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Immunomodulatory effects of probiotic
supplementation during pregnancy and infancy
in allergy prevention studies

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Front cover: Coloured scanning electron micrograph of *Lactobacillus reuteri* (blue) binding to the mucus layer (green) of the human small intestine. Alistair Walsham & Stephanie Schüller

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Life isn't about waiting for the storm to pass... It's about learning to dance in the rain

Populärvetenskaplig sammanfattning

Förekomsten av allergiska sjukdomar ökar och nya metoder för att förebygga allergier behöver utvecklas och införas kliniskt. Mer kunskap om immunologiska mekanismer är nödvändigt att införskaffa för att identifiera allergiförebyggande strategier. Vi har använt oss av probiotika (en mjölksyreproucerande bakterie kallad *Lactobacillus reuteri*) för att se om en ökad tillförsel av en normalflorbakterie kan påverka allergiutvecklingen hos barn. En extra tillförsel av probiotika skulle kunna tänkas öka stimuleringen av immunsystemet och påverka tarmfloran på ett hälsosamt sätt. Miljön under graviditet har visat sig vara betydelsefull för utvecklingen av barnets immunförsvar, då både epidemiologiska studier och experimentella djurmodeller visar att bakteriell exponering har allergiförebyggande effekt.

Vi har gett probiotika-tillskott till gravida mödrar från graviditetsvecka 36 och barnen har sedan under första levnadsåret fått samma bakterier som mamman eller placebo som kontrolltillskott.

Blodprover har samlats in från olika tidpunkter under barnets första två år, för att kunna analysera hur probiotika-tillförseln har påverkat immunsystemets utveckling. Vid två års ålder hade de barn som fått den aktiva substansen mindre allergiskt eksem än kontrollgruppen. Våra analyser av stimulerade celler visar att barn som fått probiotika har ett mer reglerat immunsvär. Detta har vi mätt genom att analysera signalsubstanser som olika celler i immunsystemet producerar när de stöter på allergener eller mikrobiella produkter. De barn som fått det aktiva tillskottet verkar ha ett mer reglerbart immunsvär än övriga barn.

Vi observerade även att probiotika verkar kunna påverka det maskineri som styr hur våra gener läses, s.k. epigenetiska förändringar.

Vi har även påbörjat en uppföljningsstudie där studietillskottet, i form av samma probiotika samt ω -3 fettsyror, ges tidigare under graviditeten för att möjligtvis kunna ge en starkare allergiförebyggande effekt. Vi har observerat vissa immunmodulerande effekter av tillskotten på mammans immunsvär under graviditeten.

Sammanfattningsvis verkar probiotika påverka immunsystemet hos barn och gravida på flera olika sätt. Mer forskning behövs dock för att bättre förstå hur probiotika modulerar immunsvär så att mer effektiva allergiförebyggande strategier kan identifieras i framtiden.

Abstract

The incidence of allergic diseases is increasing, possibly due to a reduced intensity and diversity of microbial stimulation. More knowledge is needed on the immunological mechanisms underlying the eczema preventive effect of pre- and postnatal probiotic supplementation. The pregnancy period seems to be of essential importance, since both epidemiological and experimental animal studies show the importance of microbial exposure during gestation on allergy prevention.

We have performed a study where the probiotic lactic acid producing bacteria *Lactobacillus reuteri* was supplemented to pregnant women, at risk of having an allergic infant. The pregnant mothers received the study product from gestational week 36 until delivery, and the infants then continued with the same product until one year of age. The probiotic, as compared with placebo, supplemented infants had less IgE-associated eczema at two years of age.

In order to investigate how the supplementation affected the immune system peripheral blood was collected and immune cells were stimulated with common allergens and TLR ligands. The probiotic treated group responded with a more regulated response to allergens and TLR2 ligands in comparison to the placebo supplemented group.

We also investigated how the probiotic supplementation affected the epigenetic methylation pattern in circulating T helper cells during infancy, observing the most pronounced effects at birth.

In a follow up study, supplementation was started earlier to possibly gain a stronger allergy preventive effect via changes in maternal immune regulation. Supplementation with *Lactobacillus reuteri* and ω -3 fatty acids started at gestational week 20 and throughout pregnancy. After 20 weeks of supplementation, some immunomodulatory effects among circulating activated regulatory T cells and a subpopulation of monocytes were noted. Several systemic immune modifying effects of pregnancy were observed.

In summary, probiotics show several immunomodulatory effects in infants and pregnant women. However, more research is needed to better understand the effects of the probiotic supplementation to aid future identification of more efficacious allergy preventive strategies.

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Abbreviations

α -IL-4R	anti-IL-4-receptor
AA	arachidonic acid
AD	atopic dermatitis
APC	antigen-presenting cell
ARC	allergic rhino-conjunctivitis
BSA	bovine serum albumin
CB	cord blood
CBMC	cord blood mononuclear cells
CCL	C-C motif ligand
CD	cluster of differentiation
Ct	threshold cycle
CTLA-4	cytotoxic T lymphocyte antigen 4
CV	coefficient of variance
CXCL	CXC ligand
CXCL3L	CX3C ligand
DC	dendritic cell
DHA	docosahexaenoic acid
DMSO	dimethyl sulphoxide
EBI3	Epstein-Barr virus induced 3
EPA	eicosapentaenoic acid
FCS	fetal calf serum
Fc ϵ RI	Fc ϵ receptor type 1
Foxp3	forkhead box p3
GATA-3	GATA binding protein 3
gw	gestational week
HLA	human leukocyte antigen
IFN- γ	interferon- γ
Ig	immunoglobulin
IL	interleukin
ILC2	type 2 innate lymphoid cells
LTA	Lipoteichoic acid

LPS	Lipopolysaccharides
MHC	major histocompatibility complex
NK	natural killer
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHA	phytohemagglutinin
RNA	ribonucleic acid
RORC	retinoic acid-related orphan receptor C
RT-PCR	reverse transcription polymerase chain reaction
SA-PE	streptavidin R-phycoerythrin conjugate
SPT	skin prick test
STAT	signal transducer and activator of transcription
Tbet	T-box expressed in T cells
Tbx21	T-box transcription factor 21
TCR	T cell receptor
TGF β	transforming growth factor- β
Th	T-helper
TLR	Toll-like receptor
Treg	regulatory T cells
TSLP	thymic stromal lymphopoietin

List of papers

Paper I

Forsberg A, Abrahamsson TR, Björkstén B, Jenmalm MC,

Pre- and post-natal *Lactobacillus reuteri* supplementation decreases allergen responsiveness in infancy.

Clin Exp Allergy 2013; 43: 434-42.

Paper II

Forsberg A, Abrahamsson TR, Björkstén B, Jenmalm MC,

Pre- and postnatal administration of *Lactobacillus reuteri* decreases TLR2 responses in infants.

Clin Transl Allergy 2014; 4: 21.

Paper III

Forsberg A*, Huoman J*, Bhai Mehta R, Nilsson L, Abrahamsson TR., Ernerudh J, Jenmalm, MC.

Global and Immunoregulatory epigenetic patterning in circulating CD4+ T cells of infants participating in a probiotic prevention study

Manuscript

Paper IV

Forsberg A, Abrahamsson TR., Nilsson L, Ernerudh J, Duchén K, Jenmalm MC.

Modulation of maternal immunity during pregnancy by probiotics and ω -3 fatty acids

Manuscript

Supplementary relevant publications

Tulic MK, Hodder M, **Forsberg A**, McCarthy S, Richman T, D'Vaz N, van den Biggelaar AH, Thornton CA, Prescott SL,

Differences in innate immune function between allergic and nonallergic children: New insights into immune ontogeny.

J Allergy Clin Immunol 2011; 127: 470-78 e1.

Abrahamsson TR, Sandberg Abenius M, **Forsberg A**, Björkstén B, Jenmalm MC, A Th1/Th2 associated chemokine imbalance during infancy in children developing eczema, wheeze and sensitization.

Clin Exp Allergy 2011; 41: 1729-39.

Forsberg A, Bengtsson M, Eringfalt A, Ernerudh J, Mjosberg J, Jenmalm MC, GATA binding protein 3(+) group 2 innate lymphoid cells are present in cord blood and in higher proportions in male than in female neonates.

J Allergy Clin Immunol 2014; 134: 228-30

Forsberg A, West CE, Prescott SL, Jenmalm MC,

Pre- and probiotics for allergy prevention: time to revisit recommendations?

Clin Exp Allergy 2016; 46: 1506-21.

Aims

The general aim of this thesis was to increase our knowledge on immunoregulatory mechanisms after pre- and postnatal probiotic supplementation in an allergy prevention study where the probiotic addition was associated with decreased IgE-associated eczema at two years of age. In a follow-up study we aimed to investigate the effect of prolonged prenatal probiotic and ω -3 fatty acid supplementation on maternal immunity during pregnancy.

Specific aims

Paper I

The aim of paper I was to investigate the effect of probiotic supplementation on cytokine and chemokine producing capacity in response to allergens in infants included in the allergy prevention study.

Paper II

The aim of paper II was to further characterize the effect of probiotic supplementation on the longitudinal cytokine and chemokine producing capacity in response to bacterial moieties *i.e.* TLR2/4/9 ligands and to investigate the mRNA expression of these receptors.

Paper III

The aim of paper III was to explore local and global epigenetic modifications in circulating CD4+ cells during infancy after pre- and postnatal probiotic supplementation.

Paper IV

The aim of paper IV was to investigate how maternal peripheral immune cell populations, as assessed by flow cytometry phenotyping, might be modulated during pregnancy by probiotic and ω -3 fatty acid supplementation, and what impact pregnancy itself has on these cell populations.

Introduction

This introduction will deal with the background to the use of probiotics in allergy-prevention trials, the potential immunological mechanisms of probiotic supplementation during pregnancy and in infancy to prevent allergy development. Allergic diseases have increased considerably during the latest decades and it is of great importance to try to identify efficacious prevention strategies and to put them into clinical practice.

General aspects of allergic disease

Prevalence of allergic disease and the atopic march

Allergic diseases are substantially increasing and represent a considerable socio-economic cost and a reduced quality of life. An estimated 20 % of the population worldwide is affected by allergic diseases, such as atopic dermatitis, food allergy, asthma, allergic rhinitis and conjunctivitis [1]. Allergic diseases manifest in several ways and an age-associated variation in allergy-related symptoms in childhood is typically seen in a population and is referred to as the “atopic march”. The first allergy-related symptoms are commonly eczema and food allergy, later followed by asthma and rhinoconjunctivitis. In addition to the age variation in allergic diseases, there seem to be a gender association as well. Boys have a higher incidence of allergic diseases than girls early in life [2, 3] and also a more Th2 deviated immunity with an increased susceptibility to infections [2, 3]. The stronger Th1 response in females, as evident by higher levels of inflammatory markers and more efficient infection clearance results in better protection against infection but also in increased susceptibility to autoimmune diseases [2, 3]. Allergy-related sex differences diminish at puberty, and at adult age no clear sex differences concerning allergy can be found [4]. There is a wide range in severity of the allergy related diseases. Symptoms range from very mild to disabling diseases such as severe treatment resistant asthma or eczema and life-threatening anaphylactic reactions.

To further add complexity, early life events occurring during critical windows of immune development can have long-term impact on immune-mediated diseases [5]. The immune status at birth may have a role in this also. Of importance is the maternal immunity which influence the fetus in several proposed ways [6].

Although a prevention strategy has effect on one allergic manifestation, it may fail to reduce the development of another. Sensitization and IgE-associated eczema in infancy seem to be the most reliable predictors for subsequent respiratory allergic diseases in school age.

Allergic manifestations

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 and modified by Seymour for infants are the most commonly employed in allergy research [7]. The pathogenesis of eczema is multifactorial, a loss of function in the skin-barrier seem to play a critical role in the development of eczema. These defects in skin-barrier likely results 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 protein fillaggrin are also considered a major risk factor [8].

Food allergy

While many foods can cause an allergy reaction, however, the most common food allergens are cow's milk and egg allergy in infants [9]. Common features for allergens involved in infant food allergies are heat, acid, and protease stable water-soluble glycoproteins 10 to 70 kD in size. The allergenicity is possible to modify by heating or preparing foods and thereby modifying conformational epitopes, *i.e.* reduce (cooked egg) or enhance (roasted peanut) [10]. The term food allergy can be further split into IgE and non-IgE mediated reactions. IgE mediated food allergy begins in the first 1-2 years of life with sensitisation. For an allergic reaction to occur, re-exposure is needed [11]. The symptoms of IgE mediated food allergy include angioedema, urticaria, rashes, flushing, anaphylaxis, bronchospasm, rhinoconjunctivitis and immediate gastrointestinal symptoms. The non-IgE mediated immune reactions that can arise in the gastro-intestinal tract are not so well defined and more difficult to recognize.

Asthma and Allergic rhinoconjunctivitis (ARC)

Asthma is a chronic inflammatory disease in the lower respiratory tract involving mast cells, eosinophils and T lymphocytes. Asthma is characterized by repeated attacks, induced by allergen, often furred animals, or viral infections, of wheeze and cough [12]. About 10% of children in a high risk cohort in Sweden developed IgE mediated asthma [13], also called allergic asthma. Without concomitant sensitisation or asthma in the family, infants with non-allergic asthma do not have an increased risk of asthma at 7 years of age according to the German Multicentre Allergy study [14] Rhinoconjunctivitis mediated by allergens, most often birch, is uncommon in the first two years of life but the incidence increases with age and is about 20 % in the teen-age years [15].

In summary, allergic asthma and rhinoconjunctivitis are not as common as eczema during the first two years of life. Wheeze is a frequent symptom in early childhood but it is commonly triggered by infections and does not always predispose for future asthma.

Environment and microbial exposure

The hygiene hypothesis

Allergy-related diseases were rare a hundred years ago and there has been a substantial increase in prevalence the last half of the century [16]. Even if heredity is a significant risk factor for allergy-related diseases, especially if the mother is allergic [17], the large and rapid increase cannot be explained by genetics alone. Therefore, lifestyle-related and environmental exposures are believed to interact with our genes in the development of allergy-related disease and a “Westernized” lifestyle has contributed to a substantial part. Large geographical variations in prevalence of allergy-related diseases have been demonstrated, with higher occurrence in Westernized countries [18-20]. In 1989 a researcher named Strachan discovered that there was an association between siblings, family size and hay fever [21]. Furthermore, changing climate, living conditions and urbanization have led to a biodiversity loss both at the macro and micro level [22]. Studies have shown alterations in the microbiota composition and a general microbial deprivation which characterize people

living in urban affluent environments, further enhanced by physical inactivity and a Western diet poor in fresh fruit and vegetables, which may act in synergy with dysbiosis of the gut microbiota, immune dysregulation and impaired immune tolerance. Microbial exposure in early life may educate the developing immune system, driving postnatal maturation of immune regulation as discussed in [23]. The author also suggested that the theory should be referred to as “microbial deprivation hypothesis” since the exposure to a wealth of commensal, non-pathogenic microorganisms early in life is suggested to be of benefit for proper immune maturation [24].

Farm studies

Children that lived on farms had fewer allergies than children born in urban and non-farming rural areas [24-27], as evident with a long follow up time [28]. Additionally, such environment are of course associated with an enriched microbial burden evident in several ways, children exposed to several types of animals and consumption of unprocessed raw cow milk, seem to mediate a protective effect reviewed in [25]. In fact, living with a pet during childhood may also be protective against asthma [29]. In the Protection Against Allergy: Study in Rural Environments (PASTURE) birth cohort study, 1133 pregnant women were recruited in rural areas of Austria, Finland, France, Germany, and Switzerland, demonstrating that farming-related exposures, such as raw farm milk consumption and exposure to animals, that were previously reported to decrease the risk for allergic outcomes, were associated with a change in gene expression of innate immunity receptors in early life [30]. Raw milk contains a lot of Lactobacilli strains and have a different fat composition than processed milk. Exposures to farming areas and consumption of raw milk have been associated with the up regulation of certain receptors associated with innate immunity in school-aged children [31-33]. If the mother is exposed to farm environment, in particular during pregnancy, development of allergic diseases seems to be attenuated [34, 35]. Therefore, microbial exposure in early life *i.e.* pre- and postnatal, may educate the developing immune system, driving postnatal maturation of immune regulation [16].

Farm studies and epigenetics

At least some of the protective effects of farm living may be mediated by epigenetic mechanisms. Thus, farm living has been associated with increased demethylation of the FOXP3 promoter in Treg cells. This is supposed to reflect a more efficient suppressive function in Tregs. Farm living in the same study was also associated with higher numbers of FOXP3 positive cells in cord blood mononuclear cells (CBMCs) [36]. In addition, a subsample from the PASTURE study showed hypomethylation in different regions of the ORMDL1 and signal transducer and activator of transcription 6 (STAT6) genes (genes highly associated with asthma) while regions in RAD50 and IL13 were hypermethylated in cord blood from children from farm environment [37].

Animal models of microbial exposure pre- and postnatally

The epidemiological studies are supported by animal models, demonstrating that microbial exposure during gestation can prevent allergic responses in the offspring [38, 39]. The beneficial effect of exposure to microbes have been further explored, primarily in animal models, to try to pinpoint what the mechanism on immune tolerance and protection of allergic disease might be. Of importance is the maternal environment, suggesting that maternal immunity may be transferred or at least influence the offspring. In experimental murine models the treatment of the mother with lipopolysaccharide [39-41] or the commensal *Acinetobacter lwoffii* [38] during gestation attenuates allergic sensitisation and airway inflammation in the offspring. The main receptors for bacterial products are the Toll like receptors (TLRs), which seem to be essential for the allergy protective effect of bacterial exposure during pregnancy [38]. Not only the maternal environment seem to be of importance, but the continued enhanced postnatal microbial exposure may be required for optimal allergy protection, however [42]. A reduced microbial pressure could result in insufficient induction of T cells with regulatory and/or Th1-like properties, which counteract allergy-inducing Th2 responses [25, 36, 43, 44]. Allergic diseases are known to be dependent on Th2 responses to allergens and microbial stimulation may be one way to deviate a skewed Th2 associated immunity to a more Th1/Treg associated response, as will be discussed later.

