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Lactobacillus reuteri, Infant Allergy Prevention and Childhood Immune Maturation

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Additional information is available at the end of the chapter

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Abstract

The increasing allergy prevalence in affluent countries may be caused by reduced microbial stimulation, resulting in an abnormal postnatal immune maturation. This chapter concerns the theories behind the use of probiotics in randomized prevention trials, and how this supplementation affects the immunity of pregnant women, the immune development in their children, and possibly preventing allergic diseases. Most studies investigating the underlying mechanisms have focused on postnatal microbial exposure. An increasing body of evidence from studies suggests that the maternal microbial environment during pregnancy can program the immune development of the child. In human allergy intervention studies, probiotic supplementation to the mother during pregnancy, as well as to her baby postnatally, may be important for preventive effects. Also, prenatal environmental exposures may alter gene expression via epigenetic mechanisms, aiming to induce physiological adaptations to the anticipated postnatal environment. The maternal microbial environment during pregnancy may program the immune development of the child.

Keywords: allergy, immune maturation, *Lactobacillus reuteri*, probiotics, allergy prevention, allergens, TLRs, cytokines, chemokines

1. Probiotics in allergy prevention

Different probiotic strains have been used in allergy prevention trials with successful and unsuccessful results. Why use probiotics to try and prevent childhood allergic diseases then? The increasing allergy prevalence in affluent countries may be caused by reduced microbial stimulation, reflecting an abnormal postnatal immune maturation [1], resulting in allergic diseases in children. Of course, this is a multifactorial problem where changing climate, living

conditions, and urbanization have led to a biodiversity loss. Studies show altered microbiota and general microbial deprivation which characterize people living in urban affluent environments. Consequently, this seems to be a risk factor for immune dysregulation and impaired immune tolerance. It is 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 flora [2]. Probiotics may be one way to increase microbial stimulation, enrich the gut flora and balance a skewed immune system, which will be discussed later.

2. A brief introduction to childhood allergic diseases

The cost of allergic diseases is burdening the society; reduced life quality and increased sick leave are common, asthma in children is one of the most common chronic diseases affecting children at an early age. About 20% of the population is affected by allergic disease such as atopic dermatitis, food allergy, asthma, allergic rhinitis, and conjunctivitis [3]. Furthermore, the “atopic march,” as commonly referred to, is an age-associated variation in allergy-related symptoms in childhood. The first allergy-related symptoms are often eczema and food allergy at a young age, later followed by asthma and rhinoconjunctivitis in school-aged children. In addition to the age variation in allergic diseases, there seems to be a gender issue as well. Boys early in life have a higher incidence of allergic diseases than girls [4–6] and are also more susceptible to infections maybe due to the more Th2-deviated immunity [4, 5]. Females are characterized by increased inflammatory responses and infections clearance, possibly reflecting the stronger Th1 immunity observed in girls [4, 5]. Of course, this has its pros and cons. This results not only in a better protection against infection but also in increased susceptibility to autoimmunity later in life. Allergy-related sex differences diminish at puberty; at adult age, no clear sex differences concerning allergy can be found [7].

3. The importance of the environment and the discovery of the beneficial effect of exposure to microbes

In the beginning, when elucidating the mechanism behind the increasing rates of allergic diseases, the focus was on living conditions. In 1989, a researcher named Strachan discovered that there was an association between siblings, family size, and hay fever [8]. This led to discoveries that children born in a farm had less allergies than children born in urban areas. The step after that was to focus on postnatal microbial exposure [9–12]. How infants are prepared for life outside the uterus, and how can the maternal environment be protective against allergic development in the offspring? The maternal microbial environment has been proposed to be able to program the immune development of the child, during pregnancy [13]. Especially, if the mother is exposed to farm environment, in particular during pregnancy, development of allergic diseases seems to be attenuated. Interestingly, research has showed that exposure later in life, after pregnancy and later, seems to have a weaker effect [14, 15] which opens up several other questions. For example, is it possible to program the development of immunity in the child? When is the “window of opportunity”? Can we manipulate

this and protect the child against the development of diseases? How can this be done? Numerous studies show that exposure to farm environments during infancy and even in fetal life [16, 17] reduces the incidence of allergic diseases. Furthermore, a recent report in Sweden presents data that contact with farm animals or dogs during childhood may protect against asthma development [18]. Exposures to farming areas and also consumption of raw milk have been associated with the upregulation of certain receptors associated with innate immunity. 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 and showed that farming-related exposures, such as raw farm milk consumption, 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. Raw milk of course includes many *Lactobacilli* strains among others. Therefore, it is believed that microbial exposure in early life educates the developing immune system, driving postnatal maturation of immune regulation as discussed in [19]. The author also suggests 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 of benefit. The epidemiological studies are supported by animal models, demonstrating that microbial exposure during gestation can prevent allergic responses in the offspring [20, 21].

3.1. Animal models show the benefit of microbial exposure during gestation

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 mother is treated with lipopolysaccharide which attenuated allergic disease and associated inflammation in offspring [22–24]. One study explored the effect of LPS on female BALB/c mice before conception and during pregnancy. Several weeks after birth offspring were sensitized to ovalbumin (OVA) followed by aerosol allergen challenges. LPS may operate in prenatal life in order to modulate the development of allergies in the offspring since LPS exposure prenatally enhanced Th1-associated IFN-gamma in offspring. OVA sensitization was followed with a reduction in anti-OVA IgG1 and IgE as well as unchanged IgG2a antibody responses, accompanied by a significant decrease in Th2-associated cytokine levels. This was followed by a reduction of eosinophils and macrophages in bronchoalveolar lavage fluids, which are often increased in allergic airways. However, clinical manifestations such as airway hyper-responsiveness, a hallmark of bronchial asthma, were not affected [22]. Another study also investigated the effect of LPS on pregnant mice and further explored the effect of LPS on the offspring before allergen sensitization with OVA. Prenatal and postnatal LPS exposure suppressed allergen-specific IgE production, eosinophilic airway inflammation and *in vivo* airway reactivity in response to methacholine. The suppression of allergen-mediated inflammatory responses was associated with an increased shift toward Th1 responses in culture (spleen cells) and may be mediated via Toll-like receptor (TLR) and T-bet expression by lung tissues [23]. Another group used a rat model and investigated the effect of prenatal LPS exposure on postnatal T cell differentiation and experimental allergic airway disease. The

expression of T cell-related transcription factors and cytokines was quantified in the lung, and airway hyper responsiveness was measured. Prenatal LPS exposure induced a Th1 immune milieu in the offspring of rats and also reduced OVA-induced airway inflammation, eosinophilia, and airway responsiveness [21].

The next step was to use the commensal *Acinetobacter lwoffii* [20]. *Acinetobacter lwoffii* is derived from cow shed and is non-pathogenic. The strain was used in an experimental allergic airway inflammation mouse model. Maternal intranasal exposure to *A. lwoffii* F78 protected the offspring from development of allergic disease and resulted in an induction of proinflammatory cytokines and upregulation of TLRs. On the contrary, suppression of TLRs was observed in placental tissue. To investigate if TLRs were of importance, a knockout mice was used (TLR2/3/4/7/9(-/-)). In that model, the asthma-preventive effect was completely eliminated. Additionally, the mild local and systemic inflammatory response was also absent in these *A. lwoffii* F78-exposed mothers. Therefore, it is believed that there is a direct relationship between maternal bacterial exposures, functional maternal TLR signaling and asthma protection in the progeny. The main receptors for bacterial products are the TLRs. Farm studies have also shown that these receptors can be upregulated in neonates after maternal contact with farm animals and after farm-related exposures [14, 25, 26]. One study investigated both atopic sensitization and the gene expression of receptors of innate immunity (TLRs), and how they were related to maternal exposure to stables during pregnancy. A dose-response relation was found between the upregulation of these genes and the number of different farm animal species the mother had encountered during pregnancy. Interestingly, it seemed like each additional farm animal species increased the expression of TLR2, TLR4, and CD14 [14]. In another study, it was also shown that gene expression of innate immunity receptors in cord blood was overall higher in neonates of farmers, significantly so for TLR7 and TLR8. The study further enhanced the fact that farming-related exposures, such as raw farm milk consumption, that were previously reported to decrease the risk for allergic outcomes was associated with a change in gene expression of innate immunity receptors in early life [26].

3.1.1. Toll-like receptors in the immune system

TLRs are included in the innate immune system and belong to the group of pattern recognition receptors (PRRs) which recognize the so-called pathogen-associated molecular patterns (PAMPs). These are evolutionarily conserved structures from bacteria, viruses, parasites and fungi. The PRRs are expressed on a wide variety of immune cells as well as mucosal and epithelial cells. Some subgroups of the PRRs include TLRs, NOD-like receptors (NLRs), RIG-1-like receptors (RLRs), β -glucan receptors, and other C-type lectins.

3.2. Microbial exposure to counteract the Th2 skewing in allergic diseases?

Continued enhanced postnatal microbial exposure may be required for optimal allergy protection, however [15]. 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 [16, 17, 27, 28]. Farm exposures during pregnancy increase the number and function of cord blood Treg cells associated with lower Th2 cytokine secretion and lymphocyte

