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Probiotics and prebiotics in the primary prevention of allergic diseases

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ACADEMIC DISSERTATION

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To my family

Abstract

The rapid increase in allergic diseases in developed, high-income countries during recent decades is attributed to several changes in the environment such as urbanization and improved hygiene. Such changes are followed by diminished exposure to microbes during infancy. This relative lack of microbial stimulation is connected to a delay in maturation of the infantile immune system and seems to predispose especially genetically prone infants to allergic diseases. Probiotics, which are live, ingestible health-promoting microbes, may compensate for the lack of microbial stimulation of the developing gut immune system and may thus be beneficial in prevention of allergies. Prebiotics, which are indigestible nutrients for humans, promote the growth and activity of a number of bacterial strains considered beneficial for the gut. Probiotics and prebiotics administered together are called synbiotics and are believed to act synergistically.

In a large cohort of infants at hereditary risk for allergies we studied whether probiotics and prebiotics administered in early life prevent allergic diseases from developing. We also evaluated their safety and their effects on common childhood infections, vaccine antibody responses, and intestinal immune markers.

Between November 2000 and March 2003, we randomized for a double-blind placebo-controlled trial 1 223 pregnant women living in suburban Helsinki and carrying fetuses at increased risk for allergies. These mothers used a mixture of four probiotic bacteria (*Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii* JS) or a placebo, from their 36th week of gestation. Their infants received the same probiotics plus prebiotic galacto-oligosaccharides for 6 months. The 2-year follow-up consisted of clinical examinations and allergy tests (skin prick tests and serum antigen-specific IgE-antibodies), fecal and blood sampling, and regular questionnaires.

In the probiotic group compared to placebo, fecal recovery of lactobacilli (98% *vs.* 56%, $P<0.001$) and of bifidobacteria (98% *vs.* 86%, $P=0.039$) was more frequent, and fecal counts of probiotic bacteria were higher ($P<0.001$) at 3 and 6 months, showing the success of the intervention. However, recovery of the probiotics in the feces was transient, and no differences in the colonization patterns occurred at 2 years.

A total of 925 infants participated in the 2-year follow-up. The cumulative incidence of any allergic disease (food allergy, eczema, asthma, allergic rhinitis) did not differ significantly between the probiotic (32%) and the placebo (35%) groups. However, probiotics compared to placebo tended to reduce all atopic (IgE-associated) diseases (14% *vs.* 19%), (OR 0.71, 95% CI 0.50 to 1.00; $P=0.052$).

Eczema, which was the most common manifestation (88%) of all allergic diseases by age 2 years, occurred less frequently in the probiotic (26%) than in the placebo group (32%), (OR 0.74, 95% CI 0.55 to 0.98; $P=0.035$). The preventive effect was more pronounced against atopic (IgE-associated) eczema, which, of all atopic diseases, accounted for 92%. The incidence of atopic eczema in the probiotic group (12%) was significantly lower than in the placebo group (18%), (OR 0.66, 95% CI 0.46 to 0.95; $P=0.025$). The relative risk reduction of eczema was thus 26% and of atopic eczema 34%. To prevent one case of eczema, the number of mother-infant pairs needed to treat was 16, and to prevent one case of atopic eczema, was 19.

Probiotic treatment was safe without any undesirable outcome for neonatal morbidity, feeding-related behavior, serious adverse events, or growth. Probiotics seemed to enhance the child's resistance to respiratory infections. Fewer infants in the probiotic (23%) than in the placebo (28%) group received antibiotics during their first 6 months of life (P=0.049), and thereafter to age 2 years suffered from fewer respiratory tract infections (geometric mean 3.7 *vs.* 4.2; P=0.009).

Vaccine antibodies examined in 87 infants showed no adverse effects of probiotics on tetanus, diphtheria, or *Haemophilus influenzae* type B (Hib) vaccinations. In the probiotic group compared to placebo, the first Hib-vaccine dose more frequently produced protective (≥1µg/ml) Hib IgG antibody concentrations (50% *vs.* 21%; P=0.020), and the geometric mean Hib IgG concentrations tended to be higher (P=0.064).

Studying fecal immune markers in 237 infants, we discovered that high (upper tertiles) fecal immunoglobulin A (IgA) concentrations at age 6 months protected from atopic (IgE-associated) diseases by age 2 years. Probiotics led to increased fecal α1-antitrypsin (P=0.001) and calprotectin (adjusted P=0.045) concentrations and tended to augment fecal tumor necrosis factor-α (P=0.099) and fecal IgA concentrations (P=0.085).

In conclusion, although feeding probiotics to high-risk newborn infants showed no preventive effect on the cumulative incidence of any allergic diseases by age 2, they apparently prevented eczema. This probiotic effect was more pronounced among IgE-sensitized infants. The treatment was safe and seemed to stimulate maturation of the immune system as indicated by increased resistance to respiratory infections and improved vaccine antibody responses. Stimulation of the immune system maturation offered by probiotics may occur through induction of intestinal inflammation.