Gut microbiota and associated lymphoid tissue

There are several important sites where antigens encounters occur, but since the gastrointestinal tract is the largest immunological organ in the body and the gut mucosa has a surface of approximately 400 m², this area is of essential importance [45]. The surface epithelium of the mucosa is constantly exposed to myriads of microbes and dietary constituents. This is a very important site for immune regulation and encounters with microbes and almost 70 % of the human lymphoid tissue is located there [45, 46]. The adult human intestinal canal is inhabited by an estimated 10¹⁴ microbes, mainly bacteria and mainly in the colon [47]. Earlier, the estimation of microbes was ten times the estimated number of human cells in our body, but was recently challenged and estimated to be the approximately the same number [48]. The gut microbiota has several beneficial functions for the host, including fermentation of indigestible carbohydrates into short chain fatty acids, immune system maturation and protection from invasion of exogenous microbes. There are several theories regarding the importance of the exposure to microbes. One of them, the microbial deprivation hypothesis states that 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 [23].

The gut microbiota is important in the maturation of the immune system, which is well documented in germ free animal models. These animals fail in developing normal maturation and in particular the regulatory network essential for controlling exaggerated immune responses, leading to disease states such as allergies and autoimmunity (reviewed in [49]). Development of allergic diseases in infants have been preceded by differences in composition and diversity of their gut microbiota [50-62].

Probiotics in allergy prevention

Probiotics are defined as “live microorganisms which when ingested in adequate amounts confer a beneficial effect on the host” [63]. Host interactions with microbial organisms from the environment [21] and at mucosal sites, such as the gut [50, 64, 65] have been proposed to be important for correcting the dysbiotic drift caused by modern living. Probiotics have been proposed to have immunomodulatory effects [66] and promote gut microbiota diversity

although evidence for this is scarce in allergy prevention studies (as reviewed in [67]). The intestine provides a unique environment for the development of both immunity and tolerance. Probiotic supplementation during pregnancy and early childhood could possibly provide microbial stimulation needed for normal development of immune regulatory capacity by providing a source of TLR-ligand exposure [26].

Probiotics in clinical trials

Current evidence of using probiotics for allergy prevention in pregnant women, breast-feeding mothers and/or children were summarized in [68] concluding that the most effect is observed when supplementation is given pre- and postnatal. A total of 29 trials fulfilled the criteria set up for the analysis [68]. Unfortunately, there are some factors that make the certainty of evidence low, the heterogeneity among studies (see Table Overview of Probiotic trials). There is a need for high quality studies in this area. The conclusion of this which is also stated by the World Allergy Organization (WAO) [69], is that probiotics given to pregnant women, breast-feeding mothers and/or infants reduced the risk of eczema in infants. Currently available evidence does not indicate that probiotics prevent the development of other allergies. The studies are summarized in [67] as well as investigated immune- and gut modulatory effect. However, there is a plea for future studies that consider the weakness identified regarding the risk of bias and indirectness of the evidence. To have in mind, there are several problems when advising that needs to be solved, which strain, doses and timing to use?

However, other international expert bodies including EAACI, the American Academy of Pediatrics, ESPGHAN, NIAID and FAO/WHO [70-74] do not generally recommend probiotics for allergy prevention at this time.

Table I. Summary of probiotic trials, Supplementary Table from [67]:

Overview of probiotic trials						
Study population and probiotic intervention	Effect on eczema	Effect on sensitization	Effect on respiratory symptoms	Effect on lung function measures	Immunomodulatory effects	Effects on gut microbiota
MATERNAL ADMINISTRATION ONLY						
Huurre et al, 2008 [75] Maternal allergic disease <i>L. rhamnosus</i> GG and <i>B. lactis</i> Bb-12 1x10 ¹⁰ CFU daily from first trimester and then to breastfeeding mother until cessation of exclusive breastfeeding	No Long term outcomes not reported	Not reported	Not reported	Not reported	Huurre 2008: The breast milk TGF-β2, sCD14, IFN-γ, TNF, IL-10, IL-6, IL-4 and IL-2 levels were measured from samples taken immediately after birth and 1 month after delivery. The colostrum TGF-β2 levels tended to be higher in the probiotic than the placebo group, while the other mediators were not affected [75].	Not reported
Dotterud et al, 2010 [76] and Simpson et al, 2015 [77] Unselected - about 2/3 with family history of allergic disease <i>L. rhamnosus</i> GG, <i>L. acidophilus</i> LA5, and <i>B. lactis</i> Bb-12 (5 x 10 ¹⁰ CFU of each daily) from 36 weeks gestation and then to breastfeeding mother for 3 months	Reduced cumulative incidence of eczema at 2 and 6 years	No	No	Not reported	Not reported	Dotterud 2015; Infant stool samples from 10 days, 3 months, 1 year and 2 years, were analysed by qPCR and 16S rRNA gene deep sequencing on the Illumina MiSeq platform. Only the LGG bacteria colonized the children at 10 days and at 3 months of age. There were no significant differences in the abundance of the probiotic bacteria between the groups at 1 and 2 years of age, nor for the bacterial classes and genera, alpha and beta diversity [78].
Boyle et al, 2011 [79] Any first degree relative with allergic disease <i>L. rhamnosus</i> GG 1.8 x 10 ¹⁰ CFU daily from 36 weeks gestation until delivery - no postnatal administration to mother	No at 12 months Long term outcomes not reported	No	No	Not reported	Boyle 2008; CBMCs from 73 neonates were cultured with heat-killed LGG, ovalbumin (OVA) or without stimulus. LGG treatment of pregnant women did not influence CD4+ T cell proliferation, FoxP3 expression, DC phenotype or cytokine secretion in CBMCs cultured with heat-killed LGG or OVA [80]. Boyle 2011; CBMCs from 73 neonates were examined for DC and Treg numbers and LTA and LPS induced production of TGF-β, IL-10, IL-12p40, IL-13, IFN-γ and TNF. Prenatal probiotic treatment was not associated	Lahtinen 2009; Investigated infant faecal samples from 7 and 90 days using qPCR and T-RFLP. At 90 days of age, infants whose mothers received LGG were more often colonized with species belonging to the <i>B. longum</i> group. Bifidobacterial species colonization at 7 days or <i>Bifidobacterium</i> levels did not differ between the 2 groups [81]. Ismail 2012; using T-RFLP analysis showed that prenatal LGG failed to modulate diversity of

							with any change in cord blood immune markers or cytokine secretion. Breast milk samples from the probiotic group had lower levels of total IgA at day 28 and lower sCD14 at day 7, while breast milk TGF-β1 levels were not affected [79].	early infant gut microbiota despite promoting a beneficial bifidobacteria profile [82].
Rautava et al, 2012 [83] Maternal allergic disease <i>L. rhamnosus</i> LPR and <i>B. longum</i> BL999 or <i>L. paracasei</i> and <i>B. longum</i> BL9 – each probiotic at a daily dose of 1x 10 ⁹ CFU from two months before delivery and during two months to breastfeeding mother	Reduction of eczema at 2 years in both probiotic groups Long term outcomes not reported	No	Not reported	Not reported	Not reported	Not reported	Not reported	Not reported
PERINATAL ADMINISTRATION TO MOTHER AND/OR CHILD								
Kalliomäki et al, 2001 [84] and Kalliomäki et al, 2007 [85] Any first degree relative with allergic disease <i>L. rhamnosus</i> GG 1x10 ¹⁰ CFU daily given to mothers 2-4 weeks before delivery and then to breastfeeding mothers or directly to infant, for 6 months	Reduction of eczema at 2 years which remained at 7 years	No	No	No	No	No	Rautava 2002; Probiotic administration increased the amount of anti-inflammatory TGF-β2 in the milk at three months in mothers receiving probiotics as analysed by ELISA. Infants with elevated IgE in cord blood benefited most of the probiotic supplementation [86].	Guemonde 2006; At 5 days of age, infants whose mothers received <i>L. rhamnosus</i> GG showed a significantly higher occurrence of <i>B. breve</i> and lower of <i>B. adolescentis</i> than those from the placebo group, as determined by PCR. In addition, <i>L. rhamnosus</i> GG consumption increased the bifidobacterial diversity at 3 weeks in infants [87].
Abrahamsson et al, 2007 [88] and Abrahamsson et al, 2013 [89] Any first degree relative with allergic disease	No reduction of eczema, but reduction of IgE-associated eczema in the probiotic group at 2 years	No	No	No	No	No differences between the groups when evaluated by spirometry reversibility test	Abrahamsson 2011; There were no significant difference in chemokine levels between the <i>L. reuteri</i> and the placebo-treated group, at CB, 6-, 12- and 24 months. The presence of <i>L. reuteri</i> in stool the first week of life was associated with lower CCL22 and CCL17 and higher CXCL11 levels at 6 months of age [90].	Abrahamsson 2009; The prevalence of <i>L. reuteri</i> was higher during the first year of life in the stool samples from infants in the probiotic group. The highest prevalence was recorded at 5 to 6 days of age (82% in the treated vs 20% in the placebo group). Supplementation affected neither the prevalence nor the counts of bifidobacteria or <i>C. difficile</i> , except for higher counts of bifidobacteria in the treated group at

<i>L. reuteri</i> 1 x 10 ⁸ CFU daily 2-4 weeks before delivery and then to infant for 12 months	No difference between the two groups at 7 years follow up	No		and FeNO levels at 7 years	<p>Böttcher 2008; Probiotic supplementation was associated with low levels of TGF-β2 and slightly increased levels of IL-10 in colostrum. Infants receiving breast milk with low TGF-β2 levels were less likely to become sensitized during their first 2 yr of life. The levels of total IgA, SIgA, TGF-β1, TNF, sCD14, and Na/K ratios in breast milk were not affected by <i>L. reuteri</i> intake [91].</p> <p>Forsberg 2013; Probiotic treatment was associated with low cat-induced IL-5 and IL-13 responses at 6 months, with a similar trend for IL-5 at 12 months. Cat-induced IFN-γ responses were also lower after probiotic than after placebo treatment at 24 months, with similar findings for IL-10 at birth and at 12 months. At 24 months, birch induced CCL22 levels were lower in the probiotic than in the placebo group [92].</p> <p>Forsberg 2014; Probiotic supplementation was associated with decreased LTA induced CCL4, CXCL8, IL-1β and IL-6 responses at 12 months and decreased CCL4 and IL-1β secretion at 24 months. TLR2 and TLR4 mRNA expression and responses to LPS were not affected by probiotic treatment [93].</p>	<p>2 years. At 12 months the prevalence of <i>L. reuteri</i> was lower in breast-fed than formula-fed infant. <i>L. reuteri</i> was isolated from 12% and 2% of the colostrum samples in the probiotic and placebo group, respectively [94].</p> <p>Abrahamson 2012; Probiotic supplementation did not affect gut microbiota diversity at 1 and 12 months, as determined by 16S rDNA 454-pyrosequencing [55].</p>
<p>Kukkonen et al, 2007 [95] and Kuitunen et al, 2009 [96]</p> <p>Any first degree relative with allergic disease</p> <p>Mix of <i>L. rhamnosus</i> GG and LC705 (both 5 x 10⁹) and <i>B. breve</i> Bb99 and <i>Propionibacterium freudenreichii</i> ssp. <i>shermani</i> JS (both 2 x 10⁹) plus prebiotic galactooligosaccharides; given twice daily to mother 2-4 weeks before delivery and then to infant for 6 months</p>	<p>Eczema reduction in the probiotic group at 2 years</p> <p>No eczema reduction at five years</p>	No	No	No differences in FeNO levels between the groups at 5 years in a randomized subpopulation	<p>Kukkonen 2006; In the probiotic compared with the placebo group, protective antibody concentrations to Haemophilus influenzae type b (Hib) occurred more frequently at 6 months, and geometric mean IgG titres to Hib tended to be higher. IgG titres to diphtheria and tetanus were similar in the 2 groups [97].</p> <p>Marschan 2008; Infants receiving probiotic bacteria had higher plasma levels of CRP, total IgA, total IgE, and IL-10 than infants in the placebo group [98].</p> <p>Kuitunen 2009; Probiotic supplementation caused a gut mucosal inflammation with decreased Hb values at 6 months, but Hb and the other hematologic values were similar at 2 years in the 2 groups [99].</p>	<p>Kukkonen 2007; Faecal counts of all the supplemented microbes were significantly higher at 3 and 6 months. At 2 years, no differences were observed between study groups in faecal bacterial colonization using agar culturing and PCR [95].</p> <p>Kukkonen 2009; Faecal IgA, α1-AT, TNF and calprotectin was measured at the age of 3 and 6 months. Probiotics tended to augment faecal IgA and significantly increased faecal α1-AT. High intestinal IgA associated with reduced allergy risk [102].</p>

						Kuitunen 2012 : Probiotic supplementation was associated with less IgA to casein and more IL-10 in mature BM (3 month samples) but not in colostrum, and with reduced colostrum but not mature BM TGF- β 2 levels. Probiotic supplementation did not affect colostrum or mature BM total IgA levels, nor levels of IgA antibodies to CM, BLG and OVA [100]. Savilathi 2015 : Colostrum and 3 month BM sCD14, human neutrophil peptide (HNP) 1–3 and β -defensin 2 (HBD2) levels were not affected by probiotic supplementation [101].	
Kopp et al, 2008 [103] Any first degree relative with allergic disease <i>L. rhamnosus</i> GG 1x10 ¹⁰ CFU daily given to mothers 4-6 weeks before delivery and then to breastfeeding mother for 3 months or to infant for 6 months	No at 2 years Long term outcomes not reported	No	No	Not reported	Not reported	Kopp 2008 : CBMC and PBMC of the corresponding mother were isolated from cord blood and peripheral blood (n=68). Cells were stimulated with IL-2, β -lactoglobulin or LGG and IFN- γ , IL-10 and IL-13 in the supernatants were measured with ELISA. LGG induced IL-10 and IFN- γ secretion <i>in vitro</i> , but independently of probiotic supplementation [104].	Not reported
Wickens et al, 2008 [105] and Wickens et al, 2013 [106] Any first degree relative with allergic disease <i>L. rhamnosus</i> HN001 or <i>B. lactis</i> HN019 1x10 ¹⁰ CFU daily from 2-5 weeks before delivery and then to infant directly for 2 years	Eczema reduction in the <i>L. rhamnosus</i> group at 2 years which remained until 6 years No benefit of <i>B. lactis</i>	Lower cumulative sensitisation in the group receiving <i>L. rhamnosus</i> at 6 years No benefit of <i>B. lactis</i>	No	No differences between the groups when evaluated by spirometry reversibility test and FeNO levels at 6 years	No	Prescott 2008 : Neonates of mothers who received <i>L. rhamnosus</i> but not <i>B. lactis</i> had higher CB IFN- γ levels, compared with the placebo group. Colostrum TGF- β 1 levels were increased after <i>B. lactis</i> supplementation, with a similar tendency for <i>L. rhamnosus</i> . Increased colostrum IgA levels were observed after both <i>B. lactis</i> and <i>L. rhamnosus</i> administration. Neonatal plasma sCD14 levels were lower in the <i>B. lactis</i> group compared with the placebo group [107].	Wickens 2008 : <i>L. rhamnosus</i> (71.5%) was more likely than <i>B. lactis</i> (22.6%) to be present in the faeces at 3 months, although detection rates were similar by 24 months [105].
Niers et al, 2009 [108] and Gorissen et al, 2014 [109]	Reduced cumulative incidence of	No	No	Not reported	Not reported	Niers 2009 : Reduced anti-CD2/CD28 induced IL-5 and IL-13 levels were found in whole blood cultures at 3 months of age in the probiotic compared with the placebo group. The <i>in vitro</i> lymphocyte proliferative	Niers 2009 : Using T-RFLP, qPCR and DGGE, <i>L. lactis</i> and <i>B. bifidum</i> but not <i>B. lactis</i> were more easily detectable in the probiotic compared

Allergic disease of either parent and in at least one sibling <i>Lactococcus lactis</i> W58, <i>B. lactis</i> W52 and <i>B. bifidum</i> W23 1×10^9 CFU each daily six weeks before delivery and then directly to infant for 12 months	eczema in the first three months of life No difference at 6 years					response to either anti CD2/CD28 or PHA did not differ between the groups [108]. Rutten 2015 ; Only minor and short term differences in composition of microbiota between the probiotic and placebo group were found using 16S-23S rDNA interspace region based profiling. Gut microbiota development continued between two and six years, then approaching a more adult-like composition [110].
Kim et al, 2010 [111] Any first degree relative with allergic disease <i>B. bifidum</i> BGN4, <i>B. lactis</i> AD011, and <i>L. acidophilus</i> AD031 (1.6×10^9 CFU of each daily) 4-8 weeks before delivery, 3 months to breastfeeding mother and then to infant from 4 to 6 months	Reduced cumulative incidence and prevalence of eczema at 12 months Long term outcomes not reported	Not reported	Not reported	Not reported	Not reported	Not reported
Ou et al, 2012 [112] Maternal allergic disease <i>L. rhamnosus</i> GG 1×10^{10} CFU daily from second trimester and then 6 months to mother if breastfeeding or directly to infant	No Long term outcomes not reported	No	Not reported	Not reported	Ou 2012 ; There were no significant differences in plasma IgE levels in the children at 6, 18, and 36 months of age, while mean plasma IgE was higher in the cord blood of the LGG group. Maternal plasma IL-13, IL-10, IFN- γ , CXCL10, and TGF- β levels were not affected [112].	Not reported
Allen et al, 2014 [113] Any first degree relative with allergic disease	No reduction of eczema, but a reduction of IgE-associated eczema at 2	Reduced cumulative frequency of SPT reactivity at	No	Not reported	Not reported	Not reported

<i>L. salivaris</i> CUL61, <i>L. paracasei</i> CUL08, <i>B. animalis ssp lactis</i> CUL34 and <i>B. bifidum</i> CUL20, 10 ¹⁰ CFU daily in total starting 2-4 weeks before delivery and then to the infant for six months	years of age in the probiotic group	2 years in the probiotic group				
POSTNATAL ADMINISTRATION						
Taylor et al, 2007 [114] and Jensen et al, 2012 [115] Maternal allergic disease <i>L. acidophilus</i> (LAVRI-A1) 3 x 10 ⁸ CFU given within 48 hours, and then for six months, directly to infant	No reduction at 1 year nor at the or 5 year follow-up	No Sensitisation more common in the probiotic group at 1 year, but not at the later follow-ups	No	Not reported	Taylor 2006 ; Infant cytokine (IL-5, IL-6, IL-10, IL-13, TNF or TGF-β) responses to TT, HDM, OVA, BLG, SEB and PHA were measured at 6 months of age. Probiotic supplementation was associated with reduced production of IL-5 and TGF-β in response to SEB stimulation, lower IL-10 responses to TT vaccine antigen and reduced TNF and IL-10 responses to HDM allergens[116]. Taylor 2006 ; Mononuclear cell samples were available from 118 infants and stimulated using ligands for TLR2 and TLR4/CD14, finding no effects of the probiotic supplementation on cytokine responses. Circulating DC subset frequencies and antigen presenting capacity were similar between the groups [117]. Taylor 2007 ; Infant regulatory T-cell function was examined at 6 months. Probiotic supplementation did not affect the proportion of circulating CD4+CD25+CTLA4+ cells or FoxP3 mRNA expression [118].	Taylor 2007 ; At 1 month of age, infants in the probiotic group were almost twice as likely to show culturable levels of Lactobacillus species. By 6 months of age, the rate of Lactobacillus colonization was significantly higher in the probiotic than the placebo group. The rates of colonization with Bifidobacterium not affected by the treatment [114].
Soh et al, 2009 [119] and Loo et al, 2014 [120] Any first degree relative with allergic disease, <i>L. rhamnosus</i> LPR 1 x 10 ⁹ CFU and <i>B. longum</i> (BL999) 6 x 10 ⁸ CFU daily to infant (in infant formula) for 6 months	No reduction at 2 or 5 years	No	No	Not reported	Compared with placebo, probiotic supplementation improved hepatitis B (HepB) surface antibody responses at 12 months in subjects receiving monovalent doses of HepB vaccine at 0, 1 month and a DTPa-HepB combination vaccine at 6 months, but not those who received 3 monovalent doses [121].	Not reported

<p>West et al, 2009 [122]</p> <p>West et al, 2013 [41]</p> <p>Mixed (2/3 with at least one first grade relative with allergic disease)</p> <p><i>L. paracasei ssp paracasei</i> F19 1 x 10⁹ CFU daily to infant (in infant cereal) during weaning from 4-13 months</p>	<p>Reduced cumulative incidence of eczema at 13 months</p> <p>No difference at 8 years</p>	<p>No</p>	<p>No</p>	<p>No differences between the groups when evaluated by spirometry reversibility test and FeNO levels at 8 years</p>	<p>West 2008: Antibody concentrations to Hib capsular polysaccharide (HibPS), diphtheria toxin (D) and tetanus toxoid (T) before and after the second and third doses was measured. LF19 enhanced antibody concentrations to D and T, especially in infants breastfed less than 6 months. Conversely, breastfeeding duration influenced the anti-HibPS concentrations, with no effect by LF19 [123]</p> <p>West 2009; At 13 months of age, the anti-CD3/CD28 induced IFN-γ/IL4 mRNA ratio was higher in the probiotic compared with the placebo group [122].</p> <p>West 2012; At 13 months of age, the anti-CD3/CD28 induced IFN-γ/IL-2 and IL-17A/IL-2 mRNA ratios were higher in the probiotic than the placebo group, as was the TT induced IL17A expression. No differences were observed between the two groups at 5.5 months [124].</p>	<p>West 2008: Using RAPD-PCR, <i>L. paracasei ssp paracasei</i> F19 was detected in stool in 90% of the infants in the probiotic group at 6 months and the frequency remained high throughout the intervention [123].</p>
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Immunomodulatory effects of probiotics

Probiotics may have systemic effects and may modify breastmilk composition [75, 79, 86, 91, 100, 101, 107] since the entero-mammary pathway reflect nutritional, metabolic and immunological processes in the gut and thereby the mammary glands [125]. In summary, no consistent effects on TGF- β 1, TGF- β 2 and IgA levels in breastmilk have been observed.

The effect of probiotics on peripheral immune response include several theories, including enhanced immune maturation, increased T helper 1 (Th1) associated immunity, but also induction of T regulatory cells (Tregs) and thereby increased peripheral tolerance. Effects on both the innate and adaptive immunity have been investigated, see Table I and [67]. In summary, there are some indications of reduced cytokine and chemokine responses to certain stimuli may be associated with probiotic supplementation during pregnancy and/or infancy. For example, reduced anti-CD2/CD28-induced IL-5 and IL-13 levels in whole blood cultures were noted at 3 months of age after pre- and postnatal supplementation with a mixture of *B. bifidum*, *B. lactis* and *L. lactis* as compared with placebo [108]. The same pattern with reduced responses to polyclonal stimuli with Staphylococcal Enterotoxin B (SEB) (lower IL-5 and TGF- β levels) and house dust mite (HDM) allergens (lower tumour necrosis factor (TNF) and IL-10) at 6 months was found after postnatal *L. acidophilus* as compared with placebo administration [116]. However, all studies have slightly different designs and time points for sample collection, in addition to the variation in probiotic strains and treatment duration.

Gut microbiota and probiotic supplementation

Probiotics have been proposed to have a beneficial effect on gut microbiota composition and the probiotic strain may be transiently detected during the supplementation period in most studies (Table 1)[67]. However, gut microbial diversity promoting effects early in life have not been observed generally, Table I. Although, when comparing the results from the different studies it is central to acknowledge how varying methodologies may affect the findings. Traditional culture based methods are difficult to equate with the next generation sequencing tools that are available today. There is some proof for a bifidogenic effect of probiotic supplementation [87, 126], although this has not been consistently observed [108, 114]. Also,

the probiotic strain has been detected in faeces during but not after the administration period in several studies. Long term effects remain to be investigated, as few such studies have been performed. The effects on gut microbiota composition seem to depend on choice of strain and treatment duration, which is consistent with the reported strain-specific differences also for immunomodulatory and clinical outcomes, Table I.

Lactobacillus reuteri

More and more evidence highlight the importance of choice of probiotic strain when conducting clinical trial in allergy prevention [127] and also in other areas where probiotics have had beneficial effects, such as preventing necrotizing enterocolitis (NEC) in preterm infants [128]. *Lactobacillus reuteri* is an obligate heterofermentative [129] Gram positive rod that has been isolated from the GI tract in several mammals, including humans, as well as from different food products [130-132]. In addition to glycerol, *L. reuteri* produce the antimicrobial metabolite reuterin during anaerobic conditions (272). The strain *L. reuteri* ATCC 55730 is considered safe as no adverse events have been recorded [67].

In conclusion, although promising effects of probiotics in eczema prevention has been observed, further research is required to be able to translate the WAO recommendations into practice guidelines. As specific advice, at the present, on choice of strains, dose, timing, mode of administration and duration is not possible to give due to the great heterogeneity between studies performed so far. To be able to do so more knowledge on immune modulating effects after supplementation is needed.

ω -3 fatty acids

Figure 1. Inflammatory pathways, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid

The nutritional content of the diet has changed in the Westernized world [133], epidemiological data show an increase of ω -6 fatty acids intake and a decrease in the intake of ω -3 fatty acids [134]. Several studies [135-142] indicate that fish rich in fatty acids has a protective effect on the development of allergic disease [143, 144]. Especially the ω -3 PUFAs linoleic acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the ω -6 PUFAs linoleic acid (LA) and arachidonic acid (AA), are key fatty acids, Figure 1. ω -3 fatty acids are considered to be anti-inflammatory and ω -6 pro-inflammatory. The main link between n-6 PUFAs and inflammatory processes is through AA and its metabolites. Because of their anti-inflammatory properties, n-3 PUFAs, particularly EPA and DHA, might prevent the development of asthma by antagonizing the effects of AA. The receptor of importance may be GPR120 which ω -3 signal mainly through [145]. Human studies indicate that supplementation with ω -3 PUFAs might have beneficial effect in patients with allergic asthma and atopic dermatitis [133], and also decrease the allergic response to allergens in infants [138]. In a randomized clinical trial supplementation with ω -3 fatty acids (1.6 g EPA and 1.1 g DHA or placebo) daily during pregnancy week 25 to average 3-4 months of breastfeeding

resulted in lower prevalence of food allergy and IgE- associated eczema in the treated group during the first year of life [146]. The cumulative incidence of IgE-associated disease was also decreased at two years in the treated group, although no obvious effect on the clinical symptoms of allergic diseases. Furthermore, higher mother and infant intake of DHA and EPA were associated with lower prevalence of allergic disease [147]. The effect of supplementation on the peripheral immune system was also investigated, in non-allergic infants the circulating CXCL11 levels were higher as well as the titers of IgG to diphtheria compared to non-allergic infants [148]. During pregnancy, the proportions of EPA and DHA in plasma/serum phospholipids increased in the ω -3-supplemented group, whereas AA decreased during intervention. Also, lipopolysaccharide-induced prostaglandin E2 secretion decreased in a majority of the ω -3-supplemented, with more pronounced effects among non-atopic than atopic mothers [149].

In conclusion, a higher intake of ω -3 may have an allergy preventive effect by possibly antagonize the effect of AA. The synergistic effect of supplementation with both ω -3 and probiotics for allergy prevention would be of interest to evaluate.

Immunological mechanisms

Overview of the immune system

Figure 2. Brief overview of cells associated with innate and adaptive immunity central for this thesis

Innate immunity

The immune system is traditionally divided into innate and adaptive immunity, Figure 2 [46]. The first line of defense is the innate immunity which responds rapidly to common components of microorganisms such as bacteria, viruses, parasites and fungi, structures preserved during evolution called pathogen associated molecular patterns (PAMPs). The innate immune system includes physical barriers of the mucosa, the epithelial cell layer, as well as cells responding immediately with phagocytosis of microorganisms, extinction of infected cells and cooperation with adaptive immunity. The primary sensors, pattern recognition receptors (PRRs) recognize these PAMPs. The PRRs are expressed on various cells of the immune system such as monocytes, macrophages, DCs, NK cells and innate lymphoid cells as well as mucosal epithelial and endothelial cells. Examples of PRRs are Toll-like receptors (TLR), NOD-like receptors (NLR), RIG-1-like receptors (RLR), β -glucan receptors and

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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.

Dendritic cells

Dendritic cells are the major antigen presenting cells. Microbial stimulation of DC leads to secretion of cytokines, such as IL-10 and the proinflammatory IL-12, as well as upregulation of co-stimulatory molecules, *e.g.* CD40, CD80 and CD86 [46]. The DC stimulation pattern by specific bacteria varies profoundly and bacterial strains differ in their subsequent modulation of T cells *via* DC priming [150]. DCs are also able to attract cells via secretion of chemokines, for example Th2 cells are attracted by secretion of CCL17 and CCL22. There are two major DC populations in blood, mainly characterized by their different TLR receptor expression and different function, the myeloid derived DCs and the plasmacytoid DCs. Surface expression of CD-antigens also distinguish them from each other, both subtypes lack the common lineage markers but express HLA-DR for antigen presentation [151]. Furthermore, they have different cytokine profiles, for example pDCs are the main producers of interferons [152].

Monocytes

Monocytes, bone marrow derived leukocytes with high CD14 expression, circulating in the blood [46]. When migrating to tissues, they mature and differentiate into macrophages. Gram positive and negative bacteria *via* the PRRs TLR2 and TLR4, respectively differentially activate monocytes. Macrophages can be divided into M1 and M2 subtypes. In response to bacterial moieties and IFN- γ , M1-cells produce the proliferative cytokine IL-2, and the type 1 associated chemokines CXCL9, CXCL10 and CXCL11. The M2 activation, most often associated with IL-4, leads to secretion of type 2 associated CCL17 and CCL22, as well as, the anti-inflammatory IL-10. Macrophages and DCs also produce proinflammatory cytokines such as TNF and IL-6 upon PAMP stimulation. IL-6 has several other important features besides inducing inflammatory responses such as initiating the production of C-reactive protein (CRP) and fever. IL-6 is also involved in the transition from innate to acquired immunity, including Th17 cell differentiation, inducing IgA synthesis in Peyer's patches and regulating the epidermal barrier after tissue injury. In addition, IL-6 might also have anti-inflammatory effect since it induces

the release of IL-10, IL-1 receptor antagonist and plasma cortisol in humans [46]. In blood there are several ways to define monocytes, by the expression of CD14⁺ and coexpression of CD16^{+/−}. The two subtypes have different migratory properties [153] due to their differential expression of chemokine receptors and also a divergent cytokine producing capacity [154]. The subsets seem to produce TNF at a similar degree upon TLR ligation, however only the CD14⁺CD16[−] subset may produce IL-10 [154]. CCR1 and CCR2 are more highly expressed on CD14⁺CD16[−] monocytes whereas CX3CR1 is elevated on CD14⁺CD16⁺ monocytes [155].

NK cells

NK cells recognize immune cells without the MHC I, which they kill in response to for example virus infection, they are cytotoxic and able to lyse or induce apoptosis in cells. In peripheral blood, NK cells can be divided into major subsets of CD56^{dim} CD16^{bright} (~90%) and a smaller population of CD56^{bright}CD16^{-dim} ~10% [156]. These populations differ in several aspects, including cytotoxic potential, cytokine production, and expression of cell surface markers. The CD56^{bright}CD16^{-dim} phenotype is sometimes referred to as regulatory because of its reduced cytotoxic capacity and increased cytokine producing ability.

Epithelial cells and innate lymphoid cells

It is important to remember that, although not being immune cells epithelial cells contribute to the immune response upon stimulation by PAMPs. Besides production of cytokines, such as IL-25 and -33, epithelial cells also produce CXCL8, -9, -10 and -11. TSLP and IL-31 are also important mediators released from epithelial cells, especially upon damage. Innate lymphoid cells type 2 are cells activated by those cytokines and shown to be important in the initiation of type 2 immunity [157]. There are other subtypes of innate lymphoid cells which seems to correspond to the T-helper subtypes, according to their cytokine repertoire, i.e. ILC1, ILC2 and ILC3 [158, 159].

Figure 3. T helper differentiation and associated cytokines relevant for this thesis.

Adaptive immunity

The adaptive immune system requires longer time to develop after exposure to infectious agents but is more specific and can develop memory to encountered antigens. Although these systems are generally described separately, they are closely linked and an efficient immune response is dependent on the interaction between the cellular and molecular components of the innate and the adaptive system. The adaptive part consists of T and B lymphocytes and a rich and specific antibody-repertoire. T cells include cytotoxic T cells and T helper cells with a specific TCR.

T helper cells

Antigen presentation

CD4⁺ T helper (Th) cells have a central role in adaptive immunity by orchestrating immune responses to pathogens and non-pathogenic microbes [46]. Th cells exit the thymus as naïve Th cells and recirculate between the blood and secondary lymphoid organs. Activation occurs within the secondary lymphoid organs by the recognition of antigens associated with antigen-

presenting cells (APCs). Dendritic cells (DCs) are the major APCs during the initial activation of naïve Th cells, but macrophages and B cells can also present antigens to memory Th cells. Th cells recognize antigens through their T cell receptor (TCR)-CD3 complex, which binds the antigen-major histocompatibility complex class II (MHC II) on APCs. Efficient activation is dependent on the interaction between co-stimulatory molecules on APCs, such as CD80, CD86 and CD40, and their receptors on T cells, such as CD28 and CD40 ligand. The milieu and the presence of different cytokines during the activation process drives the differentiation into distinct Th cell subsets. Th cells may differentiate into three major effector subsets, Th1, Th2 or Th17 cells.

T helper 1 cells

Th1 cells are critical for the protection against intracellular pathogens, for instance in mycobacterial infections, but they can also contribute to tissue damage during chronic inflammation and autoimmune disease [46]. The major Th1-inducing cytokines are interleukin (IL)-12, mainly produced by macrophages and DCs, in response to microbes. Cytokines induce the activation of several transcription factors, including Signal Transducer and Activator of Transcription (STAT4) and T-bet. T-bet is the master regulator of Th1 cells and promotes production of IFN- γ , which in turn serves to amplify the Th1 response. IFN- γ promotes the activation of classically activated macrophages enhancing microbicidal activity that is necessary to destroy intracellular pathogens and activate cytotoxic T cells and NK cells and B cells to produce antibodies and complement activation. Epigenetically it is possible to investigate if a T cell is differentiated to Th1 subtypes by investigating the methylated positions in the genome, i.e. closed or open for transcription [160-163].

T helper 2 cells

T helper 2 cells (Th2) are central for the protection against extracellular parasites and they are also involved in the development of allergic diseases [46]. IL-4 drives the differentiation of Th2 cells through the activation of the transcription factors STAT6 and GATA Binding Protein 3 (GATA3). GATA-3 is the master regulator of Th2 differentiation and induced expression of IL-4, IL-5 and IL-13. These cytokines promote IgE antibody responses and the activation of mast cells and eosinophils that are involved in the defense against extracellular

parasites. GATA-3 and Tbet can also act synergistically *i.e.* when Th1 genes are open for transcription, Th2 genes are closed and *vice versa* [163]. Atopy is characterized by Th2 deviated cytokine response to allergens, with high levels of IL-4, -5, -9 and -13 while Th1 cytokines, IFN- γ and IL-12 usually are observed at equal or lower levels [46].

T helper 17 cells

T helper 17 cells (Th17) cells are responsible for the immune defense against extracellular bacteria and fungi and are also involved in the development of organ-specific autoimmune diseases [46]. The differentiation of Th17 cells is induced by IL-6 and IL-1 β , while IL-23 is essential for the maturation and pathogenicity of Th17 cells [46]. In addition, TGF- β is believed to be necessary for optimal Th17 differentiation [46]. Differentiation also involves the activation of STAT3 and the master regulator retinoic acid receptor-related orphan receptors C (RORC) [46]. RORC induces the production of IL-17 (IL-17A and IL-17F) that mediates most of the effects of Th17 cells [46].

Regulatory T cells

In addition to conventional Th cells, CD4⁺ T cells can also differentiate into T regulatory (Treg) cells that are essential for the regulation of inflammatory responses to pathogens but also for peripheral tolerance and the protection against autoimmune diseases [46]. There are two main types of Treg cells, thymic (also called natural) Tregs that are generated in the thymus and are believed to protect against self-reactive immune responses. Inducible Treg cells are generated in peripheral tissues and may have specificity to self and foreign antigens [46]. Forkhead box P3 (Foxp3) is a key transcription factor for the development and function of natural CD4⁺ regulatory T cells. Different subpopulations can be defined within the Foxp3 positive cells. One definition of Treg cells are the CD4^{dim}CD25^{hi}FoxP3⁺ Treg cells, described by Sakaguchi [164]. Later on, CD45RA⁺FoxP3^{lo} resting Treg cells (rTreg cells) and CD45RA⁻FoxP3^{hi} activated Treg cells (aTreg cells) were discovered and both subtypes seem to be suppressive *in vitro* [165]. In company of these subsets, the cytokine-secreting CD45RA⁻FoxP3^{lo} non suppressive T cells were defined. Terminally differentiated aTreg cells rapidly died whereas rTreg cells proliferated and converted into aTreg cells *in vitro* and *in vivo*. rTreg reside in secondary lymphoid tissues and circulate through lymph and blood, their function may

involve inhibiting T cell priming and differentiation by consuming IL-2 which is also essential for their homeostasis. Activated Tregs reside in non-lymphoid tissue and circulate in lymph and blood. Furthermore, TCR stimulation is essential for their differentiation and for homeostasis both TCR and cytokine signals are needed. In contrast to resting Treg there is a high level of cell proliferation and cell death and their function is to suppress differentiation and function of effector T cells [166].

Taken together, the dissection of FoxP3⁺ cells into subsets enables one to analyze Treg cell differentiation dynamics and interactions in normal and disease states [166].

Treg cells have been proposed to be involved in the suppression of allergen specific responses in several ways, *i.e.* suppression of APC and Th2 effector cells regulation of B cells resulting in reduced IgE and increased IgG₄ and IgA synthesis, as well as suppression of mast cells, basophils and eosinophils.

Chemokines and cytokines

Activated Th cells migrate to sites of infection where they perform their effector functions and their specific recruitment is largely mediated by the chemokines produced at sites of infection [46, 167]. Chemokines are a large family of structurally homologous cytokines that stimulate leukocyte movement and regulate the migration of lymphocytes from the blood to the tissue. There are about 50 human chemokines, all 8- to 12- kD polypeptides that contain two internal disulphide loops [46]. The chemokines are classified into four families on the basis of the number and location of N-terminal cysteine residues. The two major families are the CC chemokine family, in which the cysteine residues are adjacent, and the CXC family, in which these residues are separated by one amino acid. The chemokine are mainly produced by leukocytes and endothelial cells, epithelial cells and fibroblasts [46]. Th1 cells preferentially express the chemokine receptor CXCR3, which binds to CXCL9, CXCL10, CXCL11, typically induced by IFN- γ [167]. Th2 cells are mainly characterized by the expression of CCR4 that binds to CCL17 and CCL22 and are induced by IL-4 and IL-13 [167].

The CCR5 ligand CCL4 is a chemoattractant for natural killer cells, and other monocytes. CXCL8 recruits neutrophils, basophils and T cells to site of infection. TNF increases vascular permeability, also important for fever induction [46].

Figure 4. Overview of mucosal immunity

Mucosal Immunity

There are several mechanisms securing a proper mucosal barrier, Figure 4. An important factor is the production of mucus from goblet cells, creating a thick barrier covering the epithelial cells [46]. Within the mucus layer there are non-specific constituents, mucins and defensins, and specific secretory components, secretory IgA (sIgA) antibodies which prevent microbial attachment to the underlying epithelium. The epithelial cell lining is also an effective barrier, as cells are joined together by tight junctions [46]. Peristalsis, low pH in the stomach, proteolytic enzymes and the normal gut microbiota are also important for the defense against harmful bacteria and breakdown of polypeptides to less immunogenic peptides. Although, there must balance between an effective barrier function against pathogens and foreign structures and beneficial microbes as well as nutrient absorption. There are several routes of uptake of antigens and microbial components in the gut, *e.g.* via M cells covering organized lymphoid tissue such as Peyer's patches, *via* DCs spreading through the epithelial cells. Increased antigen uptake is also observed when the permeability of the gut is increased, *e.g.* during inflammation [46].

Gut microbiota

The gut microbiota is primarily assigned to four phyla, the Actinobacteria with genera like *Bifidobacterium* and *Colinsella*, the Bacteroidetes, *Lactobacillus*, *Clostridium*, *Eubacteriam* and *Ruminococcus*, and the Proteobacteria, *Enterobacter spp* [168]. The composition of the microbiota changes substantially at two stages in early life: from birth to weaning, and from weaning to adulthood [169]. The pioneer species in neonates are facultative anaerobic bacteria, such as *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Enterobacter spp.* and these bacteria create an anaerobic environment that promote the growth of obligate anaerobes, such as *Bifidobacterium*, *Bacteroides*, *Clostridium* and *Eubacterium spp.* The dogma that the gut is sterile at birth is challenged [125, 170]. Now, there are consensus that delivery mode and environment at delivery affect the colonization of the infant, since lactobacilli dominate the vaginal flora and are considered beneficial [169, 171]. Furthermore, human milk forms an important continuous inoculum, and contains plenty of lactobacilli. Bacterial strains found in breast milk are postulated to translocate from the mother's intestine to the mammary gland *via* mesenteric lymph nodes, suggesting a possible route of inducing immune tolerance to these commensals [125]. Breastfed infants typically have a microbiota dominated by *bifidobacteria* while formula-fed infants have a more diverse microbiota [169].

Also, the mother 'skin microbiota and infants 'oral microbiota into the mammary gland, host genotype, gestational age, medical practices (antibiotic use), geographic origin, cultural traditions (especially regarding diets) are factors profoundly influencing the microbiota development [169]. The microbiota also influences the immune system by producing factors that promote Treg induction [172, 173].

The allergic response

There are four types of hypersensitivity reactions: immediate hypersensitivity (type I), antibody mediated (type II), immune complex mediated (type III) and T cell mediated (type IV). Type I and IV hypersensitivity reactions include allergy [46]. Type I represent IgE antibody mediated allergy with engagement of mast cells and their mediators and type IV represents Th1/Th17 cell mediated inflammation (non-IgE antibody mediated allergy) such as allergic contact dermatitis. Type II and III hypersensitivity reactions involve IgG and IgM antibodies on

the cell surface or extracellular matrix (type II) or to soluble antigens forming immune complexes (type III) and activation of the complement system [46]. In this thesis the term allergy refers to IgE mediated allergy.

The allergic reaction

Allergy can be divided into three phases, the sensitization phase, the immediate hypersensitivity reactions and the late phase reactions [46]. In the sensitization phase, the antigen presenting cells, *i.e.* 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 the 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 the high affinity FCεRI IgE receptor on mast cells in tissue and basophils in blood. Monocytes, platelets and eosinophils express IgE receptors but at lower levels. On the next encounter with the allergen, a sensitized 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, *i.e.* the late phase reaction. Eosinophilia, induced by IL-5, is particular associated with the late phase response[46].

Allergens

Allergens are innocuous environmental antigens that are able to induce immediate hypersensitivity reactions [46]. They are in general small, highly stable, soluble and glycosylated proteins, with enzymatic activity. Everyone is exposed to allergens, and antigen-specific IgG and IgM antibodies are produced. The majority of individuals do not synthesize IgE antibodies in response to allergens [46]. Atopic individuals on the other hand, have a genetic predisposition, personal and/or familial, to become sensitized and produce allergen-

specific IgE antibodies. Thus, the clinical definition of atopy is confirmation of sensitization with positive skin prick test (SPT) or presence of allergen-specific IgE antibodies in the circulation, and a Th2 skewing of the immune system with increased production of type 2 associated mediators, such as IL-4, -5, -9 and -13 [46].

Pregnancy immunology

The maternal environment during pregnancy may programme the immune development of the child in several ways [6, 170, 174, 175]. Also, women with asthma have been shown to have increased risks for preeclampsia, emergency cesarean section, and having a child small for gestational age [176]. During pregnancy, the maternal immune system is challenged by the semi allogeneic fetus, which must be tolerated without compromising fetal or maternal health. Systemic changes are induced by contact of maternal blood with placental factors and include enhanced innate immunity with increased activation of granulocytes and nonclassical monocytes [177]. Although a bias toward Th2 and Treg immunity has been associated with healthy pregnancy [177], the relationship between different circulating Th cell subsets is not straightforward. Instead, these adaptations appear most evidently at the fetal–maternal interface, where for instance Tregs are enriched and promote fetal tolerance [178, 179]. Also innate immune cells, that is, natural killer cells and macrophages, are enriched, constituting the majority of decidual leukocytes [177]. These cells not only contribute to immune regulation but also aid in establishing the placenta by promoting trophoblast recruitment and angiogenesis. The most evident changes seen in the systemic circulation are a shift from a Th1 toward a Th2 immune response and increased activation of innate immune cells [177]. Only a few studies have been performed on Th17 cells in pregnancy and these results are inconclusive. Thus, some studies have shown that circulating Th17 cell numbers are not different in pregnant compared to non-pregnant women [180], while others have reported them to be increased during pregnancy [181]. Increased numbers of nonclassical monocytes (CD14⁺CD16⁺ or CD14⁺⁺CD16⁺) and decreased numbers of classical monocytes (CD14⁺⁺CD16⁻) have been found in the third trimester of human pregnancy peripherally [182]. The number of DCs have been found to be decreased during pregnancy [183, 184]. Further, the number of circulating NK cells is decreased during pregnancy. Also, their production of IFN γ is decreased in pregnant as compared with non-pregnant women. Similar to the shift toward Th2 cells

there was also a shift toward NK2 cells (NK cells producing Type 2 cytokines) during pregnancy [185-187]. The decrease of certain populations in blood, for example, Treg cells, NK cells, and classical monocytes, may in part be explained by their recruitment to the fetal-maternal interface where these populations are enriched. Consequently, the enhanced activation of innate immunity in the circulation (*e.g.* increase in nonclassical monocytes and granulocytes) might be a compensation mechanism necessary for maintaining a protective immunity against infections, and thus maternal health. Both systemic and local changes might be due to factors produced by the placenta and/or by direct contact of immune cells with placental trophoblast cells.

In summary, there is still much to be settled in how immunity is regulated in normal pregnancy, increasing evidence suggest that failure of the immune system to adapt adequately may contribute to pregnancy complications and predispose to sickness in infants.

Development of the immune system during childhood

Immunological interactions between mother and child during pregnancy

Although, the sterile womb theory has a long time been a dogma, this has been challenged since studies suggest that infants incorporate an initial microbiome before birth and via birth and breast-feeding receive additional supplementation of maternal microbes (reviewed in [125, 170, 175]). The placental barrier was thought to keep the infants sterile throughout pregnancy, however microbes have for example been found in umbilical cord blood [188], amniotic fluid [189], and fetal membranes [189-192] without any detection of inflammation or pregnancy complications. Furthermore, an infant's first postpartum bowel movement of ingested amniotic fluid (meconium) is not sterile as previously assumed, but instead harbors a complex community of microbes, albeit less diverse than that of adults [193].

The fact that bacteria present in amniotic fluid, placenta *etc.*, was long associated with diseases may be due to that detection of microbial colonisation in the uterus was only performed when an intrauterine infection was evident or suspected. Although, bacterial infection is one important causes to preterm birth. The mechanism by which the gut bacteria gain access to the uterine environment is not well known. One possibility is that bacteria

travel to the placenta via the bloodstream after translocation over the gut epithelium. Intestinal dendritic cells may via antigen sampling transport the bacteria from the intestinal lumen throughout the body as they migrate to lymphoid organs [125, 170]. Furthermore, bacterial species normally found in the human oral cavity have also been isolated from amniotic fluid and likely enter the bloodstream during periodontal infections [194]. Interestingly, pre- and postnatal probiotic supplementation may enhance dental health, as evident in our trial with *Lactobacillus reuteri* where probiotic treated infants had significantly less caries than placebo treated infants at 9 years of age [195]. In addition to bacteria in the womb, human infants are smeared with a much larger inoculum of maternal vaginal and fecal microbes as they exit the birth canal. Many body habitats in the neonate is influenced by the mode of delivery and microbe exposure (skin, oral, nasopharyngeal, and gut) [125]. In addition, children born via C-section are significantly more likely to develop allergic rhinitis [196], asthma [196] celiac disease [197], type 1 diabetes [198], and inflammatory bowel disease [199]. Possibly, the maternally transferred vaginal and fecal microbes could induce immune maturation in the neonate. One mechanism of action could be that PRR and TLR-signaling in the intestinal mucosa is essential for the development of immunity and disruption in colonization may lead to immune mediated disorders [200].

Breastfeeding

Breastfeeding provides a secondary route of maternal microbial transmission as shown in humans. Breast milk does share many taxa with the microbiota found on sebaceous skin tissue around the nipple [125]. It has also been suggested that there may exist an entero-mammary pathway, where phagocytic dendritic cells traffic gut microbes to the mammary glands, similar to the microbial transfer to amniotic fluid as discussed earlier [125]. Supplementation with *Lactobacillus reuteri* during pregnancy have been associated with low levels of TGF- β 2 and slightly increased levels of IL-10 in colostrum. For TGF- β 2, this association was most pronounced in mothers with detectable *L. reuteri* in faeces. Infants receiving breast milk with low levels of TGF- β 2 were less likely to become sensitized during their first 2 years of life [91].

Prenatal development of the immune system

Much evidence was first gathered from murine models in prenatal development of the immune system [201]. The neonatal immune system seems immature since many cell types have poor responses in the neonates, especially to pathogenic microorganisms, as evident in both human and murine studies [202-204]. Also, there seem to be differences between infants later developing allergies and non-allergic infants [205, 206]. Initially, it was demonstrated that murine neonates were indeed competent to develop immune responses, but these responses were skewed to the Th2 lineage [202]. Th2 skewing is characterized by poor Th1 responses [207], but robust production of the Th2 cytokines IL-4, IL-5, and IL-13. It was subsequently found that human cord blood Th responses are also often Th2 biased [208]. Moreover, Treg cell function is also impaired in the neonatal period compared with that seen in adults [43, 209].

Innate immune development in infants

There is increasing evidence that differences exist between neonates and adults in multiple immune cell types [151, 204, 210]. Neonates are considered to be immature but under some circumstances able to mount fully mature immune responses. In newborns, the cells of the innate immune system – in particular, DC populations – are low in absolute and relative numbers and are not capable of fully activating both the innate and adaptive immune responses for effective antigen- specific B- and T-cell responses [211]. In cord blood, proliferation and cytokine responses to innate stimuli were less mature at birth than in adulthood, as evident when investigating innate immune responses to common TLR ligands [43]. However, TLR expression including downstream signalling molecules appears to be stable throughout ages [212]. Infants with bacterial sepsis are able to appropriately upregulate TLR expression on peripheral blood mononuclear cells. Thus, differential TLR expression appears unlikely to be a main cause for altered susceptibility to infection, inflammation, or vaccine response in early versus adult life (as reviewed in [212]). At mucosal sites, the innate lymphoid cells are of importance in initiating the type 2 immunity by responding to biomolecules released from, among others, epithelial cells. There are differences between infants and adults in ILC2 population in peripheral blood as well, possibly

indicating that these cells are important in early life when other parts of innate immunity is less developed [210].

Immune development in relation to allergy

Allergic infants have been shown to have exaggerated responses to TLR stimuli [205, 206]. In addition, it has also been shown that infants that later develop allergic diseases have an increased Th2 deviation already before clinical symptoms of disease [90, 213, 214]. Infants that later developed allergic diseases had increased systemic Th2 associated chemokine before/at birth, *i.e.* in cord blood [215]. Treg (CD4⁺CD25⁺CD127^{lo/}) function, which have been implicated in the pathogenesis of allergic diseases are different in egg-allergic and non-allergic infants already at birth [216]. DNA arrays convey several differences between allergic and non-allergic infants, mainly cytokine-genes involved in the type 2 immunity as expected but also differences in the T cell lineage pathways [217].

Environmental differences in immune maturation

Differences in immunity and responses to several types of stimuli are depending on age as previously discussed, also differences between healthy and diseased immune maturation have been presented. Upon that, there seem to be variations in immune maturation geographically. That of course include environmental differences [214], which may have an impact on immunity and other factors associated with immune maturation (bacterial colonisation for example). Furthermore, children born in more rural areas of the world seem to respond to stimulation *in vitro* differently than infants born in more Westernized areas. Children born on Papa Nya Guinea compared to Australian infants have cord blood that are functionally more quiescent than Australian born neonates [218-220]. Evidence of differential responses to BCG-vaccination have also been presented, possibly explaining the different effect of this type of vaccination between populations [221].

In conclusion, pre-symptomatic differences in immunity exist in allergic infants and it is not known what these differences are due to and further studies about how modulation by e.g. probiotics can influence immune maturation are needed.

Postnatal development of the immune system

Basic principles of epigenetics

The function and maturation of neonatal cells may be regulated by epigenetic mechanisms, for example the methylation of specific residues in the genome. Epigenetic modifications modulate and modify the accessibility of the gene transcription machinery [222]. One mechanism is by CpG methylation, directly occurring on the DNA sequence. Other mechanisms operate on the chromatin structure, such as biochemically modified histone protein tails that then subsequently allow or deny access of transcription factors to gene promoter regions. The epigenetic patterns vary between tissues and cell types, but also between individuals and over time, representing immune maturation, ageing and disease states. The epigenetic methylation pattern may also be modulated by the environment, especially by nutrition, tobacco smoke, stress and obesity [223]. There are many examples that epigenetic modifications are not permanent but changes over time. Some are implemented during only a short time to open or close chromatin state and access transcription of certain genes. In addition, the epigenetic state is reversible and with the appropriate enzymatic machinery the whole epigenome can be modified, (reviewed in [223]). DNA methylation involves the addition of a methyl group to the DNA nucleotides cytosine or adenine, mainly associated with gene silencing. DNA methylation occurs on the different CpGs clustered as islands on the majority of the genes. There are several million potentially methylated CpG islands throughout the genome of a single cell. They are clustered through the gene body and play a critical role within the promoter regions. Once these islands are methylated, gene transcription might not occur. Once removed the promoter allow interaction with various transcription factors and allow gene activation. In T cells epigenetic methylation are important for the lineage determination [160-163].

Epigenetic regulation during immune development

DNA methylation patterns in neonates appear to favor the Th2 skewing of immune responses [224, 225]. Martino *et al.* performed global DNA methylation assays and gene expression in CD4 cells collected at birth and at 12 months of age. They observed synchronized changes in gene expression and DNA methylation of immune genes involved in antigen processing and presentation, immune response, leukocyte activation, protein kinase signaling, TGFβ

signaling, and MAPK signaling [226]. In human neonatal as compared with adult CD4⁺ cells, the IFNG promoter shows greater CpG methylation levels, reflecting the maturation compared to adult cells [224, 225]. Further comparing total cord and adult mononuclear cells confirmed increased methylation at six CpG sites in the IFNG promoter in cord blood cells. Interestingly, increased methylation was also found at several CpGs in the promoters of other Th1-associated immune genes, including *IL2*, *IL3*, *IL8*, *KIR2DL4*, *TLR4* and *TNFA*. Therefore, in contrast to the situation at the Th2 locus, methylation is high in Th1-associated genes at birth and would be expected to show a decrease with age post birth.

Differential epigenetic modifications may play a role in the development of childhood-onset diseases and also later disease development [227]. One well-studied early-onset disease is allergic asthma. Environmental influences that have been epidemiologically linked with asthma include microbial exposure [36, 228].

The importance of prenatal exposure on the epigenetic methylation pattern, by e.g. probiotics, is still not known and warrants further studies.

Role of gut microbiota in immune maturation

As previously mentioned, antigen encounters and immune maturation at the mucosal sites are of essential importance for immune maturation and TLR-ligand exposure. Aberrations in the gut microbiome and intestinal homeostasis have the capacity for multisystem effects and bacterial diversity seems to be more important for appropriate immune maturation than specific bacteria taxa [229]. Prospective studies indicate that the gut microbiota composition during the first months of life influences allergy development [50-61, 230].

Colonization of the infant gut microbiota starts at delivery and the gut microbiota gradually develops up to two to three years of age when it resembles the adult one. As previously mentioned, the gut microbiota composition is influenced by heredity, but also by environmental exposures such as delivery mode, diet and antibiotics [65].

Also, here seem to be a window of opportunity, as the gut microbiota composition during the first months of life and not later in life that influences allergy development [55, 56, 59], further emphasizing that factors influencing the early maturation of the immune system might be especially important for subsequent allergic disease. It seems like the colonization of the gut

microbiota runs in parallel with the maturation of the immune system as should be regarded as an important ecosystem.

In conclusion, the role of the gut microbiota in immune development in sickness and in health and how probiotics may possibly influence the composition is not fully understood and more research in this area is needed.

Material and Methods

This thesis is based on two clinical trials with the aim to prevent allergic diseases in infants by supplementing mothers and infants with probiotics, *L. reuteri* and in the second trial *L. reuteri* and ω -3. The supplementation was given from gestational week 36 in the first trial and from gestational week 20 in the second trial.

Description of the clinical trials

Figure 5. Description of the *Lactobacillus reuteri* study

The first study was a prospective, double-blind, placebo-controlled, multicenter trial conducted at the Department of Pediatrics in the county hospitals of Jönköping, Motala, and Norrköping and the University Hospital in Linköping in southeastern Sweden [231], Figure 5. Between January 2001 and April 2003, 232 families with allergic disease (1 or more family members with eczema, asthma, gastrointestinal allergy, allergic urticaria, or allergic rhinoconjunctivitis) were recruited at antenatal clinics. The mothers started taking *L. reuteri* DSM 17938 or placebo 4 weeks before term and continued daily until delivery. After birth, the baby continued with the same study product as the mother daily up to 12 months of age. The lactobacillus preparation consisted of freeze-dried *L. reuteri* suspended in coconut oil and peanut oil containing cryoprotective components. The daily intake, of five droplets,

corresponded to 1×10^8 colony forming units (CFUs). The manufacturer of the study products guarantees, through extensive stability testing, the viability of the active study product for specified shelf-life, provided storage of the product was maintained at 2-8°C in the primary container. The study was approved by the Regional Ethics Committee for Human Research at Linköping University Dnr: 99323 (1999-11-09), Dnr: M171-07 (2007-12-06).

The primary aim was to prevent eczema-development in infants. Although no preventive effect on infant eczema occurred in this study, less IgE-associated eczema was observed at 2 years of age in infants receiving *L. reuteri* as compared with placebo. The cumulative incidence of any positive SPT was also lower, and fewer infants were sensitized at 2 years of age in the *L. reuteri* as compared with the placebo group. The effect was more pronounced when only infants whose mothers that had allergic disease were included, which may implicate the importance 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 [231].

A follow up at school age (7 years) concluded that the effect of *L. reuteri* on sensitization and IgE-associated eczema in infancy did not lead to a lower prevalence of respiratory allergic disease [89].

For this thesis blood samples were used, see sample collection overview, Table II.

Table II. Overview of sample collection in *L.reuteri*-study.

<i>L. reuteri</i> prevention study	Daily <i>L. reuteri</i> /placebo administration											
	35 w	Birth	1 mo	2 mo	3 mo	4 & 5 mo	6 mo	8 & 10 mo	1 y	1.5 y	2 y	7 y
Clinical exam, saliva					x		x		x		x	x
Questionnaire	x	x	x	x	x	x	x	x	x	x	x	x
Breast milk		x	x									
Blood sample		x					x		x		x	x
SPT							x		x		x	x
Faeces sample, weight, length	x (m)	x (5d)	x	x	x		x		x	x	x	x
Lung function												x

In order to investigate if earlier supplementation during pregnancy have a stronger preventive effect on allergic disease development and also to investigate if a synergistic effect by

supplementing with ω -3 fatty acids could be achieved, a second trial is ongoing, referred to as PROOM-3 (**P**robiotic and **O**mega-3).

Figure 6. Overview of the subpopulation of the PROOM-3 study included in this thesis.

The PROOM3-study

The other main study is a new prospective, double-blind, placebo-controlled, multicenter trial conducted at the Department of Pediatrics in the county hospitals of Motala, and the University Hospital in Linköping. Families with at least one parent or sibling with clinical symptoms or history of allergic disease were invited to participate in the double blind randomized placebo controlled PROOM-3 allergy prevention study (the same inclusion criteria as in the *Lactobacillus reuteri* study). The pregnant women were included in the study at gestational week 20. The women were randomized to four study groups, one receiving both *L. reuteri* oil drops and ω -3 PUFA capsules, the second receiving ω -3 PUFA supplementation and placebo regarding *L. reuteri*, the third receiving *L. reuteri* and placebo regarding ω -3 PUFA and the fourth group receiving placebo capsules and placebo oil drops, Figure 6. The *L. reuteri* or placebo oil drops were given to the mothers during pregnancy and

to the children during the first year of life, while the ω -3 or placebo capsules were given to the mothers during pregnancy and lactation. The ω -3 PUFA treatment comprised of maternal supplementation of three capsules of Pikasol[®] (1g capsules containing 630 mg ω -3 PUFA) twice daily during pregnancy and lactation, while the *L. reuteri* supplementation comprised of 10^9 colony forming units (CFU) *L. reuteri* DSM 17938 (corresponding to 20 drops twice daily) suspended in oil to the mothers during pregnancy and 10^8 CFU (five drops once daily) to the children during the first years of life. Blood samples were collected from the pregnant women at gestational week 20, 32 and postpartum, overview of sample collection Table III. The study was approved by the Regional Ethics Committee for Human Research at Linköping University (Dnr 2011/45-31). Registered at ClinicalTrials.gov Identifier: NCT01542970.

Table III. Overview of sample collection in PROOM3-study.

	20th week	Partus	1 m	2 m	3 m	4 m	6 m	12 m	24 m
<i>Clinical examination</i>	nurse	nurse	nur	nur	nur	nur	nurse	nurse	Pediatrician
<i>Clin History Family</i>	x								
<i>Questionnaire</i>									
Allergy	x				x		x	x	x
Dietary/	x						x		
Bowel function*	x								
<i>SPT</i>							x	x	x
<i>Milk samples</i>		1-4 d	x	x	x	x			
<i>Blod samples</i>									
Maternal	x	x							
Children		CB					x	x	x
<i>Saliva</i>							x	x	x
<i>Faeces</i>									
Maternal	x	x (1-7 d)							
Children		x (5-7 d)	x		x		x	x	x
<i>Supplementation w - 3</i>									
<i>Supplementation L reuteri</i>									
Maternal									
Children									

Clinical definitions of allergic disease (Paper I-IV)

Eczema was defined as a pruritic, chronic, or chronically relapsing non-infectious dermatitis with typical features and distribution. Eczema was classified as IgE-associated if the infant was also sensitized, *i.e.* had at least one positive SPT and/or detectable circulating IgE antibodies to allergens. At two years of age, wheeze was defined as an episode with obstructive airway

symptoms, and recurrent wheeze was defined as three or more wheezing episodes, at least once verified by a physician.

An asthma diagnosis at seven years of age required at least one of following two criteria: 1. doctor diagnosis and asthma symptoms and/or medication during the last 12 months and 2. wheeze or nocturnal cough and a positive reversibility test and/or pathological FENO value. In Sweden, most children with asthma are asymptomatic when visiting the doctor, because they are efficiently treated with inhaled corticosteroids. If the asthma diagnosis was based on doctors' diagnosis, medical records of the child were always reviewed to confirm that the diagnosis was consistent with the GINA criteria (<http://www.ginasthma.com>).

The diagnosis of ARC was based on a standard ISAAC questionnaire and required watery discharge at least twice in contact with the same allergen and no signs of infection.

Urticaria was defined as allergic if it appeared within one hour after allergen exposure, at least at two separate occasions.

A diagnosis of gastrointestinal allergy required vomiting, diarrhea, 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. Oral allergy syndrome was defined as allergic if it appeared at least at two separate occasions after ingestion of certain offending food.

Infants were regarded as sensitized if they had at least one positive SPT and/or detectable circulating allergen specific-IgE antibodies.

Allergy was defined as having at least one positive SPT and/or detectable circulating IgE, in addition to clinical allergic symptoms

Design and study population (I and II)

Sixty-one children, of whom 29 received probiotics and 32 placebo (Table IV), were selected based on availability of blood cell samples at multiple time points [7]. The infants included in this study had cells collected from at least three time points, *i.e.* birth, 6, 12 or 24 months. They were representative of the main trial with regards to the variables presented in Table IV.

Table IV. Descriptive data of children included in the study, project I and II.

	<i>Lactobacillus reuteri</i> % (n/N)	Placebo % (n/N)	p-value*
Boys	55 (16/29)	50 (16/32)	0.82
First born	55 (16/29)	63 (20/32)	0.77
Caesarean delivery	14 (4/29)	3 (1/32)	0.16
Birth weight (mean±SE)	3656±434	3469±429	0.10*
Birth length	51.3±2.0	50.6±1.9	0.18*
Parental smoking	10 (3/29)	9 (3/32)	0.91
Furred pets	7 (2/29)	13 (4/32)	0.51
Maternal atopy 79 (23/29)	75 (24/32)	0.89	
Paternal atopy 62 (18/29)	69 (22/32)	0.80	
Breastfeeding			
3 months, exclusive	59 (17/29)	78 (23/32)	0.62
6 months, partial	83 (24/29)	78 (24/32)	0.80
Antibiotics			
0-12 months 34 (10/29)	16 (5/32)	0.18	
12-24 months	48 (14/29)	53 (17/32)	0.83
Day-care			
0-12 months 0 (0/29)	3 (1/32)	0.35	
12-24 months 86 (25/29)	94 (30/32)	0.82	
Recurrent wheeze	3 (1/29)	6 (2/32)	0.63
Eczema	28 (8/29)	28 (9/32)	0.97
Allergic disease 34 (10/29)	38 (12/32)	0.87	
Sensitisation	38 (11/29)	34 (11/32)	0.84
IgE-assoc. Disease	10 (3/29)	16 (5/32)	0.59
SPT	17 (5/29)	16 (5/32)	0.89
Egg	10 (3/29)	13 (4/32)	0.81
Milk	3 (1/29)	0 (0/32)	0.30
Cat	3 (1/29)	6 (2/32)	0.63
Birch	3 (1/29)	3(1/32)	0.95
Spec IgE	34 (10/29)	31 (10/32)	0.85
Ovalbumin	7 (2/29)	22 (7/32)	0.15
b-lactoglobulin	24 (7/29)	9 (3/32)	0.19
fx5	31 (9/29)	28 (9/32)	0.85

• χ^2 test. *t-test

The eight children defined as allergic all had IgE-associated eczema. One child also had recurrent wheeze, one child had allergic urticaria and one had gastrointestinal allergy while none of the children had ARC. Non-sensitized children with symptoms (n=8) and sensitized children without symptoms (n=14) were not included in the analysis comparing allergic and non-allergic children. The difference in the prevalence of IgE-associated eczema at 2 years was significant in the main trial, 8% in the *L. reuteri* group and 20% in the placebo group [7].

Description of participants in the EILA and Illumina study

From a total of 78 individuals, (see Table V), samples from cord blood, 12 months and 24 months of age were selected based on availability of blood cell samples from the *L. reuteri* study.

Table V. Descriptive data of children from the allergy prevention trial included in the EILA and Illumina analyses.

	EILA:		Cord Blood		12 months		24 months		Illumina:		Cord Blood		12 months		24 months	
			% (n/N)		% (n/N)		% (n/N)				% (n/N)		% (n/N)		% (n/N)	
<i>Lactobacillus reuteri</i> (Yes/No)	44 (14/32)		64 (14/22)		57 (20/35)		40 (14/35)		69 (9/17)		44 (4/9)		42 (8/19)		26 (5/19)	
Boys			31 (10/32)		36 (8/22)		40 (14/35)				41 (7/17)		22 (2/9)		47 (9/19)	
First born			53 (17/32)		50 (11/22)		49 (17/35)				47 (8/17)		33 (3/9)		5 (1/19)	
Parental smoking			6 (2/32)		9 (2/22)		9 (3/35)				0 (0/17)		0 (0/9)		21 (4/19)	
Furred pets			6 (2/32)		23 (5/22)		14 (5/35)				0 (0/17)		33 (3/9)			
Maternal atopy	72 (23/32)		59 (13/22)		66 (23/35)				71 (12/17)		66 (6/9)		58 (11/19)			
Breastfeeding																
3 months, exclusive			61 (14/23)		50 (11/22)		60 (21/35)				82 (14/17)		33 (3/9)		21 (4/19)*	
6 months, partial			87 (20/23)		77 (17/22)		91 (32/35)				88 (15/17)		77 (7/9)		95 (18/19)	
Antibiotics																
0-12 months	91 (21/23)		72 (16/22)		80 (28/35)				52 (9/17)		66 (6/9)		74 (14/19)*			
12-24 months			48 (11/23)		77 (17/22)		66 (23/35)				23 (4/10)		33 (3/9)		68 (13/19)	
Day-care																
0-12 months	0 (0/23)		0 (0/22)		3 (1/35)				0 (0/10)		0 (0/9)		0 (0/19)			
12-24 months	91 (21/23)		86 (19/22)		86 (30/35)				100 (10/10)		77 (7/9)		79 (15/19)			

EILA: 9 infants lost to follow up from cord blood at 2 years but included to investigate maternal probiotic/placebo administration at birth. 1 infant was lost to follow up from 12 months to 2 years. All 24m infants followed up to 24m. 17 CB samples lost to follow up at 7 years. 8 infants from 12m samples lost to follow up at 7 years. 12 infants at 24m samples lost to follow up at 7 years.

Illumina: 7 infants lost to follow up in cord blood but included to investigate maternal probiotic/placebo administration at birth.

* No differences existed between the main trial and this sub-analysis except from data marked with *, chi-square test.

Mothers included in the PROOM3-study

The participants in this substudy of immune cell populations were recruited in Linköping and Motala and are described in Table VI.

Table VI. Characteristics of the study participants in the PROOM-3-study

	Ω3 + <i>L. reuteri</i> n=22	Ω3 + Placebo n=21	Placebo + Placebo n=22	Placebo + <i>L. reuteri</i> n=23
<i>Partus at week (mean)</i>	40	39	39	39
<i>Mothers age at inclusion (years/mean)</i>	29	30	29	29
<i>No of weeks with study product (mean)</i>	19.6	18.6	18.5	18.8
<i>Birth weight (kg/mean)</i>	3.40	3.30	3.37	3.25
<i>Birth length (cm/mean)</i>	49.5	49.5	49.0	49.5
<i>Caesarean (n/%)</i>	1 (4.3%)	3 (13.6%)	3 (13.6%)	2 (8.3%)
<i>First born (n/%)</i>	12 (40%)	17 (63%)	13 (46%)	11 (42%)
<i>Animals in household (n/%)</i>	3 (14%)	7 (37%)	3 (16%)	8 (40%)
<i>Smoking parent (n)</i>	0	0	0	0
<i>Allergic disease (n/%)</i>	16 (31%)	12 (23%)	13 (25%)	11 (21%)

22 non-pregnant age-matched women, not taking hormonal contraceptives were included as control subjects, for clinical characteristics see Table VII. The study was approved by the Regional Ethics Committee for Human Research in Linköping (Dnr 2011/45-31) and registered at ClinicalTrials.gov (Identifier: NCT01542970).

Table VII. Clinical descriptive of participating non-pregnant women

<i>Age at inclusion (mean)</i>	29.1
<i>Use of hormonal contraceptives (yes /no)</i>	0/22
<i>Menstrual cycle (luteal/follicular)^a</i>	9/11
<i>Smoking (yes/no)</i>	0/22
<i>Previous births (yes/no)</i>	6/16
<i>Any allergic disease, ARC (yes/no)</i>	3/19

Experimental design

In paper I and II we aimed to investigate the effect of probiotic supplementation on the immune cells in an *in vitro* system, therefore cells were cultured with allergens and TLR-ligands and cytokine, chemokine and RNA expression of transcription factors and TLRs were studied.

Collection of samples and preparations (Paper I-III)

Blood was collected at birth (cord blood) and venous blood samples were drawn at 6, 12 and 24 months. Cord and peripheral blood mononuclear cells (PBMC) were collected by Ficoll gradient centrifugation.

In vitro cultures of PBMCs (Paper I-II)

Figure 7. Experimental overview of paper I-II

An *in vitro* system to investigate cell function was used to explore the effect of probiotic supplementation on innate and adaptive immunity from PBMCs collected from study participants at birth (cord blood), 6-, 12- and 24 months, Figure 7. PBMCs were washed and stimulated.

To investigate adaptive immunity, cells were incubated with medium (control culture), birch (10 kSU/ml), ovalbumin (100 µg/ml), PHA (2 µg/ml), and cat (10 kSU/ml) (Allergologisk

Laboratorium København (ALK), Hørsholm, Denmark) in AIM-V and mercaptoethanol at a concentration of 1×10^6 /ml for 6 days at 37°C, except the PHA control which was incubated for 24 hours. The 6 day incubation was previously optimized due to the time it takes for APCs to process and present antigens as well as release chemokines and cytokines.

The innate stimulation were 1 ng/ml lipoteichoic acid (LTA) from *S. aureus* (InvivoGen, San Diego, USA), 1 ng/mL lipopolysaccharide (LPS) from *E. coli* K12 (Invivogen) or 1 µg/ml CpG ODN M362 (InvivoGen) in AIM V (Life Technologies AB, Täby, Sweden) with 20 µM mercaptoethanol (Sigma-Aldrich) also at a concentration of 1×10^6 /ml but incubated for 24 hours also at 37°C. Cell cultures were then centrifuged, supernatants were collected and cell pellets were lysed in RLT-buffer (Qiagen GmbH, Hilden, Germany) and stored at -70°C for later mRNA analysis. The mean viability of the freeze/thawed PBMCs was 85.2 %.

RNA extraction and Quantitative Real Time PCR (Paper I-II)

Total RNA was extracted from the control/spontaneous RLT-lysates and all RNA concentrations were measured with nanodrop-technology and 260/280 values >1.7. Reverse transcription was carried out converting approximately 120 ng of RNA to cDNA in 30 µL reactions. For real-time PCR, 1 µL of cDNA was mixed with 19 µL TaqMan Fast Universal Mastermix together with primers and probe for GATA-3 mRNA, Foxp3 mRNA, EBV-induced gene 3 (EBI3) mRNA (HS01057148_m1) or T-bet mRNA (HS00202426_m1) (Paper I). For paper II, the primers and probe used were TLR2 mRNA (HS00152932_m1), TLR4 mRNA (HS01060206_m1), TLR9 mRNA (HS00370913_s1) (Applied Biosystems). mRNA abundance was normalized to the relative levels of the internal stably expressed control gene 18S rRNA in each sample and was expressed as ratios. Absolute quantification was performed using a 5-point standard curve with fourfold dilutions of the standard, included in each run. The detection limit was <35 CT. Only one sample was below the cut off level and was given half the value of the lowest standard point.

Luminex optimization assay

Luminex is a multiplex technology which allows for simultaneous analysis of up to 100 analytes. A panel of cytokines and chemokines were set up to analyze the secretion from PBMCs stimulated with allergens (Paper I). The cytokines IL-5, -10, -13 and IFN-γ and the chemokines CXCL10 and CCL17 were analyzed with an in-house multiplexed Luminex assay.

Coupling of antibodies to microspheres

Carboxylated microspheres were bought from Luminex Corporation (Austin, TX, USA) and the coupling reaction was performed according to the manufacturer's protocol with some modifications.

Luminex assay

An eight point standard curve was created with three-fold dilutions in AIM-V, using recombinant human IL-5 (1111-4.6 pg/ml), IL-10 (833.3-0.38 pg/ml), IL-13 (3333-4.57 pg/ml) and IFN- γ (1111-13.7 pg/ml), CXCL10 (1333-5.5 pg/ml), and CCL17 (466-0.63 pg/ml) (R&D Systems, Abingdon, UK). Fifty μ l of sample and standard were added. Culture medium (AIM-V) were used as blank. Then, 50 μ l of sonicated and vortexed bead mixture (2000 beads of each subset, bead sets 17, 27, 37, 57, 77) were added to the wells. The plate was incubated in darkness for one hour in room temperature (RT) and then overnight in 4°C, to increase overall sensitivity.

The plate was washed and incubated. To each well, 50 μ l assay buffer was added before the addition of polyclonal biotinylated detection antibodies, IL-5 (500 μ g/ml, clone JES1-5A10), IFN- γ (500 μ g/ml, clone 4S.B3), CCL17 (50 μ g/ml, clone BAF364) and CXCL10 (500 μ g/ml, clone 6D4/D6/G2, BD Pharmingen) IL-10 (70 μ g/ml, clone M191004), IL-13 (50 μ g/ml, clone M191304, Sanquin, Amsterdam) and incubated for 1 hour, thereafter washed twice as previously. Then, 50 μ l assay buffer was added before 50 μ l Streptavidin-Phycoerythrin, (1 μ g/ml, Molecular Probes /VWR, Invitrogen) and incubated 30 minutes in RT on a plate shaker. The SA-PE solution was aspirated by vacuum manifold and washed. Assay buffer (75 μ l) was added to each well before analysis using a Luminex¹⁰⁰ instrument (Biosource). The data were acquired using the StarStation 3.0 software (Applied cytometry systems, Sheffield, UK).

Measurement of cytokine and chemokine secretion by Luminex (paper II)

The levels of pro-inflammatory cytokines and chemokines (IL-1 β , IL-6, IL12p70, IFN- α , TNF, CCL4 and CXCL8) and the anti-inflammatory cytokine IL-10 in the cell supernatants were analysed with multiplex assay kits, according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA). The samples were analysed on a Luminex¹⁰⁰ instrument

(Biosource, Nivelles, Belgium) and the data was analysed with the software StarStation 2.0 (Applied Cytometry Systems, Sheffield, UK). The lower detection limit was 2,34 pg/mL for IL-1 β , 33 pg/mL for IL-6, 1 pg/mL for IL-10, 0.5 pg/mL for IL12p70, 10 pg/mL for IFN- γ , 20 pg/mL for CCL4 and CXCL8 and 3 pg/mL for TNF. Undetectable samples were given the value of half the cut off level. Comparisons of the TLR ligand induced cytokine and chemokine responses were made after the control value, *i.e.* responses from cells cultured in medium alone, was withdrawn. Detectable levels of IL-6, IL-10, CCL4, CXCL8 and TNF could be measured after all stimulations. After LPS and LTA stimulation, IL-1 β levels could also be detected. Detectable IFN- γ levels were observed only after stimulation with CpG.

Enzyme linked immunosorbent assay (ELISA) (paper I)

CCL22 and CCL18 was not in the concentration-range of the multiplex assay, therefore, two separate sandwich-ELISAs were developed and optimized for quantification. Monoclonal anti-human CCL18 and CCL22 (clone 64507 and clone 57226, R&D Systems) for coating (conc. 0,5 μ g/ml and 2 μ g/ml, respectively) and biotinylated anti-human CCL18 and CCL22 antibody (BAF394 and BAF336, R&D Systems) for detection (conc. 200 ng/ml and 50 ng/ml, respectively) were used. All samples were analyzed in duplicates and the sample was re-analyzed if the CV was >15%.

Epigenetic Immune lineage Analysis to investigate immune regulatory loci in T helper cells (paper III)

Figure 8. Overview of the EILA and Illumina experimental set up.

To further investigate how maternal supplementation, environment and immunity affect the infant we wished to investigate methylation status at specific gene-loci, essential for differentiation of the respective Th subset. This was a way to explore if supplementation and development of allergic diseases affected the epigenetic regulation and the Th1/Th2/Treg/Th17 diversion often associated with allergic diseases.

DNA demethylation in regulatory CpG islands increases the transcriptional activity of lineage-specific cytokines which can be investigated with Epigenetic Immune Lineage Analysis (EILA). In accordance with studies by Jansson *et al*[160], loci of interest were promoter regions upstream the IFN- γ (position -4229[161]), IL-13 (position -46[160]), IL-17 (position -122[160]) and FoxP3 (position -77[162]) genes. From peripheral blood mononuclear cells (PBMCs), CD4⁺ T cells were isolated, and genomic DNA was extracted from these cells. The DNA was then bisulfite converted, which results in unmethylated cytosine being converted into uracil. In the following PCR-reaction, the selected loci of the IFN- γ , IL-13, IL-17 and FoxP3 genes are

amplified, and the uracil residues will be translated into thymine residues. Then the methylation-sensitive single nucleotide primer extension (Ms-SNuPE/SNaPshot) follows, in which primers bind to the DNA strand adjacent to the cytosine base intended for methylation. The primer is extended with a single, fluorescently labeled dideoxy nucleoside triphosphate. In the later capillary electrophoresis, the primers are separated due to difference in length, and the presence of cytosine or guanine indicates the presence of methylation. (Figure 7). Experimental procedures are described in detail in paper III.

Illumina 450K for global methylation analysis in circulating peripheral CD4+ cells (paper III)

The identification of differentially methylated CpGs is central in most epigenetic studies and the most studied form of epigenetic modification and results from the addition of a methyl group to cytosine (5-mC) in the context of CpG dinucleotides. Illumina Infinium Human Methylation450 BeadChip (450K) is based in the Infinium Technology and contains more than 480,000 probes, targeting 99% of genes and 96% of CpG island regions. This type of array contains two different bead types associated to two different chemical assays. Infinium I and Infinium II. Infinium I consider two bead type methylated and unmethylated for the same CpG locus, sharing the same color channel. Whereas, Infinium II utilized a single bead type and two color channels[232]. In general the analysis is based on import of raw IDAT files, a quality control, probe filtering and within array normalization is done using R and a package called RnBeads. Thereafter, a calculation and identification of differentially methylated positions or regions are executed. These are then verified and validated of differences on methylation patterns. The biological function and interpretation of data is being done with the help of DAVID and PANTHER (bioinformatics analyses tools).

Flow cytometry phenotyping of peripheral immune cell populations during pregnancy (paper IV)

A flow cytometry-method was set up to investigate the peripheral cell populations during pregnancy and to relate these to supplementation with *Lactobacillus reuteri* and ω -3 (PROOM-3 study). Samples were analyzed on a BD FACS CANTO II which consist of three

lasers, blue (488 nm), red (633 nm) and violet (405 nm). The blue laser has four detectors and can analyze/excite PE, FITC, PerCPCy5.5/PerCP and PeCy7. The red laser use two detectors and can analyze APC/eFlour660 and APC-H7, the violet laser have two detectors but we only use one, v450/Pacific Blue.

The panels set up were three different 7-color panels and one 6-color True count panel. The panels were optimized using isotype-controls and titrating of antibodies. See table VIII for antibodies used.

Table VIII. Antibodies used for flow cytometry phenotyping.

Antigen	Flourochrome	Antibody	Clone	Supplier
CD3	APC-H7	Mouse, IgG1, κ	SK7	BD
CD4	PE-Cy7	Mouse, IgG1, κ	SK3	BD
CD8	FITC	Mouse, IgG1, κ	SK1	BD
CD14	FITC	Mouse, IgG1, κ	M0P9	BD
CD16	PerCP-Cy5.5	Mouse, IgG1, κ	3G8	BD
CD19	APC	Mouse, IgG1, κ	SJ25C1	BD
CD25	PerCpCy5.5	Mouse, IgG1, κ	M-A251	BD
CD45RA	HV450	Mouse, IgG2b, κ	HI100	BD
CD56	PerCP-Cy5.5	Mouse, IgG1, κ	B159	BD
FoxP3	FITC	Rat, IgG2a	PCH101	eBiosciences
GATA3	PE	Rat, IgG2b, κ	TWAI	eBiosciences
isotype	PE	Rat, IgG2b, κ	eB149/10H5	eBiosciences
isotype	eFlour660	Mouse, IgG1, κ	P3.6.2.8.1	eBiosciences
RORC	PE	Rat, IgG2a	AFKJS-9	eBiosciences
T-bet	eFlour660	Mouse, IgG1, κ	eBio4B10	eBiosciences

Gating strategies are described in Figure 8, within the lymphocyte population (based on forward (FSC) and side scatter (SSC) characteristics), the proportion and total number of

major populations were defined in TrueCount tubes as follows (clones within brackets); B cells, APC-conjugated anti-CD19 (clone SJ25C1); NK cells, PE-conjugated anti-CD56 (clone B159, NK cells were also CD3-); T cells, APCCy7-conjugated anti-CD3 (SK7); T cytotoxic cells, FITC-conjugated anti-CD8 (clone SK1); T helper cells, PECy7-conjugated anti-CD4 (SK3). CD4 and CD8 populations were defined by co-expression of CD3. All these antibodies were from Beckton Dickinson, Franklin Lakes, New Jersey, USA. Within the CD3⁺CD4⁺ population, the naive and memory cell population was determined using v450-conjugated anti-CD45RA (HI100) (Beckton Dickinson). Expression of the intracellularly expressed T helper cell lineage markers were stained using PE-conjugated anti-GATA3 (TWAJ), eFlour 660-conjugated anti-Tbet (eBio4B10), PE-conjugated anti-RORC (AFKJS-9), FITC-conjugated anti-Foxp3 (PCH101) (all from eBiosciences) was in the CD45RA⁺ naïve (undifferentiated) and CD45RA⁻ memory (differentiated) CD3⁺CD4⁺ T helper cell population. The percentage of Tbet, GATA-3 and RORC expressing populations was determined using the CD3⁺CD4⁺CD45RA⁺ population as a population without cells expressing the markers (naïve cell population) and comparing these with CD3⁺CD4⁺CD45RA⁻ memory population. T regulatory cells (Tregs) were defined as CD4^{dim}CD25^{hi}Foxp3⁺ and subtypes of Tregs were also subdivided into CD3⁺CD4⁺CD45RA^{+/-} Foxp3^{+/++}, *i.e.* resting and activated Tregs [165], respectively. Monocytes were determined based on expression of surface antigens detected using FITC conjugated anti-CD14 (MOP9, BD) and PerCpCy5.5 conjugated anti-CD16 (3G8, BD). Monocytes were gated first on FSC and SSC and CD14⁺ expression, and subtypes of monocytes were subdivided based on CD14⁺CD16^{+/-} expression, for gating strategy see paper IV.

Statistical methods

When the data were not normally distributed, non-parametric tests were used. Comparisons between unpaired groups were analyzed with Mann-Whitney U-test, and correlations were analyzed with Spearman's rank order correlation coefficient test. Friedman's test was used to determine if there were any longitudinal changes in different cell population frequencies and cell markers of different kind during pregnancy (Paper I-IV). Wilcoxon signed rank test was used to investigate if there were any differences between the different time points.

When data was normally distributed parametric test was employed, and comparison between groups were analyzed using ANOVA and student's t-test. Correlations were compared using Pearson.

Clinical data were compared using the student's t-test and χ^2 -test in order to investigate background factors between the groups.

In the logistic regression model probiotic supplementation and IgE-associated allergic disease were included, the association between cytokine and chemokine secretion and these variables was investigated.

P-values <0.05 were considered statistically significant. Calculations were performed with a SPSS statistical package version 19.0, 20.0 and 21.0 SPSS Inc.

Results and Discussion

Probiotics

Functional characterization (cytokine and chemokine production/capacity)
There is a need for further studies investigating the immunomodulatory effect of probiotic supplementation, since clinical and experimental results are inconsistent. We investigated the effect of pre- and postnatal supplementation with *L. reuteri* on adaptive immune responses to allergens, mitogens and bacterial products. In addition, we aimed to investigate if immunoregulatory markers could be related to treatment (paper I-II).

The influence of *Lactobacillus reuteri* on allergen responsiveness (paper I)

Figure 9. Cat allergen induced (a) IL-5, (b) IL-13, (c) IL-10, (d) IFN-γ responses from cord and peripheral blood mononuclear cells during the two first years in life in probiotic and placebo treated children. Groups were compared using Mann-Whitney U-test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. The number of analysed samples is indicated (n), as well as the median.

The lower responses in the probiotic group compared to the placebo group is most evident when looking at the perennial and ubiquitously present cat allergen than the seasonal birch and food allergen ovalbumin, Figure 9. This could possibly imply different regulation upon encounter with allergens with different routes and exposure [5]. The responses to allergens were significantly lower to cat allergen at several time points in the probiotic treated group. Cat induced Th1 associated IFN- γ responses were also lower after probiotic treatment than placebo at 24 months with similar findings for the anti-inflammatory IL-10 at birth and at 12 months.

When investigating secretion after birch and ovalbumin allergen stimulation lower responses in the probiotic compared to the placebo group were found. Th2-associated chemokine CCL22 levels were lower after birch stimulation with a similar trend after ovalbumin stimulation. Lower CCL22 levels were recorded at 12 and 24 months after PHA stimulation. There were no difference in cell viability between groups, the mean viability of the freeze/thawed cells was 85.2 %.

The lower responses observed in general in the probiotic group could imply an increased capacity to downregulate exaggerated immune responses, *i.e.* an increased capability of immune regulation, possibly also proposing a reduced atopic propensity consistent with our previous findings in this cohort [88].

Similarly, lower allergen induced cytokine secretion during infancy in a country with higher microbial burden and lower prevalence of allergic disease (Estonia)[214]. Whereas, allergen-induced IL-5, -10, -13 and IFN- γ responses were lower in Estonian than Swedish infants [214]. Also, different living conditions and a different microbiota is evident in Estonia [233]. A possible mechanism of probiotic supplementation is to provide microbial stimulation needed for normal development of immune regulatory capacity, thereby providing a source of TLR-ligand exposure and a modulation of the gut microbiota [67].

Animal models have showed that a possible regulatory mechanism may be induced after probiotic treatment, since *L. reuteri* supplementation inhibits allergic airway responses in an ovalbumin-sensitized asthma model in BALB/c mice [234]. Supplementation of other strains of lactobacilli in other murine studies resulted in lower levels of both Th1 and Th2 cytokines [235, 236].

Conclusion, probiotic supplementation leads to more moderate cytokine production after allergen stimulation, especially Th2 associated but also Th1 and Treg associated.

TLR ligand responsiveness and expression (paper II)

To investigate the innate responses in PBMCs after probiotic as compared to placebo supplementation cell cultures were stimulated with TLR ligands.

Figure 10. LTA induced responses. LTA induced IL-1β (a), IL-6 (b), CCL4 (c), CXCL8 (d), responses from peripheral blood mononuclear cells during the two first years in life in probiotic (filled squares) and placebo (open circles) treated children. Groups were compared using Mann-Whitney U-test, ** p<0.01, *p<0.05. The number of analyzed samples is indicated (n), as well as the median values.

The lower responses to TLR2 ligation is in line with our previous results showing lower allergen induced responses in the probiotic treated infants, and was not dependent on differences in the TLR2 mRNA expression. The levels of the chemokines CCL4 and CXCL8 were lower after stimulation with the TLR2 ligand LTA at 12 months in the probiotic compared to the placebo group, and for CCL4 also at 24 months. The same low levels were detected for the cytokines IL-1 β and IL-6 after LTA stimulation at 12 months and for IL-1 β at 24 months (Figure 10). The lower levels of cytokine and chemokine responses were statistical significant at several time-points and showed similar trends at all ages in the probiotic group compared to the placebo treated infants. Other measured cytokines showed no difference. Although the probiotic treated infants showed the most clear and consistent difference in TLR2 responsiveness, probiotic supplementation was also associated with low CXCL8 responses to the TLR4 ligand LPS at birth. No further differences were observed in responsiveness to TLR4 and TLR9 ligands (paper II).

This is also a possible evidence for an increased immune regulatory ability, as microbial stimulation is suggested for normal development of immune regulatory capacity [6, 200]. The probiotic supplementation could then provide a source of TLR ligand exposure [6, 200, 237]. Other possible explanations for the lower TLR2 responses could be an induction of regulatory macrophages responding to stimuli with lower secretion of pro-inflammatory cytokines and chemokines [238] as well as an induction of Tregs [234, 239], which could be strain dependent [240]. Although, the production of the anti-inflammatory IL-10 was not affected by the administration of probiotics.

These results are in line with studies suggesting that immune regulatory mechanisms are established at a later age in Sweden compared to Estonia [214, 241]. In concordance, neonatal antigen presenting cells are more quiescent in children born under traditional, i.e. Papua New Guinea, compared to modern environmental conditions, i.e. Australia [218-220]. This quiescent function could potentially be a protective mechanism learned in utero and associated with an enhanced immune regulatory capacity among infants living in conditions with a higher microbial burden.

Conclusion, probiotic supplementation results in lower LTA induced responses.

Expression of T helper lineage specific transcription factors and TLR2/4/9 (paper I-II)

In order to investigate if supplementation skewed the immune response to Th1/Th2 or Treg direction lineage specific transcription factor expression was determined. From the unstimulated cell cultures the cell pellet was collected after centrifugation and lysed to obtain mRNA. In order to investigate the supplementation and not the stimulation we used the unstimulated culture. To normalize the data an internal control gene was used, 18S. Other genes was also possible but 18S have shown to be stable after stimulation with PHA and other mitogens. A control gene not affected by stimulation is important for the RNA quantity control. Probes were already optimized [242], and we created a standard curve for relative quantification. PBMCs were cultured with PHA, RNA extracted and diluted to create a standard curve (different stimulations for different genes).

The mRNA expression of the transcription factors T-bet and GATA-3, promoting Th1 and Th2 differentiation respectively, was not influenced by probiotic supplementation.

Also, neither of the TLR2 nor the TLR9 expression was affected by probiotic supplementation. However, the mRNA expression of TLR4 was significantly lower in the probiotic group compared to the placebo group at 6 months of age.

Conclusion, probiotic supplementation had no effect on mRNA expression of lineage associated transcription factors or TLR2/4/9.

Allergic disease and probiotic supplementation showed independent associations with allergen and mitogen induced responses (paper I)

To explore if there were any relation between the allergic responses to allergen stimulation and the supplementation we created a logistic regression model. The logistic regression analysis indicated that the allergen and mitogen-induced responses seemed to be independently associated with probiotic treatment and allergy development, since separate effects of treatment and allergy were indicated on immune responses. The lack of association could be due to few allergic infants included in the study. However, allergic infants showed high Th2 responses after birch and ovalbumin stimulation where probiotic supplementation showed less clear effects.

Cytokine and chemokine responses to TLR ligands are associated with probiotic treatment independently of allergy development (paper I and II)

In a logistic regression model, adjusting for probiotic treatment had no major effect on the association between probiotic treatment and cytokine responses, *i.e.* similar results as the logistic regression model for allergen responses.

Epigenetic modifications in infancy and after probiotic supplementation (paper III)

In an attempt to understand how supplementation have an impact on immunity we aimed to explore how important the fetal exposure was, *i.e.* supplementation during pregnancy. Foetal exposures *in utero* may affect the epigenetic pattern and impact immune maturation *post partum*. DNA methylation patterns in promoters of allergy related T helper cell subsets, and at a genome wide scale, were investigated utilizing Epigenetic Immune Lineage Analysis (EILA) and Illumina 450K DNA methylation assay, respectively.

In the EILA study our results showed no differences between the active and placebo group, *i.e.* the supplementation had no detectable effect on the polarisation of T helper cells.

In the Illumina part of the study, initial PCA analyses showed a tendency towards differences between the two groups in cord blood (Figure 11), whereas this was not seen at the later ages. Additionally, hierarchical clustering only successfully separated the two treatment groups at birth, based on their methylation patterns of CpGs.

Figure 12. Volcano plots depicting methylation values for CpG positions after filtration and normalisation, showing mean differences between the probiotic and placebo group in A. cord blood, B. 12 months and C. 24 months of age. The means represent the placebo group subtracted from the probiotic group. The red horizontal line indicates the $-\log_{10}$ value of $p = 0.01$ originating from a t-test, and the vertical dotted black lines indicate an effect size of $\pm 5\%$. Values stated in the right and left corners denote $n = \text{number of annotated positions (number of annotated immune related positions)} + \text{number of unannotated positions}$. Blue arrows indicate annotated immune related positions.

Furthermore, subsequent analyses showed that the majority of immune related genes that were extracted when comparing probiotic and placebo supplementation at the different time points were hypomethylated in infants treated with probiotics. The hypomethylation in the probiotic treated infants could possibly mean that the epigenome is more transcriptionally active. Among these immune related sites, the main part originates from different parts of the T cell receptor rearrangement process, such as *TRAJ21/33* and *TRAV12-3/12-1*, but other genes including *CD3g* (important for T cell receptor signal transduction) and *FAM60A* (involved in regulation of TGF β signalling), Figure 12. The amount of extracted genes diminish with age possibly indicating that the supplementation have most impact on the epigenome prenatally when the fetus have not encountered any other environmental stimuli yet. Also, in line with the steadily ongoing adaptation and developmental process of the T cells throughout immune maturation and life. Besides, speculatively the intervention-specific effects could reflect increased activation of immunity or enhanced immune maturation beginning already at birth.

When comparing age maturation in the active and placebo group several differences were observed, Figure 13 and Table IIX. In the intervention group, the number of hypermethylated sites exceeded that of the hypomethylated sites at 12 and 24 months compared to at birth. It is only possible to speculate about the importance of the hypo- and hypermethylated genes. Sites in genes such as *TRAJ48/TRAJ49* and *MBP*, involved in T cell rearrangement and induction of T cell proliferation. Furthermore, sites in genes involved in inflammation such as *S1PR3* and *SCUBE1*, *UCN*, *CCL3* were also hypermethylated relative to cord blood at 12 and 24 months, respectively, in the probiotic group. Interestingly, at 24 months a number of CpG sites unique for the treated group situated in genes taking part in T cell proliferation and homeostasis (*SPTA1*), T cell survival (*GIMAP5*), T/B cell maturation (*DNTT*) and T cell rearrangement (*DNTT/TRAJ7*), (Figure 13) were hypomethylated compared to at birth. On the contrary, *SMAD3*, known for its importance in *e.g.* TGF β signalling, was hypermethylated at two years compared to at birth.

In the placebo group, at 12 months of age, sites in genes important for the development of inflammation were hypomethylated (Figure 13, *CD97*, *LY96*, *TANK*) compared to cord blood. However, in the same comparison, a number of positions in genes with Th1 associated anti-viral immune response functions were concurrently hypermethylated (*IFNL4*, *BTBD17*, *AGT*,

GAPDH). A similar pattern was seen when comparing the two year old infants with neonates in the untreated group (Figure 13), although with less hypermethylated sites than at 12 months. Sites in genes with connection to inflammation (*TANK*) and the antiviral Th1-related response (*OAS1* and *SP110*) were more transcriptionally active in the placebo group at two years of age relative to birth, a pattern that was also seen for CpG sites in genes from processes such as antigen presentation, T cell proliferation and activation (*HLA-DMB*) and T cell rearrangement (*TRAJ19/20/21/31*).

Figure 13. Volcano plots illustrating mean methylation differences within the probiotic and placebo group separately when comparing A. 12 months vs. cord blood and B. 24 months vs. cord blood, respectively. The mean differences represent the younger age subtracted from the older age. The red horizontal line indicates the $-\log_{10}$ value of the q -value (FDR-corrected p -value) equal to 0.01. The black dotted vertical lines depict a mean difference of $\pm 10\%$. Values stated in the right and left corners denote n = number of annotated positions (number of annotated immune related positions) + number of unannotated positions. Blue arrows indicate annotated immune related and unique positions (in the comparison of probiotics vs placebo for the

Table IIX

Table of immune related genes from the comparisons of 12 months or 24 months of age vs. cord blood, respectively, in the probiotic and placebo groups separately.

12m vs CB				24m vs CB			
Probiotics		Placebo		Probiotics		Placebo	
Hyper	Hypo	Hyper	Hypo	Hyper	Hypo	Hyper	Hypo
ANGPT2	IGHD2-21	AGT	CD97	BTBD17	BST2	ANGPT2	BST2
ALOX12	IGHD6-13	ANGPT2	IGHD2-2	CCL3	CD97	BTBD17	CD97
CD9	CXCL8	BTBD17	CXCL8	CCR10	CRHBP	CCR10	GBP1
FGF2	PDCD1LG2	CD9	LY96	CD9	DNTT	CD9	HGF
GRB7		FGF2	PDCD1LG2	FGF2	GBP1	FGF2	HLA-DMB
IGDCC3		GAPDH	TANK	GAPDH	GIMAP5	GAPDH	IFI44
ITGAE		GRB7		GPR31	HGF	GRB7	IFI44L
LY86		IFNL4		GRB7	IFI44	HOXB3	IGHD2-2
MBP		IGDCC3		HOXB3	IFI44L	IFNL4	IGHD2-21
NKG7		LRRC17		IFNL4	IGHD2-2	IGDCC3	IGHD6-13
PLA2G3		LY86		IGDCC3	IGHD2-21	LY86	IGHJ5
RARA		PLA2G3		LRRC17	IGHD2-8	PLA2G3	IGHJ6
RFTN2		RARA		LY86	IGHD6-13	RARA	CXCL8
RTKN		RTKN		MBP	IGHJ5	RTKN	LY96
S1PR3		SPTBN2		PICALM	IGHJ6	SPTBN2	OAS1
SPTBN2		TRAV5		PLA2G3	CXCL8	TRAV5	PDCD1LG2
TRAJ48		UCN2		RARA	LY96	UCN2	SP110
TRAJ49				RTKN	PDCD1LG2	VTN	TANK
TRAV5				SCUBE1	SPTA1		TRAJ19
TRGV9				SMAD3	TRAJ7		TRAJ20
UCN2				SPTBN2			TRAJ21
				TRAJ48			TRAJ31
				TRAJ49			
				TRAV3			
				TRAV5			

				UCN			
				UCN2			
				VTN			

Green shades mark common genes in the 12 months vs. cord blood comparison, blue shades denote the 24 months vs. cord blood comparison. Darker shades indicate hypomethylation (mean methylation difference < -10 %), lighter shades represent hypermethylation (mean methylation difference > 10%) for the common genes. All genes in the table have a *q*-value ≤ 0.01. Unique genes, only present in one of the comparisons between the treatment groups for each age comparison, are left unmarked.

Conclusion, probiotics have an impact on the epigenetic methylation pattern in immune related genes and especially the prenatal supplementation have an influence at birth.

Circulating peripheral immune populations phenotyped with flow cytometry during pregnancy and after supplementation with probiotics and ω-3 (paper IV)

The allergy preventive effects of prenatal probiotic and ω-3 fatty acid supplementation are likely induced via changes in maternal immune regulation [42, 175]. However, most previous intervention studies started prenatal probiotic supplementation during the last trimester of pregnancy [67]. If prenatal microbial exposure is vital for the preventive effect, starting supplementation already from the second trimester of pregnancy, when circulating fetal T cells have developed, may have a more powerful allergy preventive effect [67, 175]. Furthermore, probiotic and ω-3 polyunsaturated fatty acid administration during pregnancy may act synergistically via immunoregulatory and anti-inflammatory mechanisms, respectively [42]. To further investigate the effect of supplementation maternal peripheral immunity was collected and phenotyped and after about 20 weeks of active treatment, small changes could be observed.

Supplementation with ω-3 and *L. reuteri* from gestational week 20 alters activated Treg (CD4⁺CD45RA⁺Foxp3⁺⁺) number and percentage in peripheral blood after delivery (paper IV)

Supplementation with ω-3 and probiotics during pregnancy (from week 20 to delivery) resulted in a significant change in the percentage of activated Treg cells (CD4⁺CD45RA⁺

Foxp3⁺⁺) after delivery in the different treatment groups (Figure 14A). A similar tendency was observed for the frequency of aTreg (Figure 14B). The percentage and number of aTreg cells were lowest in the *L. reuteri* treated group (placebo+placebo vs. *L. reuteri*+placebo), and significant differences were also observed between the group treated with ω -3+placebo vs *L. reuteri*+placebo. The other T cell populations were not affected by the treatment.

In line with these findings, treatment with ω -3 fatty acids should in theory be anti-inflammatory [133] due to a competitive effect on the prostaglandin signaling pathway (reviewed in [243-245]). Therefore, it can be anticipated that the anti-inflammatory compartment of the peripheral cell population is increased and noticed systemically. However, earlier research has indicated that activated Tregs may have an increased migratory capacity, as shown by their chemokine receptor expression [179]. Almost all aTregs express the chemokine receptor CCR4 in contrast to the resting Tregs (CD45RA⁺Foxp3⁺) [179]. Activated Tregs are known to reside in non-lymphoid tissue and circulate in lymph and blood and display a high level of cell proliferation and cell death with a function is to suppress differentiation and function of effector T cells [166].

Speculatively, the lower number of aTregs in the *L. reuteri* treated group could possibly indicate that these cells have already migrated to non-lymphoid tissues.

Figure 14. Activated Tregs (CD4⁺CD45RA⁺Foxp3⁺⁺) during pregnancy in the four treated groups. A) % of CD3⁺CD4⁺ cells B) number of cells. Median and IQR are shown. Mann-Whitney *U*-test and Wilcoxon test were used for statistical comparisons.

Supplementation with ω -3 and *L. reuteri* from gestational week 20 alters the frequencies of CD14⁺CD16⁻ monocytes in the pregnant women after giving birth

After delivery, the frequencies of CD14⁺CD16⁻ monocytes were lowest in the ω -3 treated group and highest in the *L. reuteri* treated group (Figure 15). Study groups given only *L. reuteri* had the highest percentage of CD14⁺16⁻ cells, (ω -3+*L. reuteri* vs. Placebo+*L. reuteri*, ω -3+Placebo vs. Placebo+Placebo, ω -3+Placebo vs. Placebo+*L. reuteri*). The other monocyte populations were not affected by the treatment. Speculatively, the results indicate an immune regulatory effect of the probiotic supplementation and an induction of regulatory monocytes.

Conclusion, probiotic supplementation during pregnancy for about 20 weeks modify the regulatory aTreg and CD14⁺CD16⁻ monocytes in peripheral blood.

Figure 15. Monocyte populations during pregnancy in the different treatment groups. Median and IQR are shown. Mann-Whitney *U*-test and Wilcoxon test were used for statistical comparisons.

Allergy

Allergic disease is associated with high Th2 related chemokines (paper I)

Allergic diseases are commonly known as a Th2 mediated/deviated disease and allergic individuals are known to respond with high levels of Th2 associated cytokines upon allergen stimulation in cell culture studies. In our study, infants defined as allergic (symptoms and sensitization) responded with higher levels of cytokines and chemokines after allergen stimulation. IgE-associated disease was associated with increased birch-induced chemokine CCL17 responses at 12 months and at 24 months, Figure 16. Trends were also observed for higher levels of birch induced Th2-associated IL-13 and CCL22 and in addition, lower levels of Th1-associated CXCL10, and anti-inflammatory IL-10. After ovalbumin stimulation, increased CXCL10 levels were observed at birth and increased CCL17 responses at 24 months, also consistent with the known Th2-deviation of allergic diseases.

Figure 16. Birch allergen induced CCL17 (a) and CCL22 (b) chemokine responses from cord and peripheral blood mononuclear cells during the two first years of life in allergic and non-allergic children. Groups were compared using Mann-Whitney U-test, *p<0.05. The number of analysed samples is indicated (n), as well as the median.

Since cytokines sometimes are produced in low levels and difficult to measure and chemokines are produced to greater extent and therefore a reasonable measurable alternative to determine the Th1/Th2 deviation. CCL17 and CCL22 are good candidates with high concentration in cell cultures to determine Th2 balance, even though these chemokines do not correlate with GATA-3. Furthermore, it is also possible to determine Th1 balance by measuring CXCL10 which correlate with T-bet.

Allergic and non-allergic infant response to TLR ligand stimulation (paper II)

We explored how allergic infants responded to TLR ligand stimulation, and no major differences were detected in the chemokine and cytokine levels. Except from that allergic infants (n=3 *L.reuteri*/n=5 placebo) compared to non-allergic infants (n=13 *L.reuteri*/n=16 placebo) secreted higher LPS-induced IL-1 β at 6 months and lower levels of CpG-induced TNF at 24 months. Also, TLR2 mRNA expression was higher at 12 months in non-allergic infants. The number of allergic infants was probably too low to discriminate a significant and consistent difference in our study.

Other studies suggest differences in TLR responsiveness between children who do and do not develop allergy [205, 206, 246]. While TLR ligand stimulation resulted in exaggerated responses at birth in children later developing allergy [206], these responses later seem to be attenuated in allergic compared to non- allergic children at the age of five years [205, 246].

Timing

Timing of age dependent changes in peripheral immunity

Both during pregnancy and infancy several changes in immunity occur, possibly due to different timing events. We have investigated some peripheral changes in relation to pregnancy trimester and age in infants.

Age dependent changes in memory and naïve T cells (paper III)

In paper III we investigated CD45RA[±]. In cord blood the percentage of memory cells was low (Fig. 17A), whereas the percentage of naïve cells was high (Fig. 16B). There was a significant increase of memory cells from cord blood to 12 months, to 24 months and to adult levels (Fig. 17B). A corresponding decrease in naïve cells could be observed, significantly so from cord blood to 12 months, to 24 months and to adult levels (Fig. 17A). These findings corroborate what is known about immune maturation in pre- and postnatal life [38, 39].

Figure 17. Flow cytometry analysis of sorted peripheral CD4+ cells in cord blood, 12 months, 24 months of age and adults showing A. naïve (CD4+CD45RA+) cells and B. memory (CD4+CD45RA-) cells. Student's t-test was employed for the analysis. *** $p < 0.001$.

Figure 18. Age related changes in specific T helper cell lineage associated CpG methylation patterns located in the transcription start site (TSS) of the respective lineages, analyzed by Epigenetic Immune Lineage analysis (EILA). DNA methylation levels in A. *IL-13*, B. *IL-17*, C. *IFN- γ* , D. *Foxp3* are displayed at birth (CB), 12 months and 24 months in infants and in a group of adults. Groups were compared using Student's t-test. CB = cord blood. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

Age associated methylation differences in immune regulatory loci during infancy (paper III)

The function and maturation of neonatal cells is regulated by epigenetic mechanisms. The methylation status of the four immunoregulatory loci *IL-13*, *IL-17*, *IFN- γ* and *FoxP3*, was not affected by probiotic supplementation, and neither did the presence of allergic disease affect the DNA methylation patterns of these loci. Although, there was an increase in the methylation levels from birth to 12 months in the *IL-13* and *Foxp3* loci (Fig. 18A and Fig. 18D), while methylation of the *IFN- γ* gene locus decreased from birth to 12 months (Fig. 18C). Additionally, *IL-13* locus methylation significantly increased from birth to 24 months as well, but was significantly less methylated in adult samples compared to all ages (Fig. 18A), with a similar pattern evident in the *Foxp3* locus (Fig. 18D). In agreement with other studies showing decreased methylation in the *IL-13* promoter region in neonatal CD4+ cells (cord blood) [247]. Possibly, pregnancy could be regarded as a Th2 polarising condition and therefore supports our findings of a Th2 biased methylation pattern early in life. The *FoxP3* DNA methylation levels follow a similar pattern to that seen in the *IL-13* promoter, with an increase in methylation from birth throughout infancy, and a relative decrease in adulthood compared to one and two years of age. The adult DNA methylation levels of *FoxP3* were also the lowest, assumingly permitting transcriptional activity of this locus to assure immunosuppression.

In contrast, the *IFN- γ* promoter became less methylated and more open for transcription during the first year of life, reflecting normal immune maturation of Th1 responses with a trajectory continuing to adult levels (Fig. 18). For the *IL-17* locus, no differences in DNA methylation could be detected in relation to age (Fig. 18B). In concordance with our results, previous studies have also observed increased DNA methylation at the *IFN- γ* promoter region in naïve CD4+ cells from neonates compared with naïve CD4+ cells from children and adults [224, 225, 248]. Likewise, Jacoby and colleagues displayed increased methylation at all six CpG sites within the *IFN- γ* promoter in CBMCs compared to adult PBMCs [249].

In general, the adult DNA methylation levels are lower than in infancy at the corresponding loci. An explanation for these findings could be the higher number of circulating memory effector cells in adults, which may be reflected in the generally lower methylation levels at all loci detected in the EILA results.

Conclusion, age dependent changes reflecting immune maturation are evident in CD4⁺ T cell loci.

Timing of peripheral immunity during pregnancy (paper IV)

Pregnancy is a unique situation in which the mother and the semi-allogeneic fetus peacefully coexist. Numerous mechanisms are needed, including fetal, maternal and placental, to protect the fetus from immunological recognition and rejection. Possibly, these mechanisms are regulated by some sort of timing mediated by cells from the local environment, such as uterine/decidual T cells [250], macrophages [238, 251], and natural killer (NK) cells [185, 252], which contribute to modulating the environment at the fetal maternal interface to sustain a successful pregnancy. In addition to mediating hormonal, nutritional and oxygen support of the fetus, the placenta also plays an important immunomodulatory role [177]. The local immune environment is also reflected in peripheral blood, from which many immune cells are recruited from to sustain local immune tolerance. Our data obtained from 3 different time points during pregnancy, *i.e.* 20 gw, 32 gw and 1 week after delivery shows dynamics in most cell populations investigated. To relate our findings to a “control state” we included non-pregnant women for comparison.

Systemic changes in subsets of lymphocytes (CD4⁺/8⁺/19⁺/56⁺) during pregnancy and compared to non-pregnant women

To determine how the systemic immunity was affected by pregnancy flow cytometry phenotyping of cell types were performed (Figure 19). The total amount of lymphocytes increased significantly from gw 20 to after delivery, and the proportions were significantly higher in non-pregnant than pregnant women (Figure 19). The percentage and total number of CD4⁺ T helper cells decreased from gw 20 to gw 32 but increased from gw 32 to after delivery (Figure 18)), and the percentage was lower in non-pregnant than pregnant women, while the absolute numbers were higher in the non-pregnant than in pregnant women at gw 20. The frequencies of CD8⁺ cytotoxic T cells increased from gw 20 to after delivery (Figure 19) and the total number of these cells were higher in non-pregnant than pregnant women (*i.e.* gw 20 and gw 32). The proportions and number of CD19⁺ B cells decreased during pregnancy, and higher percentages were observed at gw 20 and gw 32 than in non-pregnant women (Figure 18). When investigating NK cells, the CD56⁺ frequencies were higher in non-

pregnant individuals than during pregnancy (Figure 19). Within the CD56⁺ population, the proportions of CD56^{dim} cells were higher in non-pregnant than pregnant women, with similar patterns for the absolute number of cells (Figure 19). In contrast, the proportions and numbers of CD56^{hi} cells were similar in pregnant and non-pregnant women, although a slight increase in frequencies was observed from gw 20 to after delivery (Figure 19). The number of circulating NK cells was decreased during pregnancy in our study. Previous studies suggest a type 2 immune deviation also in the NK cell compartment [185, 252]. Also, their production of IFN- γ is decreased in pregnant as compared with non-pregnant women. Similar to the shift toward Th2 cells there was also a shift toward NK2 cells (NK cells producing Type 2 cytokines) during pregnancy [185-187].

Figure 19. Lymphocyte populations during pregnancy and in non-pregnant women A) percentage of lymphocytes B) number of lymphocytes C) percentage of CD4⁺ cells D) number of CD4⁺ cells E) percentage of CD8⁺ cells F) number of CD8⁺ cells G) percentage of CD19⁺ cells H) number of CD19⁺ cells I) percentage of CD56⁺ cells J) number of CD56⁺ cells K) percentage of CD56^{dim} cells L) number of CD56^{dim} cells M) percentage of CD56^{hi} cells N) number of CD56^{hi} cells. Median and IQR are shown. Mann-Whitney *U*-test and Wilcoxon test were used for statistical comparisons.

Memory T helper cells (CD4⁺CD45RA⁻) decrease during pregnancy and naïve T helper cells (CD4⁺CD45RA⁺) increase

During the course of pregnancy the memory T helper (CD3⁺CD4⁺CD45RA⁻) frequencies decreased from gw 20 to gw 32 and from gw 20 to partus, while the naïve T helper (CD3⁺CD4⁺CD45RA⁺) frequencies and numbers increased from gw 20 to gw 32 and from gw 20 to after delivery (Figure 20). Non-pregnant women had significantly higher frequencies and numbers of memory T helper cells, as compared with women at partus and at gw 32, respectively, while the frequencies and numbers of naïve T helper cells were lower in non-pregnant women as compared with women at partus (Figure 20). The percentages of naïve T helper cells were also lower in non-pregnant women as compared with pregnant women at gw 32 (Figure 20). This suggesting a state of suppression of effector T cells with capacity to strongly respond to recall antigens, possibly in order to protect the fetus.

T regulatory cell populations during pregnancy

Our study investigated three different populations of Treg cells [165, 178]. In humans, Treg cells are identified as CD4⁺ T helper cells expressing Foxp3 and high levels of the IL-2 receptor α -chain, CD25 [253, 254]. These cells are major immune regulators, suppressing many cell types including T cells [255] via mechanisms that are incompletely understood but involving cell–cell contact through, for example, CTLA-4 as well as secretion of soluble mediators such as the cytokines interleukin-10 (IL-10), transforming growth factor- β (TGF β), and IL-35 [256]. Several strategies have been used to define T regulatory cells, as the key phenotypic markers CD25 and Foxp3 also are expressed upon activation of conventional CD4⁺ T cells, complicating the enumeration of ‘true’ Treg cells [178, 254]. CD4^{dim}CD25^{bright}Foxp3 cells have previously been shown to have immune suppressive properties and to be reduced in peripheral blood in the second trimester [178], suggesting accumulation at the fetal- maternal interface [177].

Both CD4⁺CD45RA⁺Foxp3⁺ (resting Tregs) and CD4⁺CD45RA⁻Foxp3⁺⁺ (activated Tregs), as defined in [165], were lowest in percentage and number at gw 32, significantly decreased from gw 20 and significantly increased after delivery, and when combining the two populations the same pattern was found (Figure 21). In non-pregnant women, aTreg cells

were significantly higher in percentage and number compared to pregnant women at all time points (Figure 20). The same pattern was observed for rTregs (Figure 21) and for the two populations together, aTreg+rTreg (Figure 21). As previous shown, subtypes of Tregs have different roles [165], possibly due to their different chemokine expression [179].

The frequency and number of CD4^{dim}CD25^{hi}Foxp3⁺ cells were higher at gw 20 than at gw 32 and at partus. Non-pregnant control women had significantly higher percentage and total number of CD4^{dim}CD25^{hi}Foxp3⁺ cells than pregnant women in gw 32 and after delivery (Figure 21).

Figure 20. Naïve and memory cells during pregnancy and in non-pregnant women A) percentage of (CD4⁺CD45RA⁺) cells B) number of (CD4⁺CD45RA⁺) cells C) percentage of (CD4⁺CD45RA⁺) cells D) number of (CD4⁺CD45RA⁺) cells. Median and IQR are shown. Mann-Whitney U-test and Wilcoxon test were used for statistical comparisons.

Figure 21. Different Treg populations during pregnancy and in non-pregnant women A) percentage of aTreg (CD4⁺CD45RA⁺Foxp3⁺⁺) and rTreg (CD4⁺CD45RA⁺Foxp3⁺) B) number of aTreg (CD4⁺CD45RA⁺Foxp3⁺⁺) and rTreg (CD4⁺CD45RA⁺Foxp3⁺) C) percentage of aTreg (CD4⁺CD45RA⁺Foxp3⁺⁺) D) number of aTreg (CD4⁺CD45RA⁺Foxp3⁺⁺) E) percentage of rTreg (CD4⁺CD45RA⁺Foxp3⁺) F) number of rTreg (CD4⁺CD45RA⁺Foxp3⁺) G) percentage of (CD4^{dim}CD25^{hi}Foxp3⁺) H) number of (CD4^{dim}CD25^{hi}Foxp3⁺). Mann-Whitney U-test and Wilcoxon test were used for statistical comparisons.

Th1, Th2 and Th17 subpopulations during pregnancy and in comparison with non-pregnant women

To investigate Th1/Th2/Th17 skewing during pregnancy, we determined the number and proportions of CD4⁺CD45RA⁻ memory cells expressing the corresponding lineage specific transcription factors Tbet, GATA3 and RORC. The proportions of GATA3⁺ and Tbet⁺ positive CD4⁺CD45RA⁻ memory cells among CD4⁺CD45RA⁻ cells did not change significantly during pregnancy (Figure 22). Similar patterns were observed for the absolute number of cells, except that the number of CD4⁺CD45RA⁻RORC⁺ cells were significantly increased at gw 32 compared to gw 20 and after delivery (Figure 22). In addition, non-pregnant women had a significantly higher number of Tbet⁺ cells than pregnant women (Figure 22A-B), a higher number of GATA3⁺ cells compared to gw 32 (Figure 22C-D) and a lower number of RORC⁺ cells than at gw 32 and after delivery (Figure 22E-F).

The finding that the proportions and numbers of Tbet⁺ memory Th cells were higher in non-pregnant than pregnant women, is in line with a previously suggested Th2 deviation during pregnancy [177]. However, in contrast, the numbers (but not frequencies) of GATA3⁺ positive cells were also higher in non-pregnant than pregnant women at gw 32. Other studies suggest that Th17 cells may remain unchanged [180] or slightly decreased in the periphery during pregnancy [257] compared to the decidua [258].

Conclusion, the peripheral immunity during pregnancy display dynamic changes in most cell populations investigated.

Figure 22. T helper cells during pregnancy and in non-pregnant women A) percentage of memory Th1 cells ($CD4^+CD45RA^-Tbet^+$) B) number of memory Th1 cells ($CD4^+CD45RA^-Tbet^+$) C) percentage of memory Th2 cells ($CD4^+CD45RA^-GATA3^+$) D) number of memory Th2 cells ($CD4^+CD45RA^-GATA3^+$) E) percentage of memory Th17 cells ($CD4^+CD45RA^-RORC^+$) F) number of memory Th17 cells ($CD4^+CD45RA^-RORC^+$). Median and IQR are shown. Mann-Whitney *U*-test and Wilcoxon test were used for statistical comparisons.

Summary and conclusion

Probiotic supplementation during pregnancy and infancy modulates immune regulatory mechanisms and results in more moderate immune responses after stimulation with allergens and LTA.

Fetal exposure i.e. maternal probiotic supplementation, may be of essential importance for immune modulating effects since probiotic supplementation was associated with epigenetic modulations in T helper cells at birth.

Supplementation during pregnancy for around 20 weeks lead to minor modulation of peripheral regulatory immune cell populations in mothers.

Changes in immune cell populations are evident both during infancy and pregnancy.

Future perspectives

To investigate clinical effects of supplementation in the PROOM-3 study on allergic outcome in infants.

In addition, continue the analysis of other immune cell populations from mothers included in the study and relate to supplementation and allergic disease in mothers and infants.

Study epigenetic changes during pregnancy and infancy in relation to supplementation, allergic disease, pregnancy trimesters, and age in infants.

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