proliferation. Cord blood Treg cell counts were increased, with maternal farming exposures and associated with higher FOXP3 and higher lymphocyte activation gene 3 (Ppg) expressions. Furthermore, Treg cell function was more efficient, and FOXP3 demethylation in offspring of mothers with farm milk exposure was increased, possibly reflecting an increased immune regulatory capacity [17]. Also, failure to upregulate the interferon gamma (IFN γ) response during infancy is an important determinant of the risk of allergic disease. Early life exposure has also been associated with decreased IFN γ gene expression of naïve T cells [28]. 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. The immune system is generally divided into the innate and adaptive arm. The first line of defense is the innate immunity which responds rapidly to common components of bacteria, viruses, parasites and fungi, structures preserved through evolution, such as pathogen-associated molecular patterns (PAMPs). The innate immune system includes physical barriers of the mucosa, the epithelial cell layer, as well as cell responding immediately with phagocytosis of microorganisms, extinction of infected cells, and cooperation with adaptive immunity. The PRRs are expressed on various cells of the immune system such as monocytes, macrophages, DC, natural killer cells, innate lymphoid cells as well as mucosal epithelial and endothelial cells. The adaptive immune system requires longer time to develop but is more specific and can develop memory to encounter antigens. The adaptive part, on the contrary, consists of T and B lymphocytes and a rich and specific antibody repertoire.

The key players in adaptive immunity may be the CD4⁺ T helper (Th) cells that have a central role by orchestrating immune responses to pathogens. As naïve cells they exit the thymus. Th cells may differentiate into four major effector subsets, Th1, Th2, Th17, and Treg 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. It has been suggested that different species of *Lactobacillus* exert very different DC activation patterns and, furthermore, at least one species may be capable of inhibiting activities of other species in the genus [29]. DCs are also able to attract cells via secretion of chemokines, for example, Th2 cells are attracted by the secretion of CCL17 and CCL18 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 [30].

4. Probiotics and immune regulation

Probiotics have been defined as “live microorganisms which when ingested in adequate amounts confer a beneficial effect on the host” [31]. Probiotics to prevent allergic disease have gained much attention. Contacts with microbial organisms from the environment [8] and at mucosal sites, such as the gut [32, 33], may be essential in the induction of T regulatory cells after birth and have a beneficial effect on infant gut flora. The intestinal flora may vary between allergic and non-allergic infants. Also, allergic disease among children may be associated with

differences in their intestinal microflora as evident in two countries with a low (Estonia) and a high (Sweden) prevalence of allergy. Differences in the indigenous intestinal flora might affect the development and priming of the immune system in early childhood. In one study [33], feces samples were diluted and cultured and the allergic. The allergic children in Estonia and Sweden were less often colonized with lactobacilli. When comparing allergic and non-allergic infants in Sweden, it was shown that there were differences in the composition of the gut microbiota before clinical manifestations. In comparison with healthy infants, babies who developed allergy were less often colonized with enterococci during the first month of life and with bifidobacteria during the first year of life. Furthermore, allergic infants had higher counts of clostridia at 3 months, *Staphylococcus aureus* at 6 months, whereas the counts of *Bacteroides* were lower at 12 months [32]. Possibly probiotics and prebiotics may modulate the composition of the gut flora in a healthy way.

It has also been suggested that certain strains of probiotic bacteria can induce immunoregulation by modulating dendritic cells and induce Tregs [12, 34–36]. A mixture of probiotics (a combination, or selectively, of *L. acidophilus*, *L. casei*, *Lactobacillus reuteri*, *Bifidobacterium bifidum*, and *Streptococcus thermophilus*) was found to upregulate CD4⁺Foxp3⁺ regulatory T cells (Tregs). The administration of the probiotics mixture in mice models induced both T cell and B cell hyporesponsiveness and downregulated Th1, Th2, and Th17 cytokines and generated Tregs with increased suppressor activity [12]. In another study [34], BALB/c mice were treated daily with *L. reuteri* by gavage which also increased Tregs with a great capacity to suppress T effector cells [34]. O'Mahony and colleagues showed that ingestion with a probiotic strain enhanced the clearance of pathogens via the generation and function of Tregs that control excessive NFκB [35]. Lactobacilli species may have different properties to induce Tregs [36]. The intestine provides a unique environment for the development of both immunity and tolerance, and the initiated immune response is dependent on DC type and state of activation. The probiotic supplementation during pregnancy and early childhood could possibly provide microbial stimulation needed for normal development of immunoregulatory capacity, providing a source of TLR-ligand exposure [37, 38].

4.1. Treg cells in immunity

In addition to conventional Th cells, CD4⁺ T cells can also differentiate into T regulatory cells (Tregs) that are not only essential for the regulation of inflammatory responses to pathogens but also for peripheral tolerance and the protection against autoimmune diseases. There are two main types of Treg cells, thymic Tregs (also called natural) are generated in the thymus and are believed to protect against self-reactive immune responses, and peripheral Treg cells (also called inducible) that are generated in peripheral tissues and may have specificity to self- and foreign antigens. FoxP3 is a key transcription factor for the development and function of natural CD4⁺ regulatory T cells. As other cells, different subpopulations can be defined within the FoxP3-positive cells. The first definition of Treg cells is the CD4^{dim}CD25^{hi}FoxP3⁺ Treg cells, described in [39]. Later on, CD45RA⁺FoxP3^{lo} resting Treg cells (rTreg cells) and CD45RA⁺FoxP3^{hi}-activated Treg cells (aTreg cells) were discovered; both subtypes seem to be suppressive in vitro. 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. Taken together, the dissection of FoxP3⁺ cells into subsets enables one to analyze Treg cell differentiation dynamics and interactions in normal and disease states, and to control immune responses through manipulating particular FoxP3⁺ subpopulations [40].

In allergic diseases, the Th1/Th2 paradigm is useful, but it is obviously a simplification. Treg cells are important in the suppression of allergen-specific responses in several ways [41].

5. *Lactobacillus reuteri*

Lactobacillus reuteri is an obligate heterofermentative [42] Gram positive rod that has been isolated from the GI tract in several mammals, including humans, as well as from different food products [43, 44]. In addition to glycerol, *L. reuteri* produce the antimicrobial metabolite reuterin during anaerobic conditions [45]. The strain *L. reuteri* ATCC 55730 is considered to be safe, and in the USA, probiotics has started getting regulated in a similar way as pharmaceuticals [46].

6. Probiotics in human allergy prevention trials

Probiotics have been used in intervention studies with preventive effects on eczema during infancy with varying results. [47–52]. Different study design, probiotic strain, duration of follow up, etc. have resulted in different outcomes. However, there seems to be a benefit in supplementing with probiotics in prevention of childhood eczema. Randomized placebo controlled trial to prevent childhood eczema have been conducted since the first study in 2001 [53]. One of the main questions to answer is to whom you may supplement. Studies have been conducted with different modes of supplementation, supplementing only mothers during gestation, only mothers during breastfeeding, only infants after delivery or both mothers during gestation and infants after delivery [54]. Probiotic supplementation to the mother during pregnancy, as well as to her baby postnatally, may be important for preventive effects on childhood allergic disease [55]. Thus, a preventive effect on atopic eczema, the most common allergic disease at this age, has primarily been demonstrated in studies where probiotics were given both pre- and postnatally [48, 49, 56–59], whereas two studies with postnatal supplementation only failed to prevent allergic disease [60, 61].

6.1. Supplementation with *Lactobacillus reuteri* to prevent childhood IgE-associated eczema

Furthermore, in human allergy intervention studies, our study, using *Lactobacillus reuteri* supplementation, had the most clear effect on infant sensitization to allergens at 2 years of age [17]. *L. reuteri* ATCC 55730 (1×10^8 colony forming units) was given to pregnant women daily from pregnancy week 36 until delivery. The infants continued with the same product to 12 months of age and were followed up until 24 months. Primary outcome was allergic disease,

with or without positive skin prick test or circulating IgE to food allergens. The study was designed to have enough power to detect true differences between the probiotic-supplemented group and the placebo group, which was based on a 40% anticipated allergic disease risk at 2 years. The aim was a 50% reduction in frequency of allergic disease which could be detected at a 5% level of significance with 80% power. The study was also designed to allow a dropout frequency of 20%. Further considerations were done with differences in living environment and other possible confounding factors since the study was a randomized placebo-controlled multicenter trial. To achieve high-quality result study, participants were monitored regularly. At 2 years of age, children were examined by a pediatrician, 1 year after the termination of treatment. The *L. reuteri*-supplemented infants had less IgE-associated eczema during the second year, 8% versus 20% ($P = .02$). Skin prick test reactivity was also less common in the treated than in the placebo group, significantly so for infants with mothers with allergies, 14% versus 31% ($P = .02$). Wheeze and other potentially allergic diseases were not affected. A total of 184 completed a 7-year follow-up. The primary outcomes at 7 year of age were allergic disease and skin prick test reactivity. The prevalence of asthma (15% in the probiotic vs. 16% in placebo group), allergic rhinoconjunctivitis (27% vs. 20%), eczema (21% vs. 19%) and skin prick test reactivity (29% vs. 26%) was similar in the probiotic and placebo groups. No severe adverse events were reported [62]. Our study is one that has resulted in less IgE-associated eczema at 2 years of age and is one where both mother and child received the product, the mother from gestational week 32 and the child from birth to 1 year of age. 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 [63], may have a more powerful preventive effect on allergy development.

6.1.1. The importance of study design in probiotic trials

There have been implications that besides the design of the study the importance of the probiotic strain has been highlighted. To exemplify this, probiotics that are being used in trials to prevent childhood allergic disease, are also used to prevent necrotizing enterocolitis. The great importance of strain has been shown recently. Necrotizing enterocolitis is one of the most devastating diseases encountered in premature infants [64].

7. Clinical investigations and sample collection

In vitro studies examining responses to common allergens and determining the cytokine and chemokine patterns are a way to explore the effect of probiotic supplementation on immune status in infants. In vitro studies are a good complement to clinical studies in pinpointing the exact mechanism of probiotic supplementation in clinical trials. One way is to collect cord and peripheral blood from the infants included in the study at the different follow-up meetings with research nurses or doctors. From the blood, it is relatively simple to collect cells using gradient centrifugation and to store these cells for later use in liquid nitrogen. One of the benefits with this system is that you are able to analyze all samples during a limited time period instead of analyzing them over years, which is the usual time period for this kind of clinical

trials. After thawing, counting, and stimulation of cells, it is important to incubate them with a proper media and in a time period that is optimal for your experiment. To have in mind is that when you investigate innate responses they are designed to respond rather immediately, whereas adaptive responses may need antigen presentation first and then start to produce the cytokines and chemokines associated with that type of response. Commonly thought, antigen presentation and production of allergen-associated biomolecules may take 6 days to reach levels of optimal detection.

7.1. Detection of biomolecules in serum and plasma, a way to determine immune status

To be able to investigate effects that are not obvious clinically one rather convenient way is to measure biomolecules in serum and plasma. Serum and plasma are quite easily collected; venous blood samples are centrifuged and aliquoted to several small tubes that are kept in the freezer at -20 or -70°C . If you compare collecting serum and plasma samples to cell samples, the first is rather time efficient, whereas collecting cells in the laboratory from venous blood samples is rather time consuming. However, there are some drawbacks with serum and plasma samples; one is that it is relatively hard to detect cytokines in this kinds of samples since that type of biomolecules are produced at a lower concentration compared to, for example chemokines, and are acting more locally than the chemo attractive chemokines. Cells have receptors for both cytokines and chemokines on their surface [65].

The determination of circulating chemokine levels in epidemiological studies may be a tool for the identification of factors associated with the development of sensitization or allergic disease.

7.1.1. ELISA

One common method to measure biomolecules in fluids, actually all types of fluids, such as serum, plasma, blood, saliva, etc. is enzyme-linked immuno sorbent assay (ELISA). This method is based on two antibodies with two different epitopes that bind the same biomolecule, in a “sandwich” model. The first thing to do when performing an ELISA is to bind the antibodies to a surface, preferentially one in a well, in a 96-well plate used for the purpose. The binding is enhanced by adding a buffer. Thereafter, one must block the other surface to prevent unspecific binding to the plastic of the wells. This is often performed with bovine serum albumin or milk. The sample is added, and after incubation the capture antibody is added. ELISA has several ways of detection; one is to add streptavidin-conjugated horseradish peroxidase (HRP). The streptavidin will form a strong binding to the biotin-conjugated capture antibody. Then the addition of a substrate for the enzyme that is conjugated to the capture antibody; if HRP is used, TMB is a good substrate (although toxic) and HRP turns TMB to a yellow product and the reaction is incubated for about 15–30 min. The addition of a H_2SO_4 will stop the reaction and turn the liquid blue. The color of the product is relative to the concentration of the biomolecules in the sample and easily detected using an ELISA reader that measures optical density (OD). To further enhance the capability of your ELISA, you can add a standard curve with known concentration that you may relate your samples to.

7.1.2. Multiplex bead assay

Another good method to measure biomolecules in fluids is multiplex bead arrays. These bead arrays are based on the same principles as ELISA, but you couple the capture antibody to a bead instead of the well bottom. That antibody binds an epitope on the biomolecule of interest and a second detection antibody often conjugated with biotin is common; the biotin-conjugated antibody then reacts with streptavidin with a bound fluorescent molecule, often phycoerythrin (PE). The main advantage of the multiplex technology is that it enables several simultaneous analysis, that is, you are able to detect several biomolecules from the same sample at the same time by mixing beads with distinct fluorescent spectra (a mixture of two or more dyes trapped inside the beads) in the same well upon analysis. The detection method is also a bit different from ELISA, which is based on optical density, and the multiplex bead array is a flow cytometry-based system which aligns all beads in a single row to enable single-bead analysis. The single bead is excited with a laser to determine the bead emission, that is, the biomolecule you measure and the concentration of the biomolecule trapped on the bead. If you include samples with known concentration, you can create a standard curve and relate all the measured samples to that to determine the unknown concentration. Since this method can be labor and cost effective, it is possible to determine, for example, the immune status in quite large cohorts by collecting serum and plasma samples and measure biomolecules such as cytokines and chemokines.

8. What are the mechanisms behind probiotics in allergy prevention trials?

The mechanisms behind probiotic supplementation have not been totally mapped yet; various effects on the immune system have been reported after probiotic treatment in allergy prevention trials. There is no consensus among studies, possibly due to different study designs, when probiotics have been introduced prenatally, pre- and postnatally, or only postnatally. The probiotic strain is also of great importance. Evidence of increased CRP, total IgE, and IL-10 levels, which are characteristic of a low-grade inflammation has been presented [66]. Another study decreased that after supplementation during pregnancy with *L. rhamnosus* and *B. lactis*, IFN- γ in cord blood increased [67]. Anti-CD3/CD28-induced IL-2 mRNA expression at 13 months of age was showed after probiotic supplementation at weaning [68]. Although no allergy preventive effect was observed in some cohorts, an immune modulatory effect was detected [69]. Reduced TNF and IL-10 responses to house dust mite were found [70]. Boyle et al. demonstrated that prenatal *Lactobacillus GG* supplementation during the last month of pregnancy reduced heat-killed LGG-induced CD4⁺T cell proliferation [71], although no allergy preventive effects were observed [72]. Taken together, the studies indicate that several strains of *Lactobacillus* may modulate immunity in infants.

8.1. *Lactobacillus reuteri* and chemokines

The analysis of circulating chemokines is a useable tool to investigate the T helper (Th)1/Th2 imbalance in allergic disease and other diseases in vivo. Circulating levels of Th1-associated

CXC-chemokine ligand (CXCL)9, CXCL10, and CXCL11 and Th2-associated CC-chemokine ligand (CCL)17 and CCL22 have been related to allergic disease, sensitization, and probiotic supplementation [73]. Infants are born with a Th2 deviation of the immune system, which is also reflected in chemokine concentration early in life. The Th2-associated chemokines CCL17 and CCL22 have been shown to be the highest at birth and then decreased, whereas CCL18 and the Th1-associated chemokines increased with age. Allergic children have been observed to have high Th2-associated chemokine concentration, as expected. Interestingly, different allergic symptoms may be related to different chemokines. Furthermore, an imbalance in circulating Th1- and Th2-associated chemokines may precede the onset of sensitization, eczema, and recurrent wheeze from birth [73, 74]. Supplementation with specific probiotic strains [75] may be detected by the presence of strain in stool samples. The presence of *L. reuteri* in stool in the first week of life was associated with low CCL17 and CCL22 and high CXCL11 levels at 6 months of age. However, no other differences were observed between the probiotic and placebo groups. Low Th2-associated chemokine levels and high Th1-associated levels may be of benefit to counteract a Th2 deviation and could possibly imply a decreased tendency to develop allergic diseases. High Th1-associated chemokine levels were associated with day care. As discussed previously, day care is associated with reduced incidence of allergic diseases, possibly by inducing a Th1-associated immunity. Also, to keep in mind when investigating these chemokines is that Th1 and Th2 cytokines are likely important upstream mediators of these effects, as they induce the production of the respective chemokines. The names of the chemokines also indicate how they are regulated.

8.2. *Lactobacillus reuteri* and allergen responsiveness

Probiotic treatment with *Lactobacillus reuteri* [75] has been shown to be associated with lower secretion of allergen induced Th2- and Th1-related cytokines during infancy, as well as with low IL-10 and Th2-associated CCL22 responses [76]. In our study, the differences were more marked for responses to the perennial and ubiquitously present [9] cat allergen than the food allergen OVA and the seasonal birch allergen. Allergens may have different route and duration of exposure, which may imply different regulation. Also, in Sweden, it is uncommon to be allergic to house dust mite but however quite common to be allergic to birch and grass. Moreover, low mitogen induced Th2-like responses were also associated with *L. reuteri* supplementation. The lower cytokine and chemokine levels in the probiotic group could indicate an increased immune regulatory capacity, possibly implying a reduced atopic propensity, consistent with our previous findings in this cohort [75]. We also investigated if probiotic supplementation affected the pattern of cytokine release after stimulation in this study; however, the allergen- and mitogen-induced cytokine responses seemed to be independently associated with probiotic treatment and allergy development, since logistic regression indicated separate effects of treatment and allergy on immune responses. Possibly another mechanism than we were able to investigate is responsible for this effect. However, this could be due to the fact that only few allergic infants were included. In agreement with previous studies [77], allergic infants did show high Th2 responses after birch and food allergen stimulation, whereas probiotic supplementation showed less clear effects.

In this study, treatment reduced the incidence of clinical manifestations as well as sensitization, possibly reflected by the lower responses to allergen stimulation in probiotic-treated infants [75]. It is believed that some strains of probiotics can induce a Th1 immunity to counteract a deviated Th2 immunity in infants and allergic diseases. To investigate this hypothesis, Th1-associated factors were investigated in this cohort. The mRNA expression of the transcription factors T-bet and GATA-3, driving Th1 and Th2 differentiation, respectively, was not influenced by probiotic treatment, although T-bet expression correlated to the secretion of IFN- γ and the Th1-associated chemokine CXCL10. Neither Foxp3 nor Ebi3 mRNA expressions were affected by probiotic treatment, while Ebi3 and Foxp3 expressions were correlated to each other and associated with IL-10 secretion, supporting an immune regulatory role of Ebi3 [78].

The lower allergen responsiveness in the infants receiving probiotics, as compared to placebo, is similar to our previously reported observations of lower allergen-induced cytokine secretion during infancy in a country with a low incidence of allergies (Estonia) [27]. Thus, allergen-induced IL-5, -13, -10, and IFN- γ responses were lower in Estonian than in Swedish children. Living conditions are different, and besides that, lactobacilli were more often detected in fecal samples from Estonian than Swedish children [79]. A low lactobacilli colonization has been associated with allergic disease development [80].

8.3. *Lactobacillus reuteri* and Toll-like receptors

Can pre- and postnatal supplementation with *Lactobacillus reuteri* affect the innate cytokine and chemokine responses to bacterial products and the expression of associated receptors, i.e., TLR2, 4 and 9 In this study, TLR2 stimulation leads to lower IL-1 β , IL-6, IL-10, CCL4, and CXCL8 responses in the probiotic treated infants [81]. These differences were not dependent on the differences in TLR2 mRNA expression in the probiotic and placebo groups. Probiotic supplementation may thus be associated with an increased immune regulatory capacity during infancy, in line with our previous findings showing lower allergen responsiveness in the probiotic-treated children [81].

Low responsiveness to stimulation with lipoteichoic acid after previous supplementation with the Gram positive bacteria *Lactobacillus reuteri* could be related to a phenomenon referred to in the literature as lipopolysaccharide tolerance [82]. Our results could suggest that such a downregulation occurs in vivo as a consequence of long-term exposure to TLR2 ligands, that is, supplementation with the Gram positive *Lactobacillus reuteri*. The expression of TLR receptors has been shown not to be involved in this phenomenon, but studies suggest that this is dependent on a downstream effect involving IRAK [83]. This would also explain why the TLR2 mRNA expression was not affected by probiotic supplementation, whereas studies of children growing up on a farm have shown that microbial exposure upregulate these receptors [14, 25, 26]. Our data, however, indicate that the TLR2 mRNA expression and LTA-induced cytokine and chemokine responses are not correlated. The lower responses to TLR2 stimulation could be dependent on an induction of regulatory macrophages responding to stimuli with lower secretion of proinflammatory cytokines and chemokines [84]. Whether this downregulation of TLR2 responses is also related to the decreased incidence of IgE-associated disease in probiotic-treated children is not known. The logistic regression analyses suggested, however,

that the effects on TLR2 responsiveness were related to probiotic supplementation but not allergy development in this study. This could also be due to the fact that there were few allergic children included in these analyses, although we did detect higher levels of TLR2 mRNA expression in non-allergic than allergic infants at 12 months of age. Other studies suggest differences in TLR responsiveness between children who do or do not develop allergy [85–87].

That probiotic supplementation may be associated with an increased immune regulatory capacity during infancy is also in line with studies suggesting that immune regulatory mechanisms are established at a later age in Sweden compared to Estonia [27], a country with higher microbial exposure and a lower allergy prevalence than Sweden [9]. Comparatively, after allergen stimulation, Estonian infants responded with lower levels of cytokines, both Th1 and Th2, than Swedish infants [27]. Estonian infants also secrete lower levels of proinflammatory cytokines after LPS stimulation compared to Swedish infants (unpublished). Another study comparing countries with higher microbial exposure and less allergies with countries with less microbial exposure and more allergic diseases demonstrate that neonatal antigen presenting cells (APCs) are more quiescent in children born under traditional, i.e., Papua New Guinea, compared to modern environmental conditions, i.e. Australia [88]. This was reflected by less responsiveness to stimulation in vitro in APCs from newborns born in Papua New Guinea, while they exhibited higher baseline levels of activation and inhibitory markers in the resting state compared to APC from Australian neonates [88]. This quiescent function could potentially be a protective mechanism learned in utero. Thus, lower TLR-induced levels of proinflammatory cytokines and chemokines may be due to an enhanced immune regulatory capacity among infants living in conditions with a higher microbial burden.

Of course, there has been an effort in elucidating the passage ways that the bacteria colonize the body and affect the immune system. Until recently, the infant intestines were supposed to originate from perineal, vaginal, and fecal microbiota and before delivery thought to be sterile. However, the microbial colonization might already start before birth by microbial transfer through the placental barrier. DNA from a wide variety of microbial taxa in the human placenta, umbilical cord blood, amniotic fluid, and meconium have been found and sequenced. The bacteria may gain access through ascent from the vagina and/or through the blood stream for bacteria from intestinal or oral origin. So one might say that the sterile womb theory is history [89]. These findings suggest that normal colonization may already start before birth, colonization that is of benefit for the infant and not detrimental and leading to disease. Labeled *Enterococcus faecium* in an experimental animal study showed that beyond transplacental passage, bacteria can be transferred via the gastrointestinal canal [90]. As reviewed in [89], bacteria may travel via the bloodstream from the mouth, of course without causing sepsis, via the breast, external through the sebaceous skin to the breast milk. Internally, via the entero-mammary pathway that brings gut bacteria to the mammary gland via lymph and blood circulation.

Other possible effector mechanisms of probiotic supplementation could be dependent on epigenetic changes, although this needs further investigation. Thus, epigenetic regulation has been suggested as one of the underlying effector mechanisms for the allergy preventive effect of microbial exposure during pregnancy [91].

8.4. *Lactobacillus reuteri* and the composition of breast milk

Breast milk not only provides the necessary nutrients for growth and development, it also contains many important immunological components to provide the immunological immature infant for the surrounding environment and the challenges outside the womb. Such components include immune cells, antibodies (especially IgA antibodies), pro- and anti-inflammatory cytokines such as TNF, IL-10, and TGF- β , and factors that may modify immune responses to bacteria, e.g., soluble CD14 (sCD14).

How can probiotic supplementation change the composition of breast milk? Well, nutritional, metabolic, and immunological processes in the gut are reflected in the mammary gland and the milk through the entero-mammary link [89]. In addition, the immunological composition of breast milk differs between mothers, and the reasons for these differences and the consequences for the breastfed infants are not fully elucidated yet. When *Lactobacillus reuteri* was supplemented to pregnant mothers from gestational week 32 during pregnancy, supplementation was associated with low levels of TGF- β 2 and slight and increased levels of IL-10 in colostrum. The slightly higher levels of IL-10 could be due to the reason that *L. reuteri* previously had been reported to induce IL-10-producing regulatory T cells in vitro [36].

9. Probiotics and epigenetic mechanisms?

Epigenetic modifications can alter the DNA sequence without heritable changes [92] and have been shown to be important in prenatal immune programming. Epigenetic modifications can alter the DNA compaction and open/close for gene transcription [92]. The most important mechanisms are posttranslational histone modifications and methylation of DNA CpG dinucleotide [92]. The methylation pattern is thus preserved with high fidelity through cell divisions, assuring preservation of cellular inheritance [93]. The epigenetic pattern varies between tissue and cell type, and also between individuals and over time, representing immune maturation, ageing and disease states. There are many examples that epigenetic 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 [94]. Once these islands are methylated, gene transcription might not occur. Once removed, the promoter allows interaction with various transcription factors and allows gene activation. In T-cells, epigenetics are important for differentiation [95]. Of course, there are several ways of keeping/removing the epigenome. The major regulatory enzymes of DNA methylation are DNA methyltransferases (DNMTs). There are different DNMTs that play unique roles in the DNA methylation process [94]. DNA methylation patterns may be responsible for the Th2 skewing in neonates. Possibly, hypermethylation of the IFNG promoter may restrict expression of the IFNG gene. A permissive epigenetic state at the IL13 locus has been described for human naive neonatal CD4 cells as consistent with the Th2 skewing of the immune system early in life [96, 97]. Prenatal environmental exposures may also alter gene

expression via epigenetic mechanisms, aiming to induce physiological adaptations to the anticipated postnatal environment, but potentially also increasing disease susceptibility in the offspring [98]. The maternal microbial environment could possibly influence infant immune maturation [13, 14, 20] and T effector and T regulatory immunity [17, 28]. Th1, Th2, and Th17 differentiation is controlled epigenetically [95, 99, 100], and human T regulatory cell differentiation needs demethylation of the FOXP3 promoter [101]. Also, the immunological interaction between the mother infants is close during pregnancy [102, 103]. Children growing up in a traditional farming environment had a lower risk of respiratory allergic disease later in life, as discussed previously. There is increasing evidence that at least some of the protective effects are mediated through epigenetic modifications [16, 94].

10. Conclusion and future directions

Recently, the World Allergy Organisation wrote guidelines for probiotic use in prevention of allergic disease: World Allergy Organization-McMaster University Guidelines for Allergic Disease Prevention (GLAD-P): Probiotics [104]. After much research effort different strains of probiotics as supplementation during pregnancy and/or postnatal were used to prevent the development of allergic diseases in infants. There are some common guidelines to try to sum up the advances and bring them into practical guidelines in this field. Allergic diseases have a strong hereditary factor, the prevalence in infants without parents or siblings with allergic symptoms is 10% but reaches 20-30% if the first-degree has been made in trying to identify factors critical for allergy development. Of essential importance seems to be the gut microbiota. Colonization patterns differ between allergic and non-allergic infants, depending on delivery mode, and geographical factors influence this pattern. The gut microbiota and microbial environment have been reported to modulate immunity and may be one way to try to stop the escalating rate of allergic disease prevalence. WAO recommendations about probiotic supplementation for the prevention of allergy are intended to support parents, clinicians and other health care professionals in their decisions whether to use probiotics in pregnancy and during breastfeeding, and whether to give them to infants. "The WAO guideline panel determined that there is a likely net benefit from using probiotics resulting primarily from the prevention of eczema. The WAO guideline panel suggests: a) using probiotics in pregnant women at high risk of having an allergic child; b) using probiotics in women who breastfeed infants at high risk of developing allergy; and c) using probiotics in infants at high risk of developing allergy" [104].

Of course, there are many questions to answer and discuss. Several strains have been used in allergy prevention probiotic trials. There is a consensus that the strain is of importance, since different strains have different properties and also, as evident in allergy prevention trials, different outcome after use. Also, exactly when are the best "window of opportunity" and which population is the most susceptible for intervention methods?

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