Contents

Abbreviations

α1-AT:	α1-antitrypsin
cfu:	colony-forming units
CG	cytosine-guanine oligonucleotide
CpG	cytosine-phosphate-guanine oligonucleotide
CI:	confidence interval
DTwP:	diphtheria, tetanus and whole cell pertussis vaccine
HbOC:	PRP conjugated to *Corynebacterium diphtheriae* toxin CRM19
Hib:	*Haemophilus influenzae* type B
Ig:	immunoglobulin
INF-γ:	interferon- γ
IL:	interleukin
OR:	odds ratio
PRP:	polysaccharide polyribosylribitol phosphate
PRP-T:	PRP conjugated to tetanus toxoid
SCORAD:	Severity Scoring of Atopic Dermatitis
Th:	T-helper cell
TNF-α:	tumor necrosis factor- α
TLR	toll-like receptors

List of original publications

I Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, and Kuitunen M. Probiotics and prebiotic galacto-oligosaccharides in the prevention of allergic diseases: A randomized, double-blind, placebo-controlled trial. Journal of Allergy and Clinical Immunology, 2007: 119:1;192-8.

II Kukkonen K, Savilahti E, Haahtela T, Juntunen-Backman K, Korpela R, Poussa T, Tuure T, and Kuitunen M. Long-term safety and impact on infection rate of postnatal probiotic and prebiotic (synbiotic) treatment: A randomized double-blind placebo-controlled trial. Pediatrics, 2008: 122:1; 8-12

III Kukkonen K, Nieminen T, Poussa T, Savilahti E, and Kuitunen M. Effect of probiotics on vaccine antibody responses in infancy--a randomized placebo-controlled double-blind trial. Pediatric Allergy and Immunology, 2006:17:6; 416-21

IV Kukkonen K, Kuitunen M, Haahtela T, Korpela R, Poussa T, and Savilahti E. High intestinal IgA indicates reduced risk for IgE-associated allergic diseases. Submitted.

1. Introduction

The marked rise in the prevalence of allergic diseases since the 1950's parallels the postindustrial changes in lifestyle, characterized by a high standard of hygiene and reduced exposure to microbes in daily environments (Beasley *et al.*, 1998; Bach, 2002; Latvala *et al.*, 2005). The "hygiene hypothesis," which claims that unhygienic conditions and infections confer protection against the development of allergies (Gerrard *et al.*, 1976; Strachan, 1989), is supported by wider epidemiological evidence (Alfvén *et al.*, 2006). Even so, heredity is a strong positive predictive factor for allergic diseases (Prescott *et al.*, 2003).

With progressive Westernization, colonization patterns of the gut microbiota have evolved (Adlerberth *et al.*, 1991, 2006). Gut microbiota, which offer the strongest microbial stimulation throughout life, are a prerequisite for the initial maturation of our largest immunological organ, which is the gut. A mature immune system separates pathogens from harmless microbial and food antigens and maintains immune homeostasis, disruption of which may result in allergic diseases (Penders *et al.*, 2007). Differences in the composition of gut microflora between allergic and healthy infants even before establishment of any allergic disease (Björkstén *et al.*, 2001), together with the pre-symptomatic immune system dysregulation among atopics, suggests that preventive procedures should be started in early life (Prescott *et al.*, 2003).

The idea of using lactic acid bacteria to promote health is ancient. In 1910, in his search for prolongation of life, a modern immunologist, Élie Metchnikoff, at the Pasteur Institute in Paris suggested that senility caused by detrimental metabolism of intestinal microflora may be inhibited by lactic acid bacteria in fermented milk (Metchnikoff, 1910). Yet decades elapsed before the beneficial bacteria—called probiotics, based on the Greek term "for life"—became a subject of intensive research. Prebiotics are non-digestible food ingredients which promote the growth and activity of probiotics in the gut (Gibson and Roberfroid, 1995).

Live probiotic bacteria offer some compensation for a lack of microbial stimulation to our immune system. In infants who are genetically at increased risk for developing allergies, perinatal administration of probiotics has stimulated maturation of the immune function (Walker *et al.*, 2006). Probiotics have ameliorated already manifest childhood allergies, particularly eczema (Majamaa and Isolauri, 1997). Their beneficial effects have been strain-specific and have shown a more pronounced emergence in children who are sensitized to common environmental or food antigens (Viljanen *et al.*, 2005c; Rosenfeldt *et al.*, 2003; Weston *et al.*, 2005; Sistek *et al.*, 2006). In the first study on preventing eczema in children at hereditary risk for allergy, probiotics showed great promise. *Lactobacillus rhamnosus* GG, given to pregnant mothers and to their newborn babies for 6 months from birth, halved the incidence of eczema by age 2 years. However, sensitization remained unaffected (Kalliomäki *et al.*, 2001b).

The present study was started in 2000 when no information on the possibility of preventing allergies by probiotics had appeared. Since then, information on probiotics has almost exponentially increased, including doubts about and criticism of the enthusiasm for probiotics. The main purpose of this study was to discover whether probiotics and prebiotics administered to a large cohort of pregnant mothers and to their newborn infants at hereditary risk for allergy could prevent the development of allergic diseases.

2. Review of the literature

2.1 Allergy

2.1.1 Definition

Allergy, atopy, and sensitization

The term "allergy" was originally introduced in 1906 by von Pirquet to denote an antigen-induced biological response which may lead either to immunity or to allergic disease. Today, allergy denotes immunologically initiated hypersensitivity reactions to harmless antigens (Kay, 2001). Classified on the basis of mechanisms involved, allergies are further divided into immunoglobulin (Ig)E-mediated and non-IgE-mediated reactions (Johansson *et al.,* 2004).

Before the discovery of IgE in 1967, atopy was applied to allergic conditions that occur in families (Johansson, 1967). Today the term "atopy" is applied to the hereditary tendency to produce excessive IgE antibodies in response to common environmental allergens, mostly proteins, and to develop typical symptoms such as eczema, asthma, or rhino-conjunctivitis. Any allergic disease associated with IgE-sensitization is atopic (Johansson *et al.,* 2004).

Sensitization refers to excessive production of IgE antibodies during common allergen exposure measured by skin prick tests or by serum antigen-specific IgE antibodies (Johansson *et al.,* 2004). Antigens and immune modulating proteins that cross the placenta may influence the developing immune system (Jones *et al.,* 2002), but primary sensitization already developing *in utero* has not been confirmed (Bønnelykke *et al.,* 2008). Sensitized infants are at increased risk for developing symptomatic allergies and for more slowly outgrowing their childhood allergic diseases. They are the most probable subjects of the "atopic march" leading from food allergies and eczema in early childhood to airway allergies at school age (Kusel *et al.,* 2005).

Eczema

Eczema is a chronic itchy skin condition accompanied by poorly demarked erythema with scaling, crusting, or lichenified surface changes on typical sites in children, including flexural creases, the cheeks, and the outer surfaces of the legs and arms (Williams *et al.,* 1994). Since 1892 when first described by Besnier, a wealth of names have been used for the condition (Novak *et al.,* 2003). It is the first and most common manifestation of allergic diseases in childhood, affecting 16% of Finnish children before the age of 5 years (Lehtonen *et al.,* 2003). In a Danish cohort of children born to mothers with asthma, the cumulative incidence of eczema reached 31% at age 1 year, 41% at 2 years, and 44% at age 3 (Halkjaer *et al.,* 2006). Indeed, majority of eczema starts during the first year of life, but most children outgrow their eczema before adulthood (Kusel *et al.,* 2005; Gustafsson *et al.,* 2000; Novak *et al.,* 2003).

According to the revised nomenclature for allergy for global use, atopic eczema denotes eczema in IgE-sensitized individuals (Johansson *et al.,* 2004). The atopic variant of eczema occurs early in life and is a strong positive predictive factor for later development of allergic asthma and rhinitis (Kusel *et al.,* 2005; Gustafsson *et al.,* 2000). The pathophysiology of eczema involves complex interactions between genetic, environmental, and immunological mechanisms which are not yet fully understood (Novak *et al.,* 2003). Eczema may be considered a major disorder with great social, emotional, and financial effects on any family. Intensive itching, sleeping disturbances, daytime tiredness and irritability of the child, as well as parents' exhaustion and helplessness are easily underestimated (Kemp, 2003).

Food allergy

Food allergy is an early manifesting allergic disease, affecting 3 to 6 % of infants under age 3. It accompanies approximately 35% of moderate to severe eczema (Sampson, 2003). Antigens responsible for food allergy show high stability in the gastrointestinal environment and are associated with regional dietary habits (Sampson, 2003). An estimated 5 to 15% of infants show symptoms related to cow's milk ingestion (Vandenplas *et al.,* 2007), but the incidence of cow's milk allergy confirmed by challenge tests is 2 to 3% (Vanto *et al.,* 2004). Cow's milk accounts for the majority of childhood food allergies in Finland, where the incidence of cow's milk allergy in a large prospective unselected birth cohort in the 1990's was 2% (Saarinen *et al.,* 1999a).

Relatively few food antigens (cow's milk, egg, wheat, soy, fish, peanuts, and tree nuts) account for most allergic reactions. Allergic reactions to food manifest on the skin (eczema, urticaria), in the gastrointestinal tract (vomiting, diarrhea, constipation, pain), and in the airways (wheezing, swelling), and may elicit life-threatening anaphylactic reactions (Sampson, 2003; Sicherer, 2002). The majority of these hypersensitivity reactions are mediated by IgE-antibodies. The delayed non-IgE mediated reactions are more benign in nature, and children grow out of them earlier. Most children (80%) recover from their food allergies before age 4 to 5 (Vanto *et al.,* 2004; Sampson, 2003).

Asthma

Childhood asthma is a heterogeneous disease featuring repeated attacks of airway obstruction (wheezing) triggered by exercise, allergens, and viral infections (Illi *et al.,* 2006). Depending on the region studied and diagnostic criteria used, prevalence of asthma symptoms varies worldwide from 2.5 to 36%, with a male preponderance (Beasley *et al.,* 1998; Martinez *et al.,* 1995; Weinmayr *et al.,* 2007). Of Finnish school children from 1994 to 1995, 4 to 7% reported ever having had physician-diagnosed asthma (Pekkanen *et al.,* 1997). According to the Finnish Social Insurance Institution's reimbursement registry, the prevalence of chronic asthma among Finnish children was 1.9% in 1994, 3.1% in 2002, and 2.6% in 2006 (Mäkelä *et al.,* 2008).

Wheeze under the age of 3 may be classified as transient or persistent, the latter representing asthma. Early transient wheeze in common viral infections relates to maternal smoking and to small airways at birth and is outgrown before school-age. Of infants with an early frequent wheeze, the 30 to 50% sensitized to food or to airborne

allergens are at increased risk for persistent asthma, which is characterized by airway inflammation and airway remodeling and results in impairment of lung function (Illi *et al.*, 2006). In addition to atopy, early predictive factors for persistent asthma at school age are maternal asthma, exposure to tobacco smoke, eczema, and hay fever (Castro-Rodríguez *et al.*, 2000; Høst and Halken, 2000; Linneberg *et al.*, 2006; Martinez *et al.*, 1995). Morbidity from chronic asthma and its costs are both high in the European Union (Illi *et al.*, 2006).

Allergic rhinitis

Allergic rhinitis is a disorder induced by IgE-mediated inflammation in the nasal mucosa. The symptom triage includes sneezing, nasal blockage, and mucous discharge recurrently during allergen contact (Bousquet *et al.*, 2008). Allergic rhinitis is a late event in the sequence of allergies and therefore relatively rare among children under age 2. Its prevalence in pre-school-aged children varies from 5 to 7% (Grize *et al.*, 2006; Wickman *et al.*, 2002). In 1994-1995, among 11 607 schoolchildren aged 13 to14 years in Finland the self-reported prevalence of rhino-conjunctivitis during the preceding year was 16 to 23% (Remes *et al.*, 1998). Allergic rhinitis causes major illness and disability, affects sleep, school, and social life, and because of substantial indirect costs, its economic impact is often underestimated (Bousquet *et al.*, 2008).

2.1.2 Increased prevalence of allergies

The prevalence of allergic disease shows a worldwide increase that affects particularly the high-income Westernized countries (Beasley *et al.*, 1998; Weinmayr *et al.*, 2007). Among Finnish young men during 1966 to 2003, the prevalence of asthma increased 12-fold from 0.3 to 3.4%, and allergic rhinitis increased from <0.1 to 8.9%, while the prevalence of eczema remained relatively stable (Latvala *et al.*, 2005). In 1994-1995, almost half the 11 607 Finnish teenagers questioned reported ever suffering from an allergic disease (Remes *et al.*, 1998). Because of their urbanization, developing countries may be awaiting this allergy epidemic (Viinanen *et al.*, 2007; Weinmayr *et al.*, 2007); however, areas of high allergy prevalence have noticed reversing trends and plateauing incidences (von Hertzen and Haahtela, 2005; Björkstén *et al.*, 2008).

2.1.3 Immunology of allergy

Fetal immunity, which is characterized by dominance of T helper (Th) 2-type signals and weakness of Th1-type signals, is a prerequisite for normal pregnancy. The immature antigen-presenting cell system of the fetus and inhibition of Th1-responses by placental cytokines restrain fetal rejection by the mother. In infants with atopic heredity, transition from the fetal Th2-dominance to adult Th1-type immunity is delayed (Martinez and Holt, 1999).

Postnatal maturation of the Th1-type immunity depends on microbial stimulation (Martinez and Holt, 1999). The immune system must distinguish exogenous pathogens from commensal microflora (Rakoff-Nahoum *et al.*, 2004), which are capable of

attenuating and terminating pathogen-induced pro-inflammatory signaling (Neish *et al.,* 2000; Kelly *et al.,* 2004). Antigen-presenting cells in the intestinal mucosa recognize conserved microbe-associated molecular patterns, such as lipopolysaccharides, endotoxin, lipoteichoicacid, peptidoglycans, and cytosine-phosphate-guanine oligonucleotide (CpG) sequences of bacterial DNA, through molecular recognition receptors termed Toll-like receptors (TLR) (Medzhitov *et al.,* 1997; Rakoff-Nahoum *et al.,* 2004). Activated antigen-presenting cells then release a variety of signaling mediators—cytokines—which communicate between the innate and the adaptive immunity and direct the differentiation of naïve T-cells to antigen-specific memory cells.

Bacteria captured by macrophages induce interleukin (IL)-12 secretion from antigen-presenting cells, which promote differentiation of naïve T-cells in a Th1-direction. Differentiated Th1 lymphocytes produce IL-2 and interferon–γ (IFN-γ) and support pro-inflammatory reactions against viruses and bacteria. IFN-γ also promotes B-cells in producing antigen-specific IgG2, which facilitates pathogen recognition and phagocytosis (Calder, 2007).

Differentiation of naïve T-cells to Th2 lymphocytes requires IL-4. Th2-responses are targeted against helmintic worms, and the hallmarks of their activation are IL-4, IL-5 and IL-13. Th2-cytokines activate eosinophils and promote B-cells to produce IgE. Regulatory T-cells suppress both Th1 and Th2 type pro-inflammatory responses through the anti-inflammatory cytokines IL-10 and TGF-β. During certain genetic predisposition and environmental exposures, skewing of the immunity in the Th1 or Th2 direction breaks the immune homeostasis, resulting in autoimmune or allergic diseases, respectively (Calder, 2007).

In neonates with allergic heredity, sustained Th2-skewed immunity maintains IgE-responses against harmless environmental antigens (Marschan *et al.,* 2008a; Holt *et al.,* 1992). Over-expression of IL-4 results in excessive production of IgE, and IL-5 activates eosinophils, which are important effector-cells in allergic reactions (Martinez and Holt, 1999). Defective anti-inflammatory control by regulatory cells is considered central to the failure to attenuate Th2-type responses (Ling *et al.,* 2004; Hawrylowicz and O'Garra, 2005; Dunstan *et al.,* 2005). Although helminth infections strongly induce Th2-type cytokines, they are not associated with allergy. Elevated anti-inflammatory cytokines such as IL-10, observed in chronic helminth infections, correlate inversely with allergies (Yazdanbakhsh *et al.,* 2002). The atopic phenotype is also characterized by immature Th1-type immunity distinguished by deficient INF-γ secretion (Tang *et al.,* 1994; Dunstan *et al.,* 2005) and delayed maturation of adaptive immunity in the light of poor IgG responses to vaccines (Arkwright *et al.,* 2000; Prescott *et al.,* 1998).

2.1.4 Heredity

Allergy is a multifactorial disease in which heredity is the strongest predictive factor. The risk for eczema increases 2-fold with one affected parent and 3-fold if both parents are affected (Novak *et al.,* 2003). Parental asthma elevates the risk for asthma 3- to 4-fold (Illi *et al.,* 2006; Martinez *et al.,* 1995). That allergic diseases and sensitization affect boys more frequently than girls (Linneberg *et al.,* 2006; Rönmark *et al.,* 2003) and that the concordance rates for allergy are higher in monozygotic (85%) than in dizygotic (21%) twins (Novak *et al.,* 2003) supports the important role of genetic

background. Genetics alone cannot explain the recent rapid increase in allergies, but genes influence the way environment modifies early immune responses (Eder *et al.*, 2004; Bruce *et al.*, 2008; Bisgaard *et al.*, 2008)

2.1.5 Environmental factors

Mode of delivery

Cesarean delivery has been associated with childhood food allergy more among children born to allergic mothers (Eggesbø *et al.*, 2003). Cesarean section is also associated with asthma at school-age, independent of heredity (Kero *et al.*, 2002). Controversial evidence showing no association between cesarean delivery and asthma or atopy exists, as well (Maitra *et al.*, 2004). The world-wide rising trend for cesarean section—17% of deliveries in Finland (Stakes, 2008), over 30% in the US, and up to 36% in Latin America (Hamilton *et al.*, 2007; Villar *et al.*, 2007)—may account for some of the recent increase in allergies.

Exposure to tobacco smoke

Maternal smoking during pregnancy and exposure to tobacco smoke in childhood leads to increased risk for wheezing and asthma (Jaakkola and Gissler, 2004; Linneberg *et al.*, 2006; Gergen *et al.*, 1998). In Finland, 17% of fetuses are exposed to tobacco in utero (Stakes, 2008), which together with consistent exposure to environmental tobacco-smoke have an adjuvant effect on IgE-sensitization of high-risk infants during their first 3 years (Lau *et al.*, 2002). Therefore, avoiding tobacco smoke exposure is effective and important in the primary prevention of allergies.

Exposure to indoor allergens

Views conflict of whether early exposure to indoor allergens affects the development of allergies (Chen *et al.*, 2008). Indoor exposure to cat or house-dust mites has elevated risk for sensitization under the age of 3 (Wahn *et al.*, 1997) but has no effect on asthma up to age 7 (Lau *et al.*, 2002). In high-risk children, avoidance of exposure to house-dust mites from birth fails to prevent asthma, eczema, or atopy at age 5 (Marks *et al.*, 2006). Keeping indoor pets may protect from asthma and atopy (Rönmark *et al.*, 2003; von Hertzen *et al.*, 2006). Keeping dogs indoor during early life associates with less wheeze later in life in children with non-asthmatic parents (Remes *et al.*, 2001), and multiple (≥2) indoor dogs or cats during the first year of life reduces risk for sensitization later in childhood (Ownby *et al.*, 2002). The risk for allergy related to pet-keeping varies with heredity, wider environmental exposure to pet allergens, and type of exposure (Svanes *et al.*, 2003).

Benefits of pet-keeping may be mediated by the increased exposure to microbes and endotoxin (von Mutius and Schmid, 2006). The protective effect of endotoxin on sensitization depends on a certain gene polymorphism (Simpson *et al.*, 2006). Early cat

exposure causes increased eczema in those with a weakened skin barrier due to loss of function in the gene coding filaggrin (Bisgaard *et al.,* 2008). That heavy allergen exposure induces tolerance by stimulating more IgG and IgG4 antibodies than IgE antibodies (Platts-Mills *et al.,* 2001) may explain the controversy over allergen avoidance in the primary prevention of allergies. Removing pets from the home or reducing daily house-dust-mite exposure is unlikely to prevent allergies from developing at population level (Remes *et al.,* 2001; Corver *et al.,* 2006; von Mutius and Schmid, 2006).

Nutrition

The role of breast-feeding in the emergence of allergies is controversial and depends on heredity and mothers' allergies. Meta-analyses have indicated that exclusive breast-feeding for 3 months reduces risk for rhinitis and asthma, especially in children with family history of atopy (Eigenmann, 2004). Early signs of allergic diseases may prolong exclusive breast-feeding, which may mask its protective effect or even show a reversed causation between breast-feeding and allergies (Lowe *et al.,* 2006). When the onset of allergic symptoms was also considered, breast-feeding for the first 4 months of life reduced risk for asthma, especially in children without an allergic heredity in Sweden (Kull *et al.,* 2004). In a large cohort of Finnish children, exclusive breast-feeding for 3 months protected infants with atopic heredity from sensitization and allergic rhinitis at age 4 years, whereas in children without atopic heredity the risk for symptomatic allergy increased (Siltanen *et al.,* 2003). Exclusive breast-feeding ≥4 months was beneficial to lung function at school-age among Arizona children, but was associated with decreased airflows among those whose mothers had asthma (Guilbert *et al.,* 2007). In an un-selected Finnish birth cohort, prolonged exclusive breast-feeding for more than 9 months was associated with increased incidence of eczema at the age of 20 (Pesonen *et al.,* 2006).

Secretory IgA, immune cells, cytokines, galacto-oligosaccharides, and fatty acids in breast milk modify early immune responses (Brandtzaeg, 2002; Wijga *et al.,* 2006). Low contents of regulatory cytokines or secretory IgA in breast milk have been associated with increased risk for allergic diseases and sensitization (Savilahti *et al.,* 1991, Savilahti *et al.,* 2005; Saarinen *et al.,* 1999b; Oddy *et al.,* 2003), and high content of ώ-3 long-chain fatty acids have been associated with reduced risk for symptomatic allergies (Wijga *et al.,* 2006). Breast milk provides numerous health benefits to newborn infants (Brandtzaeg, 2003), among them protection against allergies when fed exclusively for the first 4 to 6 months regardless of atopic heredity (Høst *et al.,* 2008). In primary prevention of allergies, prolonged exclusive breast feeding in high-risk families is, however, is unlikely to be beneficial (Eigenmann, 2004).

Elimination diets during pregnancy have failed to prevent allergies. Such diets during lactation may delay the onset of eczema, but the disease thereafter may be more severe (Greer *et al.,* 2008; Høst *et al.,* 2008). In high-risk infants, however, when early supplementary feeding is needed, use of hydrolyzed formula instead of regular adapted cow's milk may delay the appearance of allergies (Osborn and Sinn, 2006). Although avoidance of solid foods or cow's milk in the infant's diet for the first 4 months may be beneficial, delayed introduction of solid foods (>6 months of age) is unlikely to be beneficial (Greer *et al.,* 2008; Høst *et al.,* 2008). Unnecessary elimination of foods

exposes infants to a lack of protective nutrients and delays development of tolerance to food antigens (Kankaanpää *et al.,* 1999; Arvola and Holmberg-Marttila, 1999). For example, fish is rich in ώ-3 polyunsaturated fatty acids, which are considered anti-inflammatory. When the possibility of reverse causation between parental allergy or child's early symptoms and introduction of fish to the diet was considered, regular fish consumption during the first year of life did protect against allergies and sensitization (Kull *et al.,* 2006).

Antibiotics and vaccination

Any use of antibiotics during the first months of life has been related to later development of childhood atopy or wheezing (Alm *et al.,* 2008; Johnson *et al.,* 2005; Marra *et al.,* 2006). However, opposing evidence showing the lack of any effect of antibiotics during the first year of life on later development of asthma suggests that frequent antibiotic use is a marker of risk for being diagnosed with asthma (Celedon *et al.,* 2004). Evidence from large cross-sectional studies does not support childhood vaccinations as playing any role in the allergy development. Tuberculin vaccine containing bacterial antigens may even induce tolerance against allergens. Thus, refusal of vaccination does not reduce the risk for allergies, but unnecessarily exposes the child to serious infections (Grüber, 2005).

Westernization

Based on his epidemiological observations, Gerrard *et al.* postulated in 1976 that "atopic disease is the price paid by some members of the white community…for their relative freedom from diseases due to viruses, bacteria, and helminthes." Allergies affect especially the high-income Westernized countries with clean environments and life-styles with little exposure to microbes (Wold, 1998; Weinmayr *et al.,* 2007). In the former socialist countries of Europe, atopy and allergic diseases in children were rare but have increased because of progressive Westernization (von Mutius *et al.,* 1998). In 2003, among 7- to 16-year-old school children in two regions of Finnish and Russian Karelia, geographically related but with fundamental differences in living environments, sensitization, eczema, and allergic rhinitis, occurred 3-, 6-, and 14-fold more frequently in Finland (von Hertzen *et al.,* 2006; Pekkarinen *et al.,* 2007). In Russian Karelia, less allergic and less sensitized people are exposed daily to a greater diversity of microbes than are their Finnish counterparts (von Hertzen *et al.,* 2007).

Farming environment and anthroposophic lifestyle

Living on a farm in childhood has protected Finnish 18- to 24-year-old university students from allergic rhino-conjunctivitis and asthma, but a rural non-farm environment compared to an urban milieu was not protective (Kilpeläinen *et al.,* 2000). In 2001, among 6- to 13-year-old Finnish children living currently in a rural environment, asthma and sensitization were less common in farmers' than in non-farmers' children (Remes *et al.,* 2005). In a large European study, growing up on a farm

or leading an anthroposophic life-style protected against allergies and sensitization (Alfvén *et al.,* 2006).

An anthroposophic life-style is characterized by restricted antibiotic use and less childhood vaccination, and by consumption of fermented foods plentiful in lactic acid bacteria (Alm *et al.,* 1999; Alfvén *et al.,* 2006).

Farmers' children are exposed to large quantities of microbes. Exposure to endotoxin, which is a cell-wall structure in Gram-negative bacteria, is indicative of wider bacterial contact. Increased endotoxin exposure has been associated with living on a farm and with reduced risk for atopy and for asthma (Gereda *et al.,* 2000; von Mutius *et al.,* 2000; Braun-Fahrländer *et al.,* 2002) The protective effect of livestock against allergy is also believed to be mediated through increased exposure to endotoxin (Remes *et al.,* 2003; Riedler *et al.,* 2001). Consumption of non-pasteurized farm-milk may also mediate the protective effect of farm life against atopy (Perkin and Strachan, 2006). Polymorphism in the TLR2 gene confers protection against atopy in farmers' children (Lauener *et al.,* 2002; Eder *et al.,* 2004), and a recently discovered polymorphism in an asthma candidate gene modifies the effect of livestock contact on the development allergic symptoms (Bruce *et al.,* 2008).

Childhood infections

In 1989, Strachan observed a diminished risk for hay fever with rising birth order and hypothesized that the allergy epidemic is a consequence of reduced exposure to childhood infections. Termed the "hygiene hypothesis" (Strachan, 1989), it is supported by the finding of fewer allergic diseases and sensitization among children exposed to infections in big families or at day-care in very early life (Krämer *et al.,* 1999; Ball *et al.,* 2000; Kilpi *et al.,* 2002; Turner *et al.,* 2005). Controversially, in large birth cohorts, plentiful respiratory infections later in childhood are associated with more wheeze, seasonal rhinitis (Harris *et al.,* 2007), and eczema (Paunio *et al.,* 2006). Childhood infections may thus be one cause of the atopic phenotype, characterized by delayed immune maturation and a poor defense against infections (Wjst, 2004).

Heavy exposure to orofecal and food-borne microbes have been protective against respiratory allergies (Matricardi *et al.,* 2000), but genetic factors seem to modulate the effect (McIntire *et al.,* 2003). Wild-type measles associates with less atopy in African children (Shaheen *et al.,* 1996), but not in Finnish children (Paunio *et al.,* 2000).

Chronic helminth infections are associated inversely with atopy in developing countries (Yazdanbakhsh *et al.,* 2002). They seem to stimulate anti-inflammatory regulatory cytokines and thus suppress atopy (van den Biggelaar *et al.,* 2000). This is supported by treatment studies showing that eradication of chronic helminth infections leads to increased atopic reactivity, *i.e.,* skin prick test reactivity to house dust mites (van den Biggelaar *et al.,* 2004). In developed countries, no inverse association has been observable between helminth infections and atopy (Karadag *et al.,* 2006).

It seems that more than mild viral infections or improved domestic hygiene, it is fundamental changes in lifestyle, leading to the disappearance of serious infections and reducing exposure to microbes in our daily environment, which may lie behind the allergy epidemics (Wold, 1998).

Gut microbiota in general

The gut microbiota, with up to 100 trillion microbes, provides the greatest microbial exposure throughout life. Microbes occupy all available habitats and niches from the intestinal lumen to crypts and epithelial-cell surfaces with an increasing gradient from stomach to colon (Mackie *et al.,* 1999; Ley *et al.,* 2006). The gut microbiota undergo huge metabolic activity. Fermentation of their main substrates—dietary carbohydrates (starches, dietary fiber, unabsorbed sugars, and sugar alcohols)—produces short-chain fatty acids (acetate, propionate, and butyrate), carbon dioxide, and molecular hydrogen. Organic acids such as lactic acid are important intermediates of bacterial fermentation (Blaut and Clavel, 2007).

Culturing fecal samples reveals only a diminutive fraction of the estimated whole microbiota (Ley *et al.,* 2006; Flint *et al.,* 2007). To properly understand the microbial diversity requires modern culture-independent methods based on sequence comparison of nucleic acids (DNA or RNA) (Mackie *et al.,* 1999). Using 16S rRNA oligonucleotid probes, cutoffs of 95% and 98% similarity are applied to define genus and species, respectively, and unique sequence types to identify strains (Bäckhed *et al.,* 2005).

The sterile newborn gut is gradually colonized by environmental bacteria, which outnumber human cells in the whole body within weeks (Mackie *et al.,* 1999; Favier *et al.,* 2002). Facultative anaerobes (enterobacteria, coliforms, lactobacilli and streptococci) are first detected in the feces, followed by obligate anaerobes (bifidobacteria, clostridia and *Bacteroides*) in a week, and by other strictly obligate anaerobes after weaning, to accomplish a highly diversified flora (Harmsen *et al.,* 2000; Adlerberth *et al.,* 2006; Flint *et al.,* 2007). Only a few highly dominant phylogenetic groups appear in the gut microbiota, with *Bifidobacterium* predominance in infants. After the first year of life, the commensal flora with >500 predominantly anaerobe species becomes relatively stable. It fluctuates in response to antimicrobial treatment and—to a lesser extent—to dietary changes (Salminen *et al.,* 2005; Noverr and Huffnagle, 2004; Eckburg *et al.,* 2005). The host genotype also influences the composition of gut microbiota, in view of the more similar microflora between twins than between marital couples having a similar daily environment (Forchielli and Walker, 2005; Walker *et al.,* 2006).

Mode of delivery and the type of feeding influence the initial colonization (Adlerberth *et al.,* 1991, 2006). Term, vaginally born, and exclusively breast-fed infants acquire the most beneficial microbiota with few *C. difficile* and *E.coli* and numerous bifidobacteria and lactobacilli (Penders *et al.,* 2006). Bacteria in the mother's colon are the strongest determinant of the early colonization after vaginal delivery (Salminen *et al.,* 2005). Cesarean section delays fecal colonization of bifidobacteria, lactobacilli, and *Bacteroides* (Adlerberth *et al.,* 2006; Grönlund *et al.,* 1999) and may affect composition of the gut microbiota up to school-age (Salminen *et al.,* 2004b). Human milk oligosaccharides promote the growth and activity of bifidobacteria and lactobacilli (Harmsen *et al.,* 2000; Tuohy *et al.,* 2005), which more abundantly colonize breast-fed than formula-fed infants (Brunser *et al.,* 2006).

In Westernized environments, staphylococci have replaced traditional bacteria such as *Bacteroides* in the gut flora. Recovery of skin-origin staphylococci in the feces indicates the paucity of gut microbial species and late diversification of the gut microbiota associates with the Westernized life-style (Adlerberth *et al.,* 2006). In poor, unhygienic environments, the gut flora has a high diversity and a high turnover rate

(Adlerberth *et al.,* 1998). Such conditions, related to decreased risk for allergies, provide continuous exposure to extensive array of bacteria in drinking water and in the soil and constantly stimulate the immune system (von Hertzen *et al.,* 2007).

Gut microbiota in allergies

Gut microbiota are essential for the development of the entire immune system and for the maintenance of immune homeostasis (Cebra, 1999; Kelly *et al.,* 2005). Studies on animals have revealed that gut microbiota are essential for the diversification of the antibody repertoire (Lanning *et al.,* 2000), and that without gut microbiota, oral tolerance—peripheral immunological unresponsiveness to orally administered antigens—fails to develop (Sudo *et al.,* 1997). However, in germ-free animal models, the under-developed immune function is rapidly restored after introduction of even a single bacterial strain (Cebra, 1999).

Gut microflora of allergic and healthy infants differ. In two countries with differing prevalences of allergy (low in Estonia, high in Sweden), allergic infants, when compared to healthy infants, were less frequently colonized with lactobacilli and *Bacteroides*, and more often with aerobic bacteria such as skin staphylococci and coliforms (Björkstén *et al.,* 1999). The aberrant gut microflora in allergies has been confirmed by several studies (Sepp *et al.,* 2005; Kirjavainen *et al.,* 2001; Watanabe *et al.,* 2003), although similar gut microbiota have been observed between healthy and allergic infants in European cohorts (Kendler *et al.,* 2006). Prospectively studied, early fecal samples of infants who go on to develop allergies compared to those who remain healthy grow less enterococci, bifidobacteria, and *Bacteroides*, and more clostridia and staphylococci (Björkstén *et al.,* 2001). In a Finnish study of genetically allergy-prone infants, early gut microbiota also differed between those who went on to develop sensitization and those non-sensitized by age one (Kalliomäki *et al.,* 2001a). A recent prospective European multicenter study of over 300 infants found no association between time of acquisition of any particular bacteria assessed by cultivation and development of eczema or food sensitization. However, the incidence of eczema and use of antibiotics in early life varied noticeably between centers (Adlerberth *et al.,* 2007).

In conclusion, early microflora, which are affected by the host, by environmental microbial pressure, and by early events, seem to be related to development of allergies. Several studies indicate that the gut microbiota of infants who go on to develop allergy differ from the microbiota of those who remain non-allergic.

2.1.6 Current recommendations for allergy prevention

The World Allergy Organization Guidelines for Prevention of Allergy and Allergic Asthma defines prevention of immunological sensitization in the form of specific-IgE antibodies as primary, and prevention of symptomatic allergic diseases following sensitization as secondary. It recommends avoidance of exposure to tobacco smoke during pregnancy and in childhood for all infants, and exposure to inhalant allergens for high-risk children. Avoidance diets for pregnant or lactating mothers are not recommended, however (Asher *et al.,* 2004). The European Academy of Allergy and

Clinical Immunology Pediatric Expert Group of Dietary Prevention of Allergic Diseases concludes that breast-feeding is beneficial irrespective of atopic heredity. For high-risk infants it recommends exclusive breast-feeding for at least 4 to 6 months and hypoallergenic formulas for the first 4 months if breast milk is insufficient. No dietary restrictions are recommended after the age of 4 to 6 months (Høst *et al.,* 2008). Because avoidance has failed to prevent allergies effectively, new strategies are sought to establish tolerance. Among these are immune modulation with microbial products, including bacterial extracts, mycobacteria, lipopolysaccharides, and probiotics (Matricardi *et al.,* 2003).

2.2 Probiotics

2.2.1 Definition

Probiotics are "live microorganisms, which when consumed in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). They are a heterogeneous group of bacteria with specific biological activities. After being applied in veterinary medicine for decades, probiotics were adapted to human dietary and medical needs in the 1990s. Since then, a rapid increase has occurred in probiotic research as well as in the consumption of commercially available probiotic products (Salminen *et al.,* 2002; Saxelin, 2008).

Lactobacilli, bifidobacteria, and streptococci are the probiotics most commonly selected from among human microbiota or dairy-product starters. Lactobacilli, bifidobacteria, and propionibacteria belