Moreover, CXCL10 was elevated in BAL from asthmatic patients after allergen exposure. Whether these Th1- and Th2-associated chemokines are primarily involved in the pathogenesis of allergic diseases or merely are secondary to a general immune deviation is still not known. Appropriately powered prospective studies from birth, as well as mechanistic studies, are needed to address these issues.

_In conclusion, interventions affecting either the Th1/Th2 balance or T regulatory cells, or both, may result in a reduced allergy development. Analyses of circulating chemokines offer novel tools to investigate the Th1/Th2 imbalance in allergic disease in vivo and to explore the influence of pre- and postnatal factors in infancy._
Figure 2. The chemokine receptor repertoires of leukocytes and their ligands, implicated in the pathogenesis of allergic diseases. CCR and CXCR are receptors and CCL and CXCL are ligands. The ligands employed in the present study are underlined.
Allergic responses
Allergy can be divided into three phases, the sensitisation phase, immediate hypersensitivity reactions and the late phase reactions. In the sensitisation phase, the antigen presenting cells, i.e. dendritic cells (DC) take up the allergen, process it and present it to T helper cells. Factors important for differentiation of naïve T cells to Th2 cells include the cytokine environment, the dose and route of the allergen and the presence or absence of inflammatory stimuli. The allergen specific Th2 cells induce a B cell switch to production of IgE antibodies through the secretion of the cytokines IL-4 and IL-13. IgE attaches to high affinity FceRI IgE receptors on mast cells in tissue but also to basophils in blood and activated eosinophils. Monocytes, platelets and eosinophils also express IgE receptors but at lower levels. On the next encounter with the allergen, a sensitised individual can develop an allergic immediate hypersensitivity reaction. Crosslinking of the IgE receptors on mast cells by allergens causes an activation of the cells with a subsequent release of chemical mediators such as histamine, chemokines, cytokines, prostaglandins and leukotrienes. These mediators cause an immediate allergic reaction including symptoms such as bronchoconstriction, vascular leakage from blood vessels, itch and tissue destruction. Mediators released by the mast cells and DC also attract and activate other cells such as Th2 cells, eosinophils and basophils, leading to inflammation, which may become chronic, the late phase reaction. Eosinophilia, induced by IL-5, is particularly associated with the late phase reaction.

In conclusion, although the IgE-mediated response is well described, the reason why some children get sensitised and why some sensitised children do not develop symptoms is still an unsolved conundrum.
The influence of environmental factors on the development of allergic disease

During the last century, the incidence of allergic diseases has increased dramatically. However, there is a worldwide variation in prevalence of allergic disease, and the increase seems to be limited to affluent societies, i.e. industrialised countries with market economy. Changes in the genotype cannot explain such a rapid increase. Therefore, the explanation has been sought for in the environment. Several risk factors for allergic disease have been proposed, including exposure to smoke, poorly ventilated homes, air pollution, and reduced breastfeeding. However, none of these factors can explain the large increase in affluent countries, such as Scandinavia, compared with poorer countries, such as the former socialist countries in Europe.

Consequently, factors associated with a Western lifestyle, i.e. diet, household size and improved general living conditions have been proposed to be of importance for these observations, leading to the so called hygiene hypothesis. The hygiene hypothesis proposes that the increase of allergic disease is caused by an alteration of the microbial exposure during childhood.

There has been a dramatic change in diet and the handling of food the last century. The change in how food is conserved and stored, e.g. in refrigerators and freezers, obviously affects the microbial content of the food. The nutritional content of the diet has also changed. For example, epidemiological data show an increase of ω-6 fatty acids intake and a decrease in the intake of ω-3 fatty acids over the past century in Western countries. Several studies indicate that fish, rich in fatty acids, has a protective effect on the development of allergic disease.

Also the timing and the route of allergen exposure may have changed. The first encounter with ubiquitous allergens may occur already before birth. Allergen specific immune responses have been detected in foetal blood already after gestational week 22. It has been hypothesised that allergen exposure during foetal life may be a risk factor for later sensitisation, but intervention with allergen avoidance during
pregnancy have failed to prevent allergic disease. In one study, egg elimination during pregnancy even was associated with prolonged egg intolerance in the offspring. The role of allergen avoidance in infancy as a primary preventive strategy of allergic disease is also under debate, and there are conflicting data regarding any relationship between allergen exposure during childhood and sensitisation.

Recently, it has been suggested that the route of the allergen is crucial, and that sensitisation to allergen occurs through environmental exposure to allergen through the skin, whereas consumption of food allergen, i.e. an encounter via the mucosa, induces oral tolerance. This hypothesis provides a possible explanation for the close link between eczema and the development of food allergies. Moreover, introduction of food allergen in early infancy, even before the weaning of breastfeeding, might induce tolerance, as suggested by a recent report showing that early consumption of peanuts in infancy is associated with a low prevalence of peanut allergy.

**Microbial exposure**

According to the hygiene hypothesis, or with a more appropriate term “the microbial deprivation hypothesis”, an alteration of the microbial exposure may affect the maturation of the immune system, resulting in failure of clinical tolerance development to harmless antigens, and finally, in the development of allergy.

The first to propose this theory were J.W. Gerrard and his colleagues in 1976. They related the lower prevalence of allergic disease in the Metis compared to the white community in Saskatchewan, Canada, to the increased microbial pressure in the Metis community. In 1989, Strachan showed that hay fever and eczema was inversely correlated to the number of siblings in an English population. He suggested that a reduced family size and improved living conditions associates with fewer infections and improved hygiene, and thereby may be responsible for an increased risk of developing allergic disease. Later, it has been shown that respiratory viral infections do not reduce allergy development. Consequently, the research has been focusing on normal gut microbiota and environmental microbial components such as endotoxins.
The development of the gut microbiota in infants

In the 1970s and 1980s, the infantile gut microbiota was characterized with culture-based studies. These studies revealed that the infantile gut microbiota is less complex with higher proportion of facultative bacteria than adult microbiota. Facultative bacteria, such as *E. coli*, enterococci and staphylococci, dominated the first gut microbiota in neonates as the abundance of oxygen in the neonatal gut prevents expansion of obligate anaerobes. When the facultative anaerobes expand, they consume the oxygen, creating an anaerobic environment. This favours the growth of obligate anaerobes such as bifidobacteria, clostridia and *Bacteroides*. With time, successively larger numbers of anaerobic species establish in the infantile gut. Facultatives are outnumbered by anaerobes by hundred-to-thousand-fold in adults. The opportunistic pathogen *C. difficile* also thrive less well and become less frequent. A complex microbiota dominated by obligate anaerobes provides a strong barrier against the establishment and proliferation of new bacterial strains, a phenomenon termed colonisation resistance. Colonisation resistance is poor in neonates, but increases with age. It must be pointed out, however, that most studies rely on analyses of stool samples, thus reflecting the luminal colonic microbiota. The composition of the microbiota of the small bowel is much less investigated. The higher oxygen content in the upper gut might favour facultative bacteria such as streptococci and lactobacilli.

Whether *Lactobacillus* is a common species in the infant gut is debated. The prevalence differs substantially between different studies, approximately from 20% to 90%. This might be a methodology issue, since different analyses have been employed. The specificity of conventional cultivation methods has been questioned. On the other hand, the probes of novel molecular methods might not cover the whole species. The prevalence of lactobacilli may also differ in different cohorts.

During the last decade, the introduction of molecular DNA methodology, such as 16S rRNA target probes, has revolutionised the microbiology field. Yet, the studies...
employing these techniques on infantile microbiota are few and small, especially those reaching down to the species level. The preliminary results confirm the dominance of *Bifidobacterium*, *Clostridium* and *Bacteroides* in the early microbiota. Interestingly, cloning and sequencing revealed only around 10% unidentified species the first two months of life in one study. This might be an underestimation, though, since the current technology for cloning and sequencing without cultivation has low sensitivity, detecting only bacteria whose populations exceed $10^9$/g faeces. Thus the molecular methodology is still premature and much larger studies employing more powerful methods are warranted.

Although differences in methodology preclude direct comparisons with earlier studies, it seems that colonisation by typical faecal bacteria such as *E. coli* and *Bacteroides* has decreased since the 1970s and 1980s in infant stool, indicating a reduced spread of these bacteria in contemporary western hospitals and homes. Instead, staphylococci, especially *S. aureus*, and some clostridia, including *C. difficile*, have become more common. The latter groups of bacteria have been proposed to be able to expand in the gut microbiota of the modern western infant due to reduced competition from more traditional gut microbes.

**Scientific basis for the hygiene hypothesis**

The hygiene hypothesis is supported by epidemiological as well as experimental studies. Animal models have shown that germ free mice have a prolonged Th2-deviation and do not develop oral tolerance against food allergens compared with conventional mice.

A decreased prevalence of allergic diseases has been reported in farming environments, and has been related to reduced endotoxin exposure. Endotoxin (LPS) is a component of the cell wall of Gram negative bacteria. Whether LPS is protective *per se* or if it merely reflects the microbial exposure in general is not known. Recently,
increased LPS exposure was associated with higher diversity of faecal bifidobacteria in Swedish infants \(^{141}\).

Prescription of antibiotics has been associated with an increased risk for allergic disease \(^{110,142}\). This increase seems to be limited to children receiving broad-spectrum antibiotics in early childhood. Interestingly, a recent study reported a higher incidence of asthma in children receiving antibiotics in the neonatal period \(^{143}\). Caesarean section has also been associated with a higher incidence of asthma \(^{144}\), and a delayed colonisation with bifidobacteria and \textit{Bacteroides} \(^{145,146}\).

Early day-care attendance might also be protective against allergic disease and the proposed mechanism is that these infants are more exposed to viral infection and/or commensal bacteria \(^{147,148}\). Furthermore, populations with anthroposophic lifestyles have been reported to have less allergic disease \(^{149,150}\). The anthroposophic life style includes a reduced use of antibiotics and immunisations.

Several studies show differences in the intestinal microbiota between allergic and non-allergic children \(^{112,113,151}\) and between countries with a low and high prevalence of atopic disease. For example, allergic infants have been reported to be colonised less often with \textit{Bacteroides}, bifidobacteria \(^{113,151,152}\), and lactobacilli \(^{141,151}\), and to have lower ratios of bifidobacteria to clostridia \(^{112}\). However, there have been contradictory results in other studies. Recently, two large European prospective studies could not confirm the importance of bifidobacteria and lactobacilli colonisation \(^{153,154}\). The most consistent finding seems to be the higher prevalence of \textit{C. difficile} in infants subsequently developing allergic disease. Recently, a large prospective study showed an increase risk for allergic disease and sensitisation in infants colonised with \textit{C. difficile} the first month of life \(^{155}\). Furthermore, higher IgG antibody levels to \textit{C. difficile} have been observed in allergic infants at one year of age \(^{156}\). These findings are supported by analysis of short fatty acids in stool samples from allergic and non-allergic infants. Iso-caproic acid, a short chain fatty acid associated with \textit{C. difficile} colonisation, was detected almost exclusively in allergic infants \(^{157}\). Yet, it is not
known whether *C. difficile* cause allergic disease *per se*, or if it merely reflects a disturbance of intestinal microbiota.

Whether a change in the diversity of the microbiota is more important than the prevalence of specific bacterial species or strains for the increase of allergic disease in affluent countries is debated. The acquisition and turnover of *E. coli* is much faster in Pakistani compared to Swedish infants. Recently, a study employing molecular techniques, T-RFLP and TTGE, also reported that infants developing eczema and sensitisation by 18 months had a lower diversity at one week of age. On the other hand, species with certain immunological properties might have effects *per se*. Infants colonised with super-antigen producing *S. aureus* the first week of life run a reduced risk of developing food allergy, and supplementation with this super-antigen to mice in a experimental model induced oral tolerance.

In conclusion, an altered microbial exposure in infancy may partly explain the increase of allergic disease in affluent societies. Especially the microbial stimulation during the first months of life seems to be important. Intervention studies are needed, however, to evaluate the relevance of the experimental animal and the epidemiological studies.

**The mother-baby dyad**

The immune system of the foetus and subsequently the newborn infant is influenced by maternal immunity, both during gestation and the period of breastfeeding. Thereby, the mother may influence development of the child’s immune system, not only genetically but also as an environmental factor.

**Gestation**

High levels of the anti-inflammatory cytokine IL-10 and Th2 cytokines such as IL-4 surround the foetomaternal interface during pregnancy, probably in order to divert the
maternal immune responses away from a damaging Th1-mediated response against the foetus \textsuperscript{162}. Thus, from an evolutionary perspective, Th2-skewing during gestation seems to be needed for a successful pregnancy \textsuperscript{162}. The Th2-skewed surrounding might explain why neonatal naïve cells are more easily Th2-skewed compared with adult cells \textsuperscript{163}, and transient early IgE \textsuperscript{164} and Th2 like cytokine responses against allergens are observed in both atopic- and non-atopic children \textsuperscript{63}. Newborns of atopic mothers have higher cord blood IgE levels than those with only paternal or no history of atopy \textsuperscript{165, 166}. Moreover, children of allergic mothers, as compared with children with only paternal history of allergic disease, run an increased risk to develop allergic disease in later life \textsuperscript{167}.

Allergen specific immune responses have been detected in foetal blood already in gestational week 22 \textsuperscript{168}. Possible routes for allergens could be via the amnion \textsuperscript{169, 170} and/or via immune complexes with IgG \textsuperscript{171}. Recently, an epidemiological study reported that maternal exposure to stables, \textit{i.e.} an environment with high microbial exposure, during pregnancy protected against allergic sensitisation, whereas exposures during infancy had weaker effects or no effect at all \textsuperscript{172}. Thus, sensitisation might be associated with immune programming already during the foetal period. This is supported by both experimental animal and human models. Maternal cells have been shown to cross the placenta to reside in foetal lymph nodes, inducing the development of CD4\textsuperscript{+}CD25\textsuperscript{high} FoxP3\textsuperscript{+} T regulatory cells \textsuperscript{173}. Moreover, tolerance was transferred to the offspring from mothers who were tolerised against ovalbumin before conception in a mice model \textsuperscript{174}. This process was dependent on IFN-\gamma production by T memory cells in the offspring.

Maternal IgG antibodies are transferred to the foetus over the placenta and provide the baby with protection against infections the first months of life \textsuperscript{175}. Whether these antibodies also influence the development of allergic disease in the offspring has not been clearly established, but high cord blood levels of IgG antibodies may be associated with less development of allergic disease in school age \textsuperscript{176}.
The amniotic fluid and the foetal gastrointestinal tract have been considered to be sterile. Recently, however, there are reports that amniotic fluid, umbilical cord blood and meconium from the newborn contain bacteria, suggesting that a prenatal mother-to-child efflux of commensal bacteria may exist. If these preliminary and controversial results are correct, they implicate that the gut immune system are exposed to commensal bacteria during gestation.

The origin of the bacteria in breast milk is also discussed. Contamination from the maternal or infantile gastrointestinal tract externally has been considered to be the most plausible explanation. However, bacterial translocation, or at least bacterial DNA and antigen, from the gut endogenously, has been suggested recently. Furthermore, the bifidobacteria counts in maternal faeces at gestational week 35 have been reported to correlate with the count in breast milk and subsequently with the faecal *Bifidobacterium* levels of the offspring, suggesting breast-milk bacteria as an important source of bacteria in the establishment of infantile intestinal microbiota.

**Breastfeeding**

Breast milk not only provides the necessary nutrients for growth and development, it also contains numerous immunological components compensating the immature and inexperienced neonatal mucosal immune system. Such components include immune cells, antibodies (especially IgA antibodies), pro- and anti-inflammatory cytokines such as TNF, IL-10 and TGF-β, and factors that may modify immune responses to bacteria, e.g., soluble CD14 (sCD14). The immunological composition of breast milk differs considerably between mothers, however, and the factors contributing to the precise composition is not fully understood. Maternal allergy, infections, inflammation, stress and supplementation of fish oil and probiotics during pregnancy have all been suggested to affect the composition of breast milk.

Nutritional, metabolic and immunological processes in the gut are reflected in the mammary gland and the milk through the enteromammary link. Regarding specific
IgA in breast milk, antigens from the mother’s gut are taken up by M-cells and passed to the antigen presenting cells for Th cell activation after antigen processing and presentation. IgA switching is then induced in antigen specific B cells with help of T helper cells. Recent data indicates also that DCs can induce IgA switching in a T cell-independent fashion. Recognition of bacterial signatures by TLRs at the intestinal epithelial barrier induces the release of innate IgA switch-inducing factors such as BAFF and APRIL by DC and epithelial cells. This T cell-independent pathway preferentially yields low-affinity, polyreactive IgA antibodies to commensal bacteria. Subsequently, the B cells migrate to mucosal membranes and exocrine glands, e.g. mammary glands, and secrete the specific IgA. The secreted IgA enter the breast milk via a receptor mediated transport and may prevent antigens and bacteria to penetrate the baby’s gut.

The controversial results regarding the allergy preventive role of breast feeding have been suggested to be, at least partly, due to differences in breast milk composition. For instance, low levels of breast milk IgA and TGF-β have been suggested to increase the risk for allergy development in the child, although this is still controversial.

**Epigenetics**

Epigenetics may be defined as a change in state of the expression of a gene that does not involve a mutation, but is nevertheless inherited in the absence of the event that initiated the change. Thus, epigenetic information is not encoded by changes in the sequence of the DNA but by differential methylation of the DNA and modifications of chromatin, affecting whether, when and to what level specific genes are expressed in a given cell. Because the DNA sequence remains unchanged, epigenetic modifications can be heritable but plastic. Thus, the progeny cells retain the potential for change in response to altered environmental signals. Recent data suggest that epigenetic processes help to regulate the fate and function of T regulatory cells as well as Th1, Th2 and Th17 cells.
Consequently, microbial exposures to the mother during her childhood may influence the immune system of her offspring. Analyses in a birth cohort study revealed an inverse association of cord blood sensitisation to seasonal allergens with established maternal immunity against rubella and *Toxoplasma gondii*\(^{203}\). Moreover, a study assessing cytokines in breast milk among mothers resident in Sweden, of whom half of them were immigrants, revealed that the maternal country of birth influenced the breast milk composition\(^{204}\).

**In conclusion, the mother may influence the child’s immune system, not only genetically but also as an environmental factor. Microbial exposures to the mother during gestation, or even before conception, may affect the immune system of her offspring. Sensitisation might be associated with immune programming already during the fetal period. Therefore, the gestational period might be crucial in allergy prevention strategies.**

**Probiotics**

Fermenting foods to enhance their taste and nutritional value is an ancient and widespread practice. A century ago, the Nobel Prize winner Elie Metchnikoff proposed that soured milk could antagonise harmful bacteria in the lower gut and that regular ingestion of soured milk impacted upon the longevity of Bulgarians. His contemporary, Henri Tissier demonstrated that bifidobacteria were predominant in the gut microbiota of breastfed infants. He then proposed that administration of these bifid bacteria could restore gut microbial balance and resolve diarrheal disease. The concept of probiotics was born\(^{205}\).

According to the latest revision, the FAO/WHO defines probiotics as “live microorganisms which when ingested in adequate amounts confer a beneficial effect on the host”\(^{206}\). The most commonly used species are lactobacilli and bifidobacteria, but other bacterial strains have also been used as well as the yeast *Saccharomyces*
**Review of the literature**

*B. boulardi*. Lactobacilli are non-sporing gram-positive rods and belong to the Lactic Acid Bacteria (LAB), including several bacterial genus such as *Streptococcus*, *Enterococcus* and *Lactococcus* \(^{207}\). The genus *Lactobacillus* is heterogeneous with over 60 species. Before 1990, the taxonomy was based on phenotypic analysis, *e.g.* their fermentative characteristics. Even before 1990, the taxonomy developed rapidly. Species were renamed or divided into new one based on their phenotypic characteristics. However, the introduction of molecular methods, such as 16S rRNA analyses, has made comparison with older reports even more difficult, since new groupings cross established taxonomic lines \(^{207}\).

In 2002, a joint FAO/WHO working group launched guidelines regarding evaluation of bacterial strains and defined data needed to be available to substantiate health claims as a probiotic strain (Table 1) \(^{208}\).

**Table 1. FAO/WHO guidelines for evaluation of probiotic strains**

- Identification to the genus, species and strain level by phenotypic and genotypic methods
- *In vitro* tests of resistance to gastric acidity and bile acids, adherence properties, antimicrobial activity, ability to reduce pathogen adhesion to surfaces and bile salt hydrolase activity
- Determination of antibiotic resistance patterns
- Assessment of certain metabolic activities, *e.g.* D-lactate production and bile salt deconjugation
- Assessment of side-effects in human studies
- Post-market surveillance of adverse effects in consumers
Safety
Lactobacilli and bifidobacteria are generally considered to be safe\textsuperscript{208}. They have been used in various types of foods for a long time, and they rarely cause infections in humans. The numbers of reported lactobacilli-induced bacteremia have not increased, despite the rapid increase of probiotic use the last decade\textsuperscript{209}. In a Swedish study, the incidence of lactobacilli-induced bacteremia and the presence in blood cultures of three commercially available probiotic strains were followed for five years. The incidence of bacteremia caused by lactobacilli constituted $<$1% of the total number of bacteremia cases with no increase during this five year-period. Lactobacilli-induced bacteremia was not caused by any of the three commercially available strains in any of the reported cases\textsuperscript{210}. In a Finnish study, 89 patients with lactobacilli sepsis were identified between 1990 and 2000. The most common species were \textit{L. rhamnosus} (28%), including \textit{L. rhamnosus GG} (12%), \textit{L. fermentum} (10%) and \textit{L. casei} (8%). No sepsis was caused by \textit{L. reuteri}. In 82% of the cases there was an underlying severe condition such as malignancies or serious gastrointestinal disorders. The mortality within one month was 26%, and the mortality was reduced if adequate antibiotics were employed\textsuperscript{211}.

It has been suggested that probiotics should be used with caution in patients who are immunocompromised, have cardiac valvular disease, short bowel, jejunostomy or a central venous catheter\textsuperscript{212,213}. Recently, a Dutch double-blind placebo controlled (DBPC) study in patients with severe acute pancreatitis revealed a higher mortality rate in the probiotic treated than in the placebo group\textsuperscript{214}. Notably, the formula consisted of six different species in a relatively high dose of $10^{10}$ bacteria per day\textsuperscript{214}. No severe adverse events have been reported in any of the intervention studies performed in full term neonates and healthy infants\textsuperscript{215-217}.

Concerns have been raised that probiotic strains can potentially act as reservoirs of antibiotic resistance genes\textsuperscript{218,219}. Lactobacilli are consistently resistant to vancomycin, but this resistance gene is intrinsic and is assumed to be non-transferable. They are also often resistant to cephalosporins and penicillins, but usually sensitive to
erythromycin, clindamycin, imipenem and aminoglycosides. The EU PROSAFE project recommended that no future probiotics should contain known antibiotic resistance traits. Recently, plasmids carrying resistance genes against tetracycline and linkomycin were removed from a commercial probiotic strain, *L. reuteri* ATCC 55730, resulting in the daughter strain *L. reuteri* DSM 17938.

**Proposed mechanisms of probiotics**

Proposed modes of action by probiotics include degradation of ingested food protein, improved intestinal barrier function, effects on the gut microbiota, and influence on the gut immune system.

Probiotic bacteria have been reported to enhance murine and human intestinal epithelial barrier function in experimental models. In an intervention trial, in which a probiotic product consisting of a *L. rhamnosus* and a *L. reuteri* strain reduced the symptoms of eczema, the probiotic treatment also enhanced the intestinal barrier.

Human *in vitro* studies have indicated that lactobacilli stimulate Th1-like responses with IFN-γ, IL-12 and IL-18 activation in human PBMC and monocytes. Furthermore, lactobacilli can promote the Th1-inducing capacity of human DCs and reduce allergen specific Th2 cytokine production from PBMCs of allergic individuals. However, lactobacilli strains seem to differ in their immunological properties. For example, substantial differences were found among strains in the capacity to induce IL-12 and TNF production in murine DCs in *in vitro*. Interestingly, a *L. reuteri* strain, a poor IL-12 inducer in this study, inhibited IL-12, IL-6 and TNF induction by *L. casei*, while IL-10 production remained unaltered. Moreover, other strains of *L. reuteri* and *L. casei*, but not a strain of *L. plantarum*, have been reported to prime human monocyte-derived DCs to drive the development of Treg cells *in vitro*. These Treg cells inhibited the proliferation of bystander T cells in an IL-10-dependent fashion. The study further implied that the *L. reuteri* and *L. casei* strains targeted the C-type lectin DC-SIGN on the DCs. Furthermore, live but
not killed *L. reuteri* up-regulated the anti-inflammatory Nerve growth factor (NGF) and inhibited TNF and *Salmonella* induced IL-8 synthesis by human epithelial cell lines \(^{235}\). The effect required pre-incubation with the lactobacilli.

Several animal models have suggested that lactobacilli induce Th1 polarization *in vivo*. In ovalbumin-primed mice orally fed with lactobacilli, IgE to ovalbumin was down-regulated through induction of IL-12 and IFN-γ and suppression of IL-4 and IL-5 \(^{236-238}\). Other murine models have revealed anti-inflammatory properties of lactobacilli. The reduction of eczema by administration with *L. rhamnosus* GG in a mice model was accompanied with elevated IL-10 levels in lymph nodes and Peyer’s patches \(^{239}\). *Lactobacillus* GG also induced murine T regulatory Fox P3+ and TGF-β+ cells and reduced sensitisation and airway inflammation \(^{240}\). Interestingly, *L. rhamnosus* GG administrated to mice during pregnancy also reduced airway response in the offspring \(^{241}\). Although placental TNF expression was enhanced, TNF, IL-5, IFN-γ and IL-10 were decreased in the spleen of the offspring of the LGG treated mice in this study. Moreover, a *L. reuteri* but not a *L. salivarius* strain, attenuated influx of murine eosinophils into the airways and reduced airway responses in a mice model via an TLR9 dependent mechanism \(^{242}\), which was associated with increased percentage of CD4+CD25+FoxP3+ T regulatory cells in the spleen \(^{243}\). Lactobacilli, including *L. reuteri* strains, have also been reported to diminish inflammatory bowel disease in murine models \(^{244, 245}\). Interestingly, TLR9 signaling has been reported to mediate the anti-inflammatory effects in murine experimental colitis \(^{246}\).

Immunological analyses have also been performed in some of the intervention studies with lactobacilli in infants with eczema. The clinical effects in these studies have been attributed to increased IFN-γ production by PBMC from the lactobacilli treated infants \(^{247, 248}\). In one of these studies, *L. rhamnosus* GG increased IL-6, CRP and soluble E-selectin in plasma *in vivo*, whereas another probiotic study product, comprising four different strains, increased IL-10 \(^{249}\).
Recently, elevated circulating CRP, IL-10 and IgA levels were also associated with probiotic supplementation to infants in an allergy prevention study. Reduced CRP levels were also noted in infants developing eczema in this study. Moreover, neonates of mothers supplemented with a \textit{L. rhamnosus} strain during pregnancy had elevated levels of IFN-\(\gamma\) in cord blood in another allergy prevention study. Subsequently, infants in the probiotic group had a lower incidence of eczema until two years of age in this study. Probiotic supplementation has also been reported to increase vaccine antibody responses in infants in intervention studies. On the other hand, in an allergy prevention study without any effect on allergic disease, there was no effect on immunological parameters such as regulatory markers, innate immune function or allergen specific response.

Another mode of action of probiotics could be an indirect effect on the immune system and/or the intestinal barrier through an influence on the composition of the intestinal microbiota. For example, \textit{L. reuteri} strains produce the antimicrobial metabolite reuterin and inhibit pathogenic bacteria, without inhibiting normal bacterial residents of the gastrointestinal tract \textit{in vitro}. There is, however, no evidence that probiotic administration affect the composition of the gut microbiota \textit{in vivo} in human studies.

**Probiotics in clinical trials**

Several clinical trials have evaluated the efficacy of probiotics in the treatment of infectious diarrhoea. A Cochrane review in 2004 identified 23 randomised controlled trials with a total of 1917 participants, evaluating probiotic treatment of infectious diarrhoea. Probiotics reduced the risk of having diarrhoea at the third day after onset and the mean duration of diarrhoea with about one day. The authors concluded that probiotics appear to be a useful addition to oral rehydration therapy in the treatment of acute infectious diarrhoea in children and adult. There are also promising results from prevention trials in preterms. A recent meta-analysis indicated that probiotics might reduce the risk for necrotising enterocolitis and mortality in preterm neonates with less than 33 weeks gestation. On the other hand, although animal studies have
been promising, meta-analyses on probiotic treatment of inflammatory bowel disease (IDB) have been discouraging so far 259, 260.

The initial intervention trials with probiotics in treatment of eczema yielded promising results with reduced symptoms in the treated infants 228, 261-263. Subsequently, these initial results have not been confirmed, and in the most recent meta-analysis, it was concluded that the current evidence suggests that probiotics are not an effective treatment for eczema 215.

Hitherto, six allergy prevention studies with probiotics have been reported 20, 251, 264-267, besides the study included in this thesis 268. Three randomised controlled trials have demonstrated a lower incidence of eczema after perinatal administration of probiotics 20, 251, 264, whereas three studies demonstrated no preventive effect 265-267. These allergy prevention studies will be discussed more thoroughly in the “Results and Discussion” section below. In a Cochrane report in 2007, comprising four of these seven trials, it was concluded that there is insufficient evidence to recommend the addition of probiotics to infant feeds for prevention of allergic disease 217.

In conclusion, probiotics possess characteristics making them appropriate candidates for intervention studies addressing the microbial deprivation hypothesis. Yet, there is still insufficient evidence to recommend probiotics for allergy prevention.

**Lactobacillus reuteri**

*Lactobacillus reuteri* is an obligate heterofermentative 207 Gram positive rod that has been isolated from the GI tract in several mammals, including humans, as well as from different food products 124, 269-271. In addition of glycerol, *L. reuteri* produce the antimicrobial metabolite reuterin during anaerobic conditions 272. Hitherto, it has not been possible to distinguish different *L. reuteri* strains from each other with molecular 16S rRNA technique.
**Lactobacillus reuteri** strains have been reported to affect the immune response in several *in vitro* and animal studies and seem to possess significant anti-inflammatory properties, as reviewed in the previous section.

The strain *L. reuteri* ATCC 55730, which was employed in the actual study, was originally isolated from the breast milk of a Peruvian mother (personal communication, BioGaia AB, Stockholm, Sweden). Safety and tolerance have been evaluated in healthy individuals, with doses up to $1 \times 10^{11}$ bacteria/dose\(^2\), as well as immune-compromised adults\(^3\). Safety assessments have also been included in clinical trials with children\(^4-8\). No severe adverse events have been reported, and all standard blood laboratory parameters were within the normal range in the study on healthy adults\(^9\).

Administration of doses from $1 \times 10^8$ bacteria and higher have been reported to lead to efficient colonisation in human trials, but the colonisation is transient and lost after two months wash-out\(^10,11\). In all these studies conventional cultivation methods were employed. In one of the studies *L. reuteri* was also identified in intestinal biopsy specimen by using fluorescence in situ hybridization with a molecular beacon probe\(^12\). The method section in this report, however, referred to an unpublished manuscript. I have therefore asked for the manuscript and consulted a microbiologist in a research group at the Swedish University of Agricultural Science, who previously has performed research on *L. reuteri*\(^13,14,15-18\). He concluded that the method was accurate (personal communication, Stefan Roos).

Recently, the survival of *L. reuteri* ATCC 55730 after a sudden shift in environmental acidity to a pH close to the conditions in the human stomach was examined. More than 80% of the *L. reuteri* cells survived at pH 2.7 for one hour\(^19\).

A preparation consisting of freeze-dried *L. reuteri* ATCC 55730 suspended in refined coconut and peanut oil has been developed for diet supplementation to infants. The daily intake, five oil droplets, corresponds to $1 \times 10^8$ colony forming units (CFU).
Clinical studies have confirmed beneficial effects in acute diarrhoea in children\textsuperscript{275, 276, 287} and reduction of infections in a day-care setting\textsuperscript{277}. Furthermore, administration of this strain have been reported to improve feeding tolerance in formula fed premature neonates\textsuperscript{280} and colicky symptoms in breastfed infants\textsuperscript{279}. The colic study was not blinded, however, and has to be confirmed by a double-blind placebo-controlled study.

\textit{In conclusion, \textit{L. reuteri} possesses immunological properties which hypothetically might favour tolerance development and reduction of allergic disease. The strain \textit{Lactobacillus reuteri} ATCC 55730 is considered to be safe, is easy to administer to infants and has had a documented effect in clinical trials in children. Thus, \textit{L. reuteri} ATCC 55730 was an appropriate candidate in a prospective allergy prevention trial in early childhood.}
Aims of the thesis

The general aim of this thesis was to assess the effect of oral supplementation with *Lactobacillus reuteri* in infancy on the development of allergic disease and sensitisation during the first two years of life and to examine possible mechanisms underlying the effect on allergic manifestations.

The specific aims of each individual paper were:

I  To address if eczema and sensitisation could be prevented in infants with a family history of allergic disease by oral supplementation with *Lactobacillus reuteri*.

II  To identify factors affecting the prevalence of *Lactobacillus reuteri* in maternal faeces and breast milk and infant faeces after oral supplementation of this probiotic bacterium and to assess the influence on microbial ecology, particularly *Clostridium difficile* and *Bifidobacterium* colonisation.

III  To evaluate the effect of *Lactobacillus reuteri* supplementation on the immunological composition of breast milk in relation to sensitisation and eczema in the babies.

IV  To relate circulating Th1- and Th2-associated chemokines to the development of allergic disease, pre- and postnatal factors and probiotic supplementation in infancy.
Material and Methods

Study design

The results of the all papers included in this thesis are based on a prospective DBPC multi-centre trial conducted at the Department of Paediatrics in the county hospitals of Jönköping, Motala and Norrköping and the University Hospital in Linköping in South Eastern Sweden. Between January 2001 and April 2003, 282 families with allergic disease (i.e. one or more family members with eczema, asthma, gastrointestinal allergy, allergic urticaria, or allergic rhinoconjunctivitis) were asked at antenatal clinics to participate in the study. The family history of allergic disease was obtained by a structured interview preceding the inclusion of the family. In total 232 of these families were enrolled to the study. The reasons for absent inclusion are displayed in Figure 3.

Randomisation was stratified for each study centre. Each centre was provided an allocation list with unique ID-numbers for each subject. Prior to the delivery, each bottle was labelled and randomly mixed by an independent contract manufacturer. The code was kept in sealed envelopes at the investigator site until the end of the study. The material was double-blinded until the statistical analyses were completed for Paper I-III.

The mothers started taking L. reuteri or placebo four weeks before term and continued daily until delivery. The duration of the maternal supplementation was similar in the two study groups [median (range; 95% CI): 4.0 weeks (0-7; 3.5-4.1) vs. 4.0 weeks (1-7; 3.5-4.1), p=0.84]. After birth, within 1-3 days, the baby continued with the same study product as the mother, daily up to 12 months of age. Neonates admitted to the neonatal ward during the first week of life were excluded from the study. The infants were followed until two years of age. Follow-up visits was performed by research nurses at 1, 3, 6, 12 and 24 months of age, and structured telephone interviews with parents at 2, 4, 5, 8, 10 and 18 months. A final follow up was done by a paediatrician at two years of age.
Material and Methods

Figure 3. Participation rate and reason for absent inclusion and discontinuation.

Six out of the 17 families not meeting the inclusion criteria did not have an allergic disease, while nine had coconut allergy and two were expecting twins. Sixteen out of the 19 families declining discontinuation gave no reason, while three (two in the L. reuteri group) did so because of abdominal discomfort/colic.

In total 188 infants completed the study, 95 in the probiotic and 93 in the placebo group. Sixty-six out of 74 completed the study in Jönköping, 39 out of 55 in Linköping, 29 out of 32 in Motala and 54 out of 71 in Norrköping. The participation rate and the reason for discontinuation are displayed in Figure 3.

Families were requested not to use any other probiotic products during the study period and received a list of available probiotics on the market. Compliance to the
treatment regime was assessed by interviews at each visit or scheduled telephone interview and by collecting used study product bottles. Insufficient compliance, i.e. a consumption of the study product below half of the expected, led to the exclusion of the participant from the study. The mothers were encouraged to breastfeed. At weaning, their babies were offered a hypoallergenic whey hydrolysate, Profylac™ (ALK, Hørsholm, Denmark), as formula, until 6 months of age. Thirty-nine (41%) in the L reuteri and 43 (47%) in the placebo group received Profylac™.

The lactobacillus preparation consisted of freeze-dried L. reuteri (strain American Type Culture Collection 55730, BioGaia AB, Stockholm, Sweden), suspended in 3/4 refined coconut oil and 1/4 refined peanut oil containing cryo-protective components. The refined oil did not contain peanut proteins (detection level < 0.005%). As the content of coconut protein was unknown, nine mothers who reported coconut allergy were not included in the study. The daily intake, five oil droplets, corresponded to $1 \times 10^8$ colony forming units (CFU). The placebo consisted of the same oil without any bacteria and was not possible to differentiate from the active product by smell, taste or visual appearance.

**Study subjects**

**Paper I**

All families completing the DBPC trial were included in this paper. Infants were excluded from subanalyses, if data were missing from any time point until two years age. This was the case for the analyses of sensitisation and IgE-associated allergic disease, since blood samples were not obtained from all infants. For the analyses of the cumulative incidence of these manifestations until two years of age, 75 in the L. reuteri and 75 in the placebo group were available. For the analyses of the point prevalence of IgE-associated eczema at two years of age 88 in the L. reuteri and 85 in the placebo group were available.
Paper II
All families completing the DBPC trial were included in this paper. Stool samples were obtained from the mothers at gestational week 35 and the infants at 5-6 days, 1, 3, 6, 12 and 24 months of age. Breast milk samples were obtained at 1-3 days (colostrum) and one month postpartum. Stool samples were not available from all mothers and infants at all sampling occasions. The number of analysed samples at each age is displayed in Table 1 and 2 in Paper II. At least three samples, however, were analysed from 159 of the infants (85%) during their first year of life, 82 (86%) in the active and 77 (83%) in the placebo group.

Paper III
One hundred and nine mother/infant pairs from the DBPC study were included in this paper. Inclusion was based on the availability of colostrum samples, obtained within the first three days after delivery, and mature milk samples, obtained one month after delivery, when the laboratory analyses started. Fifty-four of the mothers belonged to the *L. reuteri* group and 55 to the placebo group. Fifty-one of the mother/infants pair in the *L. reuteri* and 53 in the placebo group completed the study until two years of age.

In Table 1, paper III, too few infants were included in the statistical analyses of descriptive data. The correct number should be 51 in the *L. reuteri* and 53 in the placebo group. The statistical analyses have been remade with the right number, and still there were no significant differences between the study groups.

Paper IV
The chemokine analyses in this paper were confined to 179 infants in the DBPC trial, from whom blood was available from any time point during the first two years of life. Venous blood was collected from the umbilical cord (n=109) and at 6 (n=104), 12 (n=116) and 24 months (n=123). The samples consisted of both plasma (n=205) and
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serum (n=233) samples. Ninety children were supplemented with probiotics and 89 received placebo.

In this paper, there were also analyses relating allergic manifestation to different exposures, as a complement to the results in Paper I. In these analyses, all 188 infants completing the DBPC trial were included.

Clinical methodology

The clinical evaluation was similar in Paper I-IV. The visits consisted of structured interviews related to symptoms of allergic disease, adverse events, infections, use of antibiotics and possible confounding factors as well as an inspection of the skin. The SCORAD index was used to assess the severity of the eczema. Records from primary care units, private paediatricians and the paediatric clinics were examined if the parents reported that the child had been seen by a physician. All children with suspected eczema were re-examined by a physician belonging to the study team.

Diagnostic criteria

Allergic disease included eczema, gastrointestinal allergy, asthma, allergic rhinoconjunctivitis (ARC) and allergic urticaria. Eczema was defined as a pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution, as suggested by Hanifin and Rajka and modified by Seymour for infants. Since all the infants had a family history of allergic disease, the criteria were further modified as displayed in Table I in Paper I. Internal workshops were arranged for the investigators in the study before the study commenced in order to achieve uniform diagnostic criteria of eczema in all the study centres. Eczema was classified as IgE-associated if the infant was also sensitised.

Wheeze was defined as an episode with obstructive airway symptoms. Asthma was defined as ≥ 3 wheezing episodes, at least once verified by a physician.
A diagnosis of ARC required watery discharge at least twice in contact with the same allergen and no signs of infection. Urticaria was defined as allergic when appearing at least twice in conjunction with a certain food. A diagnosis of gastrointestinal allergy required vomiting, diarrhoea or systemic reaction after ingestion of a potentially allergenic food and a confirmation by challenge, unless there was a clear history of a severe systemic reaction.

Infants were regarded as sensitised if they had at least one positive SPT and/or detectable circulating allergen specific IgE antibodies against food allergens. Allergic disease was classified as IgE-associated if the symptomatic infant also was sensitised.

**Laboratory methodology**

The laboratory methods used in the papers included in this thesis are listed below. The methods are described in more detail in the “Material and Methods” sections in the respective papers.

- **ELISA**
  Analyses of cytokines, chemokines, IgA and sCD14 (paper III and IV)
- **Luminex**
  Analyses of chemokines (paper IV)
- **UniCap**
  Total IgE, circulating IgE against allergens (paper I, III and IV)
- **Skin prick testing** (paper I, III and IV)
- **Conventional cultivation of bacteria** (II, III and IV)
- **Ion selective electrodes**
  Sodium and potassium levels in breast milk (paper III)
**Statistical methods**

The power calculation to estimate the sample size needed to detect true differences between the *L. reuteri* and the placebo group was made based upon an anticipated 40% frequency of allergic disease in the placebo group at two years of age. With at least 91 subjects in each group, a 50% reduction in frequency of allergic disease could be detected at a 5% level of significance with 80% power. With a drop out frequency of 20%, a total sample size of 228 families was desirable.

The $\chi^2$ test was used to compare the prevalence of outcome variables and background factors between the groups. Fisher’s exact test was used when the expected frequency for any cell was less than five. Logistic regression was made for adjusting for the influence on prevalence figures from possible confounders. For variables that were not normally distributed, such as SCORAD scores, the bacterial count and for most of the laboratory variables, correlations were analysed with Spearman’s rank order correlation coefficient test, comparisons between unpaired groups with Mann–Whitney U-test, and paired groups with Wilcoxon signed-rank test. When values were undetectable, they were given the value of half the cut off before the statistical analyses. It was indicated when student t-test was employed. ANOVA was used for adjusting influences on continuous variables from possible confounders. Friedman’s test was employed in analyses of multiple longitudinal measures obtained from the same subject. Repeated-measures ANOVA was used in analyses of multiple longitudinal measures of specific chemokines in subjects in two different groups. When a sample was missing from a subject at any age, the value corresponding to the median value for the specific chemokine at that age and group was given before repeated-measures ANOVA was performed.

Only families that completed the study were included in the analyses in paper I, II and IV. In the analyses of breast milk in paper III, all available samples were included, since the cytokine levels in breast milk were primary variables in this paper. As the mother received the study product in late pregnancy, the effect of the treatment was
also assessed by stratifying by parental status of allergic disease. A probability level of <0.05 was considered to be statistically significant.

The calculations were performed with the computer program Stata version 8.2 (Stata Corp LP, Texas, USA) in Paper I-III, StatView version 5.9 for PC (SAS Institute Inc. North Carolina, USA) in Paper III and SPSS 16.0 for Windows (SPSS Inc, Chicago, IL, USA) in Paper IV.

**Ethical considerations**

Research including children is crucial in order to achieve knowledge of possible causes underlying the development of allergic diseases and finding preventive strategies in this age group. Commercial probiotic products are widely used and are claimed to have beneficial effect on a wide range of diseases without thorough evidence. Intervention studies are needed to refute or confirm their suggested effects. We did not perceive any major health hazard with this study design. *L. reuteri* is considered to be safe and could conceivably be used for simple, safe and effective prevention of allergic disease. An informed consent was obtained from both parents before inclusion. Pain connected with blood sampling was minimised with topical anesthesia. The close medical surveillance may also have been a potential benefit for the infants. We concluded that the potential benefits outweighed the discomfort to the infants and their families. The Regional Ethics Committee for Human Research at Linköping University approved the study.
Results and discussion

The results will be discussed in detail below. Briefly, the cumulative incidence of eczema was similar, 36% in the treated versus 34% in the placebo group. The *L. reuteri* group had a lower cumulative incidence of IgE-associated allergic disease, 20% versus 35% (p=0.04), and less IgE-associated eczema during the second year, 8% versus 20% (p=0.02). The prevalence of *L. reuteri* was higher during the first year of life in stool samples from infants, as well as in colostrum, in the active as compared with the placebo treated group. Colostrum from *L. reuteri* supplemented mothers had reduced levels of TGF-β2, and low levels of this cytokine were associated with less sensitisation. Low Th1- and high Th2-associated chemokine levels preceded allergic disease. The presence of *L. reuteri* in stool during the first year of life was associated with lower levels of the Th2-associated chemokines CCL17 and CCL22 and higher levels of the Th1-associated chemokine CXCL11.

Methodological aspects

Study design

The DBPC design meant a reduce risk for distortion by systematic and random bias, which was supported by the fact that the randomisation seemed to be balanced with similar baseline characteristics in the two treatment groups (Table II, Paper I). Moreover, the adjustments for potential confounders did not affect the outcome of the primary variables (Paper I). Day-care attendance was not included in the adjustment model in paper I, but inclusion with this variable did not affect the comparison between the study groups significantly with regard of the incidence of eczema (OR=1.04, p=0.91), IgE-associated allergic disease (OR=0.42, p=0.04), IgE-associated eczema (OR=0.48, p=0.09) and any SPT (OR=0.47, p=0.05) or the prevalence of IgE-associated eczema at 24 months (OR=0.26, p=0.01). An advantage with the study design was the close surveillance of the infants, especially the first year of life, which enhanced the reliability of the clinical data. It reduced the risk for recall bias for such information as infections, antibiotics, breastfeeding, day-care etc.
The power of the study became sufficient for the incidence of eczema as well as for IgE-associated allergic disease. However, the incidence of IgE-associated eczema was too low for sufficient power, which might explain why this analysis did not reach statistical significance. The daily dose was lower in our study (1 x 10^8 CFU) compared with those prevention studies showing an effect on eczema with probiotics (1 x 10^9 - 1 x 10^10 CFU, Table 3). In a previous study on gastroenteritis, the effect of *L. reuteri* was dose dependent. Thus, it cannot be excluded that a higher dose of *L. reuteri* may also have had an effect on eczema in non-sensitised infants and not only in sensitised ones as in our study.

Because both the mother and her offspring were supplemented with the study product, it is impossible to conclude what period was most important for the outcome. However, since cord blood, colostrum and maternal stool samples were obtained, influences by the maternal supplementation on the immunological parameters could be evaluated. Nevertheless, new intervention trials with supplementation only during pregnancy are warranted.

**Diagnosis of allergic disease**

The allergic diseases are heterogeneous as was pointed out in the “Review of the literature” section. Eczema is the dominating allergic disease in infants and, consequently, has a decisive role in this trial. However, the pathogenesis of eczema is multifactorial and heterogeneous. In a community based cohort, such as in our study, the severity of eczema is much lower than in a cohort based on referrals to a paediatrician. The low SCORAD scores in our study are consistent with that. Furthermore, a substantial part of the infant eczema is non-IgE mediated. Since non-sensitised infants with eczema do not run increased risk for later respiratory allergic disease, it has been questioned whether such eczema belong to the allergic diseases. Thus the validity of infant eczema for predicting subsequent allergic disease may be insufficient.
The eczema criteria available at the time of planning of this study did not distinguish between eczema with different underlying pathogenesis. We modified the criteria of Hanifin and Rajka for infants. Furthermore, we added SCORAD to evaluate the severity of the eczema. Internal workshops were arranged for the investigators before the study commenced in order to achieve uniform diagnostic criteria of eczema. Unfortunately, a Kappa analysis in order to further evaluate the reliability of our method was not made. The fact that three different investigators examined the infants did probably not affect the comparisons between active and placebo group, however, since randomisation was stratified for each study centre and investigator.

As pointed out in the “Review of the literature” section, asthma is a heterogeneous disease as well, and the dominating underlying pathogenesis changes depending on the age of the patients. In infancy, asthma is seldom IgE-mediated, but the dominating trigger is respiratory infections. However, infections may unmask an underlying susceptibility that often is related to subsequent atopy, especially in those with repeated symptoms. Since a single episode of wheezing is very common in infancy and seldom associated with atopy, at least three episodes were required for fulfilling the diagnosis in our study. In addition, all infants had a family history of allergic disease. Also, obstructive symptoms had to be diagnosed at least once by a doctor for the asthma diagnosis. In conclusion, the validity of asthma assessments in infancy has been questioned. However, the quite rigid criteria employed in our study might have enhanced the validity, as possibly indicated by our chemokine results.

The incidence of allergic urticaria, ARC and gastrointestinal symptoms due to allergy was too low for achieving enough power in a study with the size and follow-up time of ours.

Sensitisation in combination with eczema, *i.e.* IgE-associated eczema, has been suggested to be the most valid predictive factors for subsequent development of
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...respiratory allergic disease\textsuperscript{3, 6-8}. Therefore, a subanalysis with IgE-associated eczema was performed in Paper I. Subsequently, we also assessed the incidence of IgE-associated allergic disease in Paper IV, since this analysis was suggested in another intervention study\textsuperscript{20}. One should bear in mind, however, that IgE-associated disease should be regarded as a predictive factor and not a disease in itself. It might be affected either by reducing sensitisation, eczema, or both. The effect of \textit{L. reuteri} on IgE-associated disease seems to be mediated mainly by a reduction in sensitisation in our study.

Compliance
An important issue in intervention studies in neonates and infants is how easy the study product can be administered. The preparation employed in the actual study is easy to administer in any age group, which probably increased the compliance to the treatment in the study. The close surveillance of the families by the research nurses probably also further stimulated the parents to adhere to the treatment. Compliance to the treatment regime was high according to interviews at each contact and by assessing used study product bottles (Figure 4). However, obviously these measurements do not exclude that some families did not adhere to the treatment during the whole study. The unexpected decline in the prevalence of \textit{L. reuteri} in infant stool after the first week of life might be explained by this, although several more plausible explanations are suggested in Paper II.

Compliance to the treatment also aimed at abstinence from probiotic products in the placebo group. Fortunately, there was no product with \textit{L. reuteri} on the commercial market in Sweden during the study period. Neither were there any other probiotics for infants below four months of age.
Figure 4. Compliance rate in infants receiving oral supplementation of L. reuteri (lines, closed symbols) or placebo (hatched lines, open symbols) daily during the first 12 months of life, as assessed by interviews at each contact and survey of used study product bottles.

Statistical analyses
An important statistical issue is whether correction for multiple comparisons should be employed when using several outcomes. Some statisticians would correct for multiple comparisons in a study like this, whereas others advocate that it is possible to see each outcome as a separate study, and in that case one should not correct for multiple comparisons. We have taken the latter approach in this study. For the interpretation of the results, the pattern over time or within a group of relating variables has been important. In addition, Friedman’s test and repeated-measures ANOVA were employed in analyses of multiple longitudinal measures in paper IV. We have not drawn any conclusions from solitary statistically significant results without an underlying well-grounded hypothesis.

Bacteriological analyses
The bacterial analyses in this study had certain limitations. For ethical reasons only stool samples could be used, while the most significant site of action of probiotics may be the mucosa in the small intestine. In another study with adults receiving L. reuteri,
analyses of biopsy samples from the mucosa of small intestine indicated that the colonisation rate is close to 100%, and that *L. reuteri* influences immune cells in the mucosa\(^{281}\). As the authors of this study referred to an unpublished manuscript in their method section, their bacteriological method has been evaluated as described in the “review of the literature section”. Furthermore, in order to maintain a high level of compliance and because of logistical reasons, the samples had to be frozen and stored until evaluation in our study, which might also have affected the result, especially the concentration figures\(^{290}\). A cultivation independent method might have increased the sensitivity.

Conventional culture methods are probably not as specific as molecular methods\(^{112}, 124\), but on the other hand, the probes of molecular methods might not cover the whole species. Moreover, it might be a disadvantage that also dead bacteria are included in molecular analysis. Although a recent report indicated that the specificity of conventional cultivation might be low for lactobacilli\(^{124}\), the specificity for *L. reuteri* with the present method employing reuterin as a biochemical marker, is considered to be high\(^{272,291}\). As our method did not allow analysis of *L. reuteri* at a strain level, it is possible that some of the *L. reuteri* demonstrated in faeces in the treated infants are wild strains. Consequently, the validity of our *L. reuteri* analysis as a measure of compliance may be questioned. On an individual level, isolation of *L. reuteri* was not taken as conclusive evidence for colonisation of the infant with the administered strain. Yet, on a group level, the prevalence of live *L. reuteri* after oral supplementation could be assessed, and factors affecting the prevalence could be identified. The differences between the active and placebo group at several repeated occasions suggest that most *L. reuteri* isolated from the treated infants were the administered strain, and that *L. reuteri* is able to survive in infants.

The limitations pointed out above also affected the results of the bifidobacteria and *C. difficile* analyses. The prevalence data agree with other studies employing conventional methods, however\(^{112,126}\). Furthermore, the influence of breastfeeding on
Results and discussion

Microbial ecology, with lower *C. difficile* and higher bifidobacteria and lactobacilli counts in breastfed infants, concords well with previous reports 126, 292.

Immunological analyses in breast milk

The sample size in this study was based on the number of mothers needed to detect true differences between the *L. reuteri* and the placebo group in cytokine levels in breast milk. The power for the analysis of the relationship between IgE-associated eczema and cytokines become too low, however.

In a substantial part of the colostrum samples the IL-10 and TNF levels were below the detection levels (23% and 32%, respectively), which might affect the result. None of the TGF-β1 and TGF-β2 analyses were below detection levels. Since it has been suggested that breast milk components inhibit cytokines, this have been evaluated in a previous publication 184. Recovery of exogenous cytokine was measured after addition to breast milk. The recovery was between 93-98%. The inter-assay CVs were 5-20% and the intra-assay CVs 6-12%.

Chemokine analyses

The chemokines CXCL9, CXCL10, CXCL11, CCL17 and CCL22 were analyzed with an in-house multiplexed Luminex assay. The specificity and the possibility of cross-reactivity between the different microsphere sets was investigated by comparing the median fluorescence intensity (MFI) generated from a standard curve of the monoplex assay with the MFI generated from the corresponding standard curve of the pentaplex assay. Spiking with different chemokines resulted only in signal from the right beads. No cross-reactivity between the assay components was observed in the present setting. Moreover, the inter-assay CVs were evaluated by using a high and low concentration control for each chemokine. The inter-assay CV was for CXCL9 40% and 20%, CXCL10 17% and 20%, CXCL11 9% and 12%, CCL17 31% and 30%, CCL18 23%
Results and discussion

and 24%, CCL22 27% and 27% for the high and low control, respectively. The intra-assay CV was never above 10%. Since samples from the study groups consisted of both plasma and serum samples, the correlation between plasma and serum samples was evaluated. Both serum and plasma samples were collected at the same time point from eleven children. Chemokine levels correlated mostly well in these samples: CXCL9 (p<0.001, ρ=0.90), CXCL10 (p=0.12, ρ=0.48), CXCL11 (p=0.03, ρ=0.63) CCL17 (p=0.02, ρ=0.68), CCL18 (p=0.01, ρ=0.84), CCL22 (p=0.048, ρ=0.58).

Almost all chemokine analyses were above the detection limit, except to CXCL10 at four occasions. This implicates that chemokine analyses are appropriate for in vivo assessments of the immune system in infants. Yet, assessment in peripheral blood only reflects a general chemokine pattern. The levels locally in various tissues cannot be determined with this method.

Clinical outcome

The cumulative incidence of eczema was similar in the treated and the placebo group (36% vs. 34%). Neither were the other potentially allergic diseases affected by the L. reuteri supplementation (Table 2). The cumulative incidence of any symptom of allergic disease, including wheeze, was 46% vs. 45%. The cumulative incidence of IgE-associated allergic disease was lower, 20% vs. 35% (p=0.04), and there was less IgE-associated eczema at two years of age, in infants receiving L. reuteri (8% vs. 20%, p=0.02, Figure 5), however. There were also a statistical trend for lower cumulative incidence of any positive skin prick test (18% vs. 29%, p=0.07), and fewer sensitised infants in the L. reuteri group at two years of age (Figure 6). The effect was more pronounced when only infants whose mothers have allergic disease were included (Paper I, Table V), possibly implicating significance of the supplementation to the mothers in late pregnancy. The effect of L. reuteri treatment may also have become more evident in infants of mothers with allergic disease, as they run an increased risk for allergic disease 167.
RESULTS AND DISCUSSION

Table 2. The cumulative incidence of allergic disease, skin prick test >3mm, specific IgE levels >0.35 kU/L and use of topical corticosteroids in infants completing the study until two years of age.

<table>
<thead>
<tr>
<th></th>
<th>Lactobacillus reuteri</th>
<th>Placebo</th>
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<tbody>
<tr>
<td></td>
<td>% (n)</td>
<td>% (n)</td>
</tr>
<tr>
<td>Eczema</td>
<td>36 (34)</td>
<td>34 (32)</td>
</tr>
<tr>
<td>IgE-associated eczema</td>
<td>17 (13)</td>
<td>28 (21)</td>
</tr>
<tr>
<td>Asthma</td>
<td>7 (7)</td>
<td>11 (10)</td>
</tr>
<tr>
<td>Wheeze incl. asthma</td>
<td>18 (17)</td>
<td>16 (15)</td>
</tr>
<tr>
<td>ARC</td>
<td>1 (1)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Gastrointestinal allergy</td>
<td>2 (2)</td>
<td>2 (2)</td>
</tr>
<tr>
<td>Allergic urticaria</td>
<td>3 (3)</td>
<td>1 (1)</td>
</tr>
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<td>Any allergic disease</td>
<td>46 (44)</td>
<td>45 (42)</td>
</tr>
<tr>
<td>IgE-associated allergic disease</td>
<td>20 (15)*</td>
<td>35 (26)*</td>
</tr>
</tbody>
</table>

Skin prick test (≥3mm)
- egg: 14 (13) vs 23 (21)
- milk: 4 (4) vs 7 (6)
- cat: 2 (2) vs 5 (5)
- birch: 2 (2) vs 4 (4)
- grass: 0 (0) vs 1 (1)
- any allergen: 18 (17) vs 29 (27)

Specific IgE (>0.35kU/L)
- ovalbumin: 16 (12) vs 27 (20)
- beta-lactoglobulin: 19 (14) vs 13 (9)
- food allergens (fx5): 34 (26) vs 36 (26)
- Sensitisedc: 37 (28) vs 48 (36)

Topical corticosteroids: 41 (39) vs 49 (46)

Infants were excluded from subanalyses if data were missing from any time point.

a Sensitised infants with eczema.  b Sensitised infants with any allergic disease.

c Infants with either a positive skin prick test and/or circulating IgE to food allergens.

* p=0.04 with chi-2 test.

Since sensitised infants with eczema are at increased risk for later development of allergic asthma and ARC, these observations warrant further clinical follow up in school age. Interestingly, the children included in the first allergy prevention study by Kalliomäki et al. have now been followed up until seven years of age. Although the difference in cumulative incidence of eczema still was significant, there was no difference in asthma or ARC prevalence at seven years of age. Indeed, the effect at
two years of age in this trial was restricted to eczema and there was no reduction in the incidence of sensitisation or IgE associated eczema\(^{264}\), which further supports the importance of sensitisation as a predictor for subsequent respiratory allergic disease.

**Figure 5.** Prevalence of any eczema (solid lines) and IgE-associated eczema (hatched lines) during the first 24 months of life in infants receiving daily oral supplementation of *L. reuteri* (closed symbols) or placebo (open symbols) during the first 12 months of life. *p* = 0.02 with Chi-2 test.

Hitherto, six allergy prevention studies with probiotics have been reported\(^{20, 251, 264-267}\), besides the study included in this thesis (Table 3)\(^{268}\). Three of these randomised controlled trials have demonstrated a lower incidence of eczema after probiotic supplementation\(^{20, 251, 264}\), whereas three studies demonstrated no preventive effect at all\(^{265-267}\). None of the studies reported any preventive effect on sensitisation, but the cumulative incidence was lower in the probiotic compared with the placebo group in our study in the subanalysis, in which only infants to mothers with allergic disease were included (28% vs. 52%, \(p=0.009\)).
Figure 6. Prevalence of sensitisation, i.e. at least one positive skin prick test and/or detectable circulating allergen specific IgE antibodies (lines), and any positive skin prick test (hatched lines) during the first 24 months of life in infants receiving daily oral supplementation of L. reuteri (closed symbols) or placebo (open symbols) during the first 12 months of life. Statistical analyses with Chi-2 test.

Different probiotic strains were used in all studies, except for the studies by Kalliomäki et al.\textsuperscript{264} and Kopp et al.\textsuperscript{266}, which both used L. rhamnosus GG.

Unfortunately, the study by Kopp et al. did not reach enough statistical power because a low inclusion rate, which makes comparisons with this study difficult. Probiotic strains differ in important characteristics such as immunological properties, survival rate in the gastrointestinal tract, and the influence on the gut microbiota (described in a previous section), which might explain the contradictory results in these prevention studies. Interestingly, in an Australian study by Wickens et al there was a preventive effect on eczema by a L. rhamnosus but not a B. animalis lactis strain, although the design was exactly the same in all other aspects\textsuperscript{251}.

Besides the use of a different bacterial strain, there were other differences in the study designs. In all studies but two, the mother received the study product in late pregnancy (Table 3)\textsuperscript{20,251,264,266,268}. In the Australian study by Taylor et al and the study from
Singapore by Soh et al., only the infants were supplemented. These trials were negative, possibly implicating that the supplementation during pregnancy was important for the preventive effect. This is supported by an epidemiological study, which reported that maternal exposure to stables, i.e. an environment with high microbial exposure, during pregnancy protected against allergic sensitisation, whereas exposures during infancy had weaker or no effect at all. Moreover, the duration of the supplementation of the infants differed between the reported allergy prevention studies: from six to 24 months (Table 3). However, the duration did not seem to determine the preventive effect of the probiotics in these studies.

An important issue in this study was safety assessments. No severe adverse events were reported. Neither were there any differences in the cumulative incidence of mild adverse events such as colic and constipation during supplementation (Paper I, Table IV). At one and two months of age more infants in the *L. reuteri* group were reported having spitting-ups than in the placebo group (26% vs. 14%, \( p=0.04 \), at one month, and 33% vs. 19%, \( p=0.04 \), at two months), however, but there were no differences when the parents were asked whether their infants had any gastrointestinal problems (13% vs. 9%, \( p=0.37 \), at one month, and 6% vs. 11%, \( p=0.28 \), at two months). Furthermore, the infants were heavier in the *L reuteri* than the placebo group at three months (6.4 kg vs. 6.1 kg, \( p=0.03 \), t-test), but not at any other time point (Table 4). The cumulative incidence of infants reported to have gastrointestinal problems during the first 12 months was 21% in the *L. reuteri* group and 23% in the placebo group.
Table 3. Allergy prevention studies with probiotics. Odds ratio for the cumulative incidence of allergic manifestations until two years of age (95% ci). Follow up at one year of age is indicated with *1y.
The adjusted OR has been used when available. * p<0.05; ** p<0.01.

<table>
<thead>
<tr>
<th>Study product (daily dose)</th>
<th>Supplementation</th>
<th>Eczema</th>
<th>Any SPT</th>
<th>Any SPT + spec. IgE</th>
<th>IgE-assoc. eczema</th>
<th>IgE-assoc. disease</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGG 132 completed (1 x 10¹⁰)</td>
<td>Yes 6 m</td>
<td>0.37** (0.16-0.82)</td>
<td>1.72 (0.69-4.4)</td>
<td></td>
<td></td>
<td></td>
<td>Kalliomäki et al, 2001</td>
</tr>
<tr>
<td>MIX inc. LGG+prebiotics 925 completed (4 x 10⁵)</td>
<td>Yes 6 m</td>
<td>0.69* (0.52-0.93)</td>
<td>0.82 (0.61-1.10)</td>
<td>0.61* (0.42-0.90)</td>
<td>0.65* (0.45-0.94)</td>
<td></td>
<td>Kukkonen et al, 2007</td>
</tr>
<tr>
<td><em>L. acidophilus</em> 178 completed (3 x 10⁵)</td>
<td>No 6 m</td>
<td>1.18 (0.62-2.26)</td>
<td>2.04* (1.01-4.5)</td>
<td>2.18 (0.95-5.20)</td>
<td></td>
<td></td>
<td>Taylor et al, 2007</td>
</tr>
<tr>
<td><em>L. reuteri</em> 188 completed (1 x 10⁵)</td>
<td>Yes 12 m</td>
<td>1.04 (0.55-1.95)</td>
<td>0.47 (0.22-1.00)</td>
<td>0.63 (0.31-1.27)</td>
<td>0.48 (0.20-1.13)</td>
<td>0.42* (0.19-0.94)</td>
<td>Abrahamsson et al, the present study</td>
</tr>
<tr>
<td>LGG 94 completed (1 x 10¹⁰)</td>
<td>Yes 6 m</td>
<td>0.96 (0.38-2.33)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kopp et al, 2008</td>
</tr>
<tr>
<td><em>L. rhamnosus</em> 294 completed (6 x 10⁵)</td>
<td>Yes 24 m</td>
<td>0.51* (0.30-0.85)</td>
<td>0.74 (0.46-1.18)</td>
<td></td>
<td>0.51* (0.27-0.97)</td>
<td></td>
<td>Wickens et al, 2008</td>
</tr>
<tr>
<td><em>B. animalis subs. lactis</em> 302 completed (9 x 10⁵)</td>
<td>Yes 24 m</td>
<td>0.90 (0.58-1.41)</td>
<td>0.82 (0.52-1.28)</td>
<td></td>
<td>0.69 (0.38-1.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. rhamnosus+B. longum</em> 245 completed (2.8 x 10⁸)</td>
<td>No 6 m</td>
<td>0.82 (0.44-1.52)</td>
<td>1.37 (0.71-2.66)</td>
<td>1.43 (0.76-2.70)</td>
<td>1.08 (0.44-2.65)</td>
<td></td>
<td>Soh et al, 2009</td>
</tr>
</tbody>
</table>
Oral supplementation with the *L. reuteri* strain used in the present study has been reported to decrease febrile episodes, episodes with diarrhoea and antibiotic prescriptions, but not respiratory illness in Israeli infants in child care centres. In contrast, in our study antibiotics were prescribed more often in the *L. reuteri* group during the first year of life (37% vs. 20%, p=0.01), although the infection rate was similar in the two groups (Paper I, Table IV). Antibiotics were mostly prescribed for acute otitis media in the present study (70%). Although it cannot be excluded completely, it is not likely that oral supplementation with *L. reuteri* increases the risk for acute otitis media. On the other hand, antibiotics have been associated with a higher risk for allergic disease in previous studies. This higher risk, however, seems to be restricted to broad spectrum antibiotics, which seldom are prescribed for acute otitis media in Sweden, possibly explaining why adjustments for antibiotics did not affect the effect of *L. reuteri* in our study.

### Table 4. Weight and length (mean and 95% confidence interval) until 24 months of age in infants supplemented with *L. reuteri* or placebo the first year of life.

<table>
<thead>
<tr>
<th></th>
<th>Weight (kg)</th>
<th>Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>L. reuteri</em></td>
<td><em>Placebo</em></td>
</tr>
<tr>
<td><strong>Birth</strong></td>
<td>3.66 (3.56-3.75)</td>
<td>3.60 (3.50-3.71)</td>
</tr>
<tr>
<td><strong>3 months</strong></td>
<td>6.35 (6.19-6.51)*</td>
<td>6.10 (5.95-6.25)*</td>
</tr>
<tr>
<td><strong>6 months</strong></td>
<td>8.13 (7.92-8.34)</td>
<td>7.88 (7.69-8.07)</td>
</tr>
<tr>
<td><strong>12 months</strong></td>
<td>10.23 (10.00-10.46)</td>
<td>10.07 (9.87-10.26)</td>
</tr>
<tr>
<td><strong>24 months</strong></td>
<td>13.06 (12.77-13.35)</td>
<td>13.03 (12.73-13.33)</td>
</tr>
</tbody>
</table>

n= 95 in the *L. reuteri* and 93 in the placebo group except at 24 months when data was obtained from 94 and 92, respectively, for weight analysis, and 91 and 90, respectively, for length analysis. T-test. *p=0.02.

The incidence of allergic disease was also related to factors that previously have been suggested to influence allergy development (Paper IV), and significant results are displayed in Table 5. Interestingly, day-care attendance was associated with lower incidence of sensitisation and IgE-associated eczema, which is in concordance with
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previous reports 147,148. On the other hand, day-care attendance tended to be significantly associated with asthma (p=0.08), possibly because asthma is predominantly triggered by infections in infancy. In contrast to previous reports 110,142, antibiotics prescription did not reduce sensitisation, but was associated with an increase incidence of eczema.

The role of breastfeeding in prevention of allergic disease remains controversial 193. Infants who were, as compared to were not, still breastfed at six months had a lower incidence of SPT positivity and tended to have a lower incidence of IgE-associated eczema (p=0.06) until two years of age, whereas breastfeeding at 12 months or exclusive breastfeeding at three months did not have any influence. Interestingly, the recommendation to mothers in Sweden during the study period was to introduce food from four months of age, while still breastfeeding. Almost all mothers still breastfeeding at six months did so, as only one infant was exclusively breastfed at six months of age. This might indicate a protective effect of breastfeeding during food introduction, in consistence with a recent report 109. The influence of delivery mode, birth weight, birth length, parental smoking, older siblings, furred pets, total number of infections and antibiotics the second year of life was also assessed but was not significantly associated with allergic disease.
Table 5. The cumulative incidence, % (n), of allergic disease and skin prick test > 3mm in relation with sex, day-care attendance, antibiotics and breastfeeding.

<table>
<thead>
<tr>
<th></th>
<th>Day-care 12-24 months</th>
<th>Antibiotics 0-12 months</th>
<th>3 months (excl.)</th>
<th>6 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m</td>
<td>f</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>Exema</td>
<td>33 (32)</td>
<td>38 (34)</td>
<td>33 (51)</td>
<td>47 (15)</td>
<td>22 (12)*</td>
</tr>
<tr>
<td>Asthma</td>
<td>13 (13)*</td>
<td>4 (4)*</td>
<td>11 (17)</td>
<td>0 (0)</td>
<td>13 (7)</td>
</tr>
<tr>
<td>Any SPT</td>
<td>17 (16)*</td>
<td>31 (28)*</td>
<td>20 (30)**</td>
<td>44 (14)**</td>
<td>23 (12)</td>
</tr>
<tr>
<td>IgE-assoc. eczema</td>
<td>18 (14)</td>
<td>28 (20)</td>
<td>19 (24)*</td>
<td>39 (10)*</td>
<td>11 (5)*</td>
</tr>
<tr>
<td>IgE assoc. disease</td>
<td>21 (16)</td>
<td>35 (25)</td>
<td>24 (30)</td>
<td>42 (11)</td>
<td>18 (8)</td>
</tr>
</tbody>
</table>

Infants were excluded from subanalyses if data were missing from any time point. * p<0.05, ** p<0.01. Chi-2-test. The differences for sex, day-care and antibiotics remained statistically significant also after adjustment for study group affiliation with logistic regression.
**Bacteriological analyses**

The prevalence of *L. reuteri* in infant stool was higher in the actively treated than in the placebo group on all sampling occasions during the supplementation period (Figure 7). The highest prevalence was recorded 5-6 days after birth in the active group (82%) and it then declined despite continuous supplementation. Our method did not allow analysis of *L. reuteri* at a strain level. Thus, it is possible that some of the *L. reuteri* demonstrated in faeces in the treated infants were wild strains. The difference between the active and placebo group at repeated occasions suggests that most *L. reuteri* isolated from the treated infants were the administered strain, however.

**Figure 7.** Prevalence of any lactobacilli (closed symbols) and *L. reuteri* (open symbols) in stool from infants receiving daily oral supplementation of *L. reuteri* (lines) or placebo (hatched lines) during the first 12 months of life. † *p*<0.01; ‡ *p*<0.001 with Chi-2 test.
In concordance with previous reports showing that *L. reuteri* may belong to the commensal microbiota in some infants\(^{124, 270, 271}\), *L. reuteri* was also isolated from infants in the placebo group.

Treatment was associated with a higher prevalence of *L. reuteri* in maternal stool and colostrum but not in breast milk obtained one month after the mother had stopped the intake of the study product (Paper II, Table 2). The most likely origin of *L. reuteri* in colostrum is external contamination from the gastrointestinal tract, although bacterial translocation from the gut endogenously has been suggested recently\(^ {181}\). Although higher in the active than in the placebo group, the prevalence of *L. reuteri* in colostrum was low (12%) and probably not important for the clinical effect.

There are also recent reports that amnion fluid\(^ {177-179}\), umbilical cord blood and meconium from the newborn\(^ {180}\) contain bacteria, suggesting that a prenatal mother-to-child efflux of commensal bacteria may exist. If these preliminary and controversial results are correct, the fetal gut immune system may have been exposed to *L. reuteri* antigen already during gestation. In the placebo group, infants of mothers with *L. reuteri* in stool perinatally had higher prevalence of *L. reuteri* the first week of life, suggesting transmission from the mother to the newborn. The difference remained until six months of age (paper II).

The prevalence of *L. reuteri* infant stool was in concordance with intervention studies with other probiotic strains (Figure 8)\(^ {20, 251, 293, 294}\). In contrast to the previous intervention studies with lactobacilli\(^ {20, 293, 265, 294}\), however, we assessed stool samples at several time points during the study period and also faecal and breast milk samples from the mothers. Thus, the dynamics of the faecal *L. reuteri* could be evaluated more accurately. Unexpectedly, the prevalence of *L. reuteri* declined after the first week of life despite continuous supplementation. Possible explanations include decreased compliance, competition from other bacterial species\(^ {292}\), a lower proportion of bacteria surviving passage through the stomach due to decreasing pH level with age, and immune recognition of *L. reuteri* resulting in an increased shedding of the bacteria.
Since lactobacilli, including \textit{L. reuteri}, stimulate IgA production in human and mouse models, an IgA-mediated inhibitory effect is possible \cite{295,296}. Immunoglobulin A against \textit{L. reuteri} or other inhibitory factors might also explain why breastfeeding was associated with lower faecal prevalence of \textit{L. reuteri} (Paper II, Figure).

\textbf{Figure 8.} Prevalence of probiotic bacteria in infant stool samples in allergy prevention studies after supplementation with the indicated probiotic bacteria (closed bars) or placebo (open bars). If the bacterial analyses were reported in a separate paper, the paper with the clinical outcome is displayed in parenthesis beneath. The difference between active and placebo group was statistically significant in all studies.

The six allergy prevention studies with colonisation data available are displayed in Figure 8. Interestingly, the two probiotic strains that had the lowest faecal prevalence in the active group, the \textit{L. acidophilus} strain in the study by Taylor \textit{et al} \cite{265} and the \textit{B. animalis lactis} strain in the study by Wickens \textit{et al} \cite{251}, were also associated with no
preventive effect. In the study by Wickens et al the *L. rhamnosus* strain, which had a preventive effect on eczema, were significantly more common in infants faeces than the *B. animalis lactis* strain. Thus, the survival rate in the gastrointestinal tract may be of importance for the preventive effect.

![Graph showing prevalence of bifidobacteria and C. difficile](image)

**Figure 9.** Prevalence of bifidobacteria (closed symbols) and *C. difficile* (open symbols) during the first two years of life in infants receiving oral supplementation with *L. reuteri* (lines) or placebo (hatched lines) daily during the first 12 months of life. The prevalence was similar in the two treatment groups.

*Lactobacillus reuteri* strains produce the antimicrobial metabolite reuterin and have been shown to inhibit pathogenic bacteria, without inhibiting normal bacterial residents of the gastrointestinal tract *in vitro*. Supplementation with *L. reuteri* affected neither the prevalence nor the counts of bifidobacteria or *C. difficile* (Figure 9, Paper II, Table 2 and 3), however. Nor was there any correlation between bifidobacteria and *C. difficile* counts, regardless of treatment (data not shown). Thus, we could not confirm that the lower incidence of IgE-associated allergic disease in *L.*
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*L. reuteri* treated infants was mediated by a modification of the intestinal microbiota, at least for the presently analysed bacterial species. Our findings are supported by a mouse model, in which probiotics reduced eczema and increased IL-10 in plasma and Peyer’s patches, although it did not affect the intestinal microbiota²³⁹. Yet, obviously, *L. reuteri* might have affected the gut microbiota, although it was not revealed by our method.

**Immunological analyses in breast milk.**

Supplementation with *L. reuteri* during pregnancy was associated with lower colostral TGF-β2 and increased IL-10 levels. No such association was observed in mature milk collected one month after the treatment had ceased (Paper III, Table 3). The levels of TGF-β2 in colostrum were also lower in mothers with as compared to without detectable *L. reuteri* in faeces, in the *L. reuteri*-treated group (Paper III, Figure 1).

![Image](image-url)

**Figure 10.** Infants who were sensitised at 6, 12, and/or 24 months of age had received colostrum with higher levels of TGF-β2 than infants that were not sensitised.
Furthermore, low colostral TGF-β2 was associated with less sensitisation up to two years of age in the breast fed infants (Figure 10). Interestingly, our research group have observed similar findings in another cohort including Estonian and Swedish children followed prospectively from birth up to five years of age. In that study, low breast milk TGF-β2 levels were associated with less SPT positivity up to two years of age (unpublished data).

These findings challenge the general idea of TGF-β as an anti-inflammatory mediator that suppresses IgE responses. High concentration of TGF-β1 and TGF-β2 in colostrum has been associated with post-weaning onset of atopic diseases, whereas low TGF-β1 and TGF-β2 was associated with pre-weaning onset. In two other clinical studies, there was no clear association between breast milk TGF-β1 and TGF-β2 and development of atopic manifestations, however.

Figure 11. The figure shows a positive correlation between the TGF-β2 levels and Na/K ratio in colostrum (Spearman’s rank order correlation coefficient test).
Results and discussion

Also high colostral Na/K ratios were associated with increased risk of sensitisation (Paper III). This is in agreement with a previous study, where high breast milk Na/K ratios in atopic mothers were associated with development of positive SPT and symptoms of atopic disease up to 18 months of age \(^{301}\). Elevated Na/K ratios in breast milk are considered indicative of increased epithelial permeability caused by inflammation in the mammary epithelium and have been reported to correlate well with IL-8 and TGF-\(\beta\)2 levels, \textit{i.e.} two cytokines with the potential to both promote and down-regulate inflammation \(^{188}\). Interestingly, epithelial cells produce more TGF-\(\beta\)2 than TGF-\(\beta\)1 upon \textit{e.g.} IL-13 stimulation, whereas TGF-\(\beta\)1 is the predominant isoform expressed by immune cells, including regulatory T cells \(^{302-305}\). Although the breast milk Na/K ratios were not affected by \textit{L. reuteri} supplementation, the TGF-\(\beta\)2 levels and Na/K ratios correlated (Figure 11). This suggests that the relation between \textit{L. reuteri} supplementation, breast milk TGF-\(\beta\)2 levels and childhood sensitisation might be explained by effects on epithelial barrier function.

An obvious question is whether the association between TGF-\(\beta\)2 and infant sensitisation really is attributed to the effect of \textit{L. reuteri} on TGF-\(\beta\)2 in colostrum, or if it is due to a separate mechanism associated with the \textit{L. reuteri} supplementation to the infants the first year of life. The relationship between low levels of TGF-\(\beta\)2 in colostrum and subsequent sensitisation in the offspring remained in a logistic regression model adjusting for treatment regime and Na/K ratios, however, indicating that one explanatory mechanism to the clinical effect of \textit{L. reuteri} may be via lowering breast milk TGF-\(\beta\)2 levels (Paper III, Table 4). The mechanism is unknown, and it is still possible that TGF-\(\beta\)2 only is a marker for a co-existing effect on the mother and, via the mother, on the foetus.

In contrast, supplementation with \textit{L. rhamnosus} GG to Finnish mother during pregnancy and lactation was associated with increased TGF-\(\beta\)2 levels in mature breast milk obtained at three months after delivery \(^{190}\). No direct association between breast milk TGF-\(\beta\)2 levels and development of sensitisation or atopic disease in the infant was reported in the Finnish study. Notably, in contrast to our prevention study, \textit{L.}}
\textit{rhamnosus} GG supplementation did not have any preventive effect on sensitisation in the two Finnish prevention studies, while there was an effect on the incidence of eczema symptoms in their studies but not in ours \textsuperscript{20,264}.

The levels of IL-10 in colostrum were slightly higher in the \textit{L. reuteri} than placebo treated mothers. \textit{Lactobacillus reuteri} has been reported to induce IL-10 producing regulatory T cells \textit{in vitro} \textsuperscript{41} and an IL-10 inducing capacity by \textit{L reuteri} supplementation is possible. The breast milk IL-10 levels were not associated with sensitisation or development of eczema in the infants. None of the other analysed breast milk parameters, \textit{i.e.} IgA, SIgA, TGF-\(\beta\)1, TNF and sCD14 differed between the \textit{L. reuteri} and placebo treated mothers and none were they related to sensitisation or development of eczema in the infants, in agreement with our previous findings \textsuperscript{199}.

\textbf{Chemokine analyses}

The chemokine analyses revealed that an imbalance in circulating Th1- and Th2-associated chemokines precedes the onset of allergic disease, implicating that these chemokines are primarily involved in the pathogenesis of allergic diseases and not only secondary to a general immune deviation. Our results also suggest that circulating chemokine analyses can be employed as novel tools for discriminating between factors possibly influencing allergy development in infancy.

In agreement with previous reports \textsuperscript{75,76,79,80,306}, eczema and asthma were related to elevated levels of Th2-associated chemokines (Paper IV, Figure 2 and 3), although these mediators have not previously been studied before disease onset. In contrast, the Th1-associated chemokines correlated inversely with eczema, although only significantly so for CXCL10 at 12 months (Paper IV, Figure 2). This subsequent delay in maturation of the immune system with a prolonged Th2-deviation in children developing allergic disease supports and extends previous reports \textsuperscript{62,63}.
The clear association between asthma and the Th2-associated chemokines might be somewhat unexpected, since most asthma/recurrent wheeze in infancy are non-IgE-mediated and triggered by viral infections. Possibly, our rigid definition of asthma has selected infants with an underlying susceptibility for subsequent development of atopy. CCL18, previously named pulmonary-and activation-regulated chemokine, has been suggested to play a predominant role in asthma. It is preferentially expressed in the lung by APCs and is induced by Th2 cytokines. In contrast to the other Th2-associated chemokines, the CCL18 levels increased during infancy (Paper IV, Figure 3). Furthermore, CCL18 but not CCL17 and CCL22 levels were also elevated in non-sensitised infants with asthma (Figure 12).

![Figure 12](image)

**Figure 12.** The levels of CCL18 in cord blood and in peripheral blood at 6, 12 and 24 months in non-sensitised infants with (closed bars) and without (open bars) asthma (a) or any wheezing episode (b) until two years of age. The 10th, 25th, 50th, 75th and 90th percentiles as well as outliers are indicated.
The underlying mechanisms seem to differ between the various allergic manifestations as well as for the expression of immunological mediators. For instance, in contrast to eczema and sensitisation, asthma was related to higher levels of the Th1-associated chemokines the second year of life. In support of this, there are reports that high exposure to bacterial lipopolysaccharides, which e.g. induces CXCL10 expression, decreases the risk for sensitisation, but increases risk for asthma exacerbations. CXCL10 is elevated in BAL from asthmatic patients after allergen exposure, and although CXCL10 favoured Th1-like response in lymph nodes in a mouse model, it attracted Th2-cells and eosinophils locally in lung at a late stage of airway inflammation. Thus, when the subject once has become asthmatic, Th1-inducing factors might aggravate the asthmatic inflammation.

Interestingly, only two of the chemokines were significantly related to sensitisation. The inverse relationship between CXCL11 already at birth and subsequent skin prick test and IgE-associated disease (Paper IV, Figure 4 and E1) suggests that this chemokine might have a key role in tolerance development. Notably, none of the neonates with CXCL11 levels above the upper quartile at birth developed a positive SPT. Upon IFN-γ-stimulation, CXCL11 is expressed by several cell types, e.g. intestinal epithelium, endothelium and monocytes. This chemokine is also a potent chemoattractant for IL-2 activated T-cells during immune responses to foreign antigens. CXCL11 have higher affinity to the CXCR3 receptor and was found at higher circulating levels than CXCL10 in our material. If CXCL11 is important for the early programming of the immune system, mechanisms associated with this chemokine might result in subsequent inhibition of Th2-associated chemokines and a decreased risk for allergic disease in late infancy.

The high CCL22 levels at birth in infants with subsequent sensitisation (Paper IV, Figure 4) further support the theory that sensitisation might be associated with immune programming during the fetal period. CCL22, which is expressed by dendritic cells and macrophages in lymph nodes and attracts Th2-cells, is induced by prostaglandin E₂ and Th2 cytokines, which are accumulated in the decidua during
Interestingly, in contrast to CCL17 and CCL18, CCL22 expression is reduced by the anti-inflammatory cytokine IL-10. Thus, increased CCL22 may be a marker for reduced immunoregulatory capacity in utero.

Figure 13. The levels of CCL22 (a) and CXCL11 (b) in peripheral blood at 6, 12 and 24 months in infants with (open bars) and without (closed bars) L. reuteri in stool the first week of life. The 10th, 25th, 50th, 75th and 90th percentiles as well as outliers are indicated.
The chemokine levels were similar in the *L. reuteri* and placebo treated group, although CXCL11 tended to be higher in cord blood in the probiotic group (Paper IV, p=0.06).

As has been described in previous sections, perinatal supplementation to the mother and child might be essential for the effect of probiotics. Faecal prevalence of *L. reuteri* in infant stool the first week of life was associated with low CCL17 and CCL22 and high CXCL11 levels at 6 months, suggesting a more rapid maturation of the immune system after birth (Figure 13 and Paper IV, Figure 5). Furthermore, the CXCL9 and CXCL10 levels were higher at 24 months in infants to mothers with *L. reuteri* in stool perinatally (Paper IV), also supporting the importance of the perinatal treatment.

The moderate relationship between faecal prevalence of *L. reuteri* on one hand and low CCL22 and high CXCL11 levels on the other hand possibly implies that *L. reuteri* acts via a mechanism involving these specific chemokines, as they were the only chemokines significantly associated with sensitisation.

The chemokine analyses also confirm that humans are born Th2-skewed and gradually develop a Th1/Th2-balance (Figure 14 and Paper IV, Figure 1). This process is stimulated by environmental factors, such as exposure to commensal microflora. From an evolutionary perspective, Th2 skewing during gestation seems to be needed for a successful pregnancy. In agreement with this, reduced levels of CCL18 were associated with lower birth weight and length in our study (Paper IV, Figure E2).
Results and discussion

Figure 14. The chemokine analyses confirmed that humans are born Th2-skewed and that the Th1/Th2-ratio increase with age. The median of circulating levels of the Th2-associated CCL22 (hatched lines) and Th2-associated CXCL 10 (lines) from birth until 24 months of age.

Attendance to day-care was the most Th1-stimulating postnatal factor (Figure 15). Accordingly, this was also related to a lower incidence of sensitisation and IgE-associated eczema (Table 5), in accordance with previous reports 147, 148.
Results and discussion

Figure 15. The levels of CXCL10 (a) CXCL11 (b) and CCL22/CXCL10 ratio (c) in peripheral blood at 24 months in infants attending (open bars) and not attending (closed bars) day-care the second year of life. The 10th, 25th, 50th, 75th and 90th percentiles as well as outliers are indicated.

Unexpectedly, day-care tended to be associated with a higher incidence of asthma, i.e. recurrent wheeze (Table 5, p=0.08). This is consistent with the relationship between asthma and CXCL11 levels at 24 months of age, however. Day-care probably influences the immune system by enhanced microbial exposure, and the absent correlation between infection rate and the Th1-associated chemokines (Paper IV) suggests that the effect of day-care most likely is mediated by enhanced exposure to commensal microbiota.

Infants who were still breastfed at six months had a lower incidence of SPT reactivity until two years of age than infants who were not (Table 5). Yet, breastfeeding was associated with elevated Th2-associated chemokines. Exclusive breastfeeding at three months of age was related to subsequent elevated CCL18 and CCL22 levels, and
Results and discussion

Infants still breastfeeding at six months had higher CCL17 and CCL22 levels at 12 months of age compared to those that did not (Paper IV, Figure E3). This indicates that a different mechanism than a generalised effect on the Th1/Th2-balance underlies the preventive effect of breastfeeding in this material.
Future perspectives

The difference in IgE-associated allergic disease between the two study groups warrants an evaluation of respiratory allergic disease in school age in this trial. Therefore, a seven-year follow up is being performed at present.

Numerous immunological analyses in the two-year material still remain to be done in this trial. For the time being, functional analyses of mononuclear cells from umbilical and peripheral blood from the infants are being performed. Cytokine and chemokine production is assessed after the cells have been stimulated with TLR-ligands such as LTA, LPS and CpG, as well as with various allergens. Additional in vivo analyses from peripheral blood have also been done. Preliminarily, we can confirm the result from a recent Finnish study, that low CRP levels in the first year of life are associated with a higher risk for eczema and IgE-associated allergic disease, indicating the importance of innate immune activation in the maturation of the immune system. Interestingly, infants to mothers with L. reuteri in colostrum had higher CRP at birth (p=0.03), and infants to mothers with L. reuteri in stool perinatally had higher CRP at 24 months of age (p=0.002). Analyses of circulating IL-10 levels, vaccine antibody responses and salivary IgA will also be reported.

Hitherto, only conventional cultivation methods have been employed for the bacterial analyses in this trial. Recently, collaboration with several research groups within the microbiology field has been established in order to analyse faecal and breast milk samples with cultivation independent techniques. Together with researchers at Linköping University and the Swedish University of Agricultural Sciences in Uppsala, methods to detect the supplemented L. reuteri strain are examined. Since it has been difficult to distinguish the supplemented strain from other L. reuteri strains via 16S rRNA, other targets such as penicillin binding protein genes, surface protein genes and plasmid genes are under investigation. Whole genome comparison with the closely related L. reuteri strain CF48-3A may also reveal regions that could be used for strain specific PCR. Draft genome sequences are publicly available for both these strains.
Future Perspectives

The influence on the lactobacilli and bifidobacteria populations are also being analysed. Moreover, assessments of the diversity of the microbiota are performed in collaboration with a research group at the Karolinska Institute in Stockholm with a new powerful methodology, 454 pyrosequencing.

Since the results from our study and those of others imply that prenatal treatment may be important for the outcome, new interventions addressing this hypothesis are warranted. As the dose in our study was relatively low, the design of a new intervention should also include a higher dose.

Recently, there have been very promising results from prevention trials in prematures. A recent meta-analysis indicated that probiotics might reduce the risk for necrotising enterocolitis (NEC) and mortality in preterm neonates with less than 33 weeks gestation. As a neonatologist, it really would be exciting to evaluate probiotics in prevention of severe conditions such as NEC, sepsis, food intolerance etc. Furthermore, chemokines seems to be promising novel tools to evaluate immune response in prematures in vivo.
Summary and concluding remarks

In this thesis, a DBPC intervention with *L. reuteri* in infancy offered the opportunity to test certain aspects of the hygiene hypothesis and to evaluate a novel strategy for allergy prevention. We evaluated the effect of *L. reuteri* supplementation on allergic disease and sensitisation, examined possible underlying mechanisms, and evaluated the safety of a long-term administration with *L. reuteri* in infancy. Furthermore, the influence of other pre- and postnatal factors on allergy development was assessed, as well as the relationship between the Th1/Th2-associated chemokine balance and allergic disease.

We could not confirm the preventive effect of probiotics on infant eczema in previous reports. The cumulative incidence of IgE-associated allergic disease and the prevalence of IgE-associated eczema during the second year were lower in the treated group, however. As sensitised infants with eczema have increased risk for later development of allergic asthma and rhinoconjunctivitis, the children in the *L. reuteri* group possibly run a reduced risk to develop later respiratory allergic disease. The effect was more pronounced when only infants with history of maternal allergic disease were included in the statistical analyses, possibly implicating the significance of the supplementation to the mothers in late pregnancy. This theory is consistent with the results from other allergy prevention studies, where studies without prenatal supplementation have given negative results.

The importance of the perinatal supplementation was further supported by the laboratory assessments examining possible underlying mechanisms by *L. reuteri*. Supplementation of *L. reuteri* during pregnancy was associated with low levels of the cytokine TGF-β2 and slightly increased levels of IL-10 in colostrum, and low colostral levels of TGF-β2 were associated with less sensitisation in breast fed infants. The moderate association between *L. reuteri* and the Th1- and Th2- associated chemokines may also be confined to the perinatal supplementation. The faecal prevalence of *L. reuteri* in infant stool the first week of life was associated with lower levels of the
SUMMARY AND CONCLUDING REMARKS

Th2-associated chemokines CCL17 and CCL22 and higher levels of the Th1-associated chemokine CXCL11 at six months of age, suggesting a more rapid maturation of the immune system after birth in these infants.

The bacterial analyses of faeces samples indicated that L. reuteri was able to survive in most infants after oral supplementation during the first year of life, and may also be detected in colostrum after oral supplementation to the mother. The highest prevalence was recorded 5-6 days after birth in the active group and it then declined despite continuous supplementation. The colonisation data from other allergy prevention with probiotics indicates that the survival rate in the gastrointestinal tract is of importance for the preventive effect. The prevalence of L. reuteri in the actively treated infants seemed to be inhibited by breastfeeding but not by antibiotics. Supplementation with L. reuteri affected neither the prevalence nor the counts of bifidobacteria and C. difficile.

The safety assessments did not reveal any severe side-effects and nor were there any differences in the cumulative incidence of mild adverse events such as colic, constipation and mild to moderate infections between the L. reuteri and placebo group. At one and two months of age, however, more infants in the L. reuteri group were reported having spitting-ups than in the placebo group, although there were no differences when the parents were asked whether their infants had any gastrointestinal problems. Furthermore, the infants were heavier in the L. reuteri than the placebo group at 3 months. Maybe the increased prevalence of spitting-up at that age was due to an increase food intake in the L. reuteri group? The weight and length was similar in the two study groups at all other time points until two years of age.

The chemokine analyses also revealed that an imbalance in circulating Th1- and Th2-associated chemokines precedes the onset of allergic disease, implicating that these chemokines are primarily involved in the pathogenesis of allergic diseases and not only secondary to a general immune deviation. Sensitisation was preceded by low levels of the Th1-associated chemokine CXCL11 and high levels of the Th2-
associated chemokine CCL22 already at birth. The subsequent delay in maturation of the immune system with a prolonged Th2-deviation in children developing allergic disease supports and extends previous reports. The relationship to pre- and postnatal factors was also examined. High Th2-associated chemokine levels were associated with increased birth length and weight and long duration of breastfeeding, and high Th1-associated chemokine levels to day-care attendance. Our results suggest that circulating chemokine analyses can be employed as novel tools for discriminating between factors possibly influencing allergy development in infancy.

The incidence of allergic disease was also related to factors that previously have been suggested to influence allergy development. Day-care attendance was associated with lower incidence of sensitisation and IgE-associated eczema, which may be attributed to an enhanced microbial exposure of these infants. The role of breastfeeding in prevention of allergic disease remains controversial. In our study, infants who were still breastfed at six months had a lower incidence of skin prick test positivity until two years of age than infants who were not. This may indicate a protective effect of breastfeeding during food introduction. Yet, any conclusion from these relationships has to be drawn with caution, since the study was not primarily designed for these analyses. Half of the infants received probiotics and only families with history of allergic disease were included.

In conclusion, this intervention study on infants with a family history of allergic disease could not confirm a preventive effect of probiotics on infant eczema. The L. reuteri treated infants had lower cumulative incidence of IgE-associated allergic disease at two years of age, however, and therefore possibly run a reduced risk to develop later respiratory allergic disease. The mechanisms underlying this effect require further elucidation. There is still insufficient evidence to recommend probiotics in the prevention of allergic disease.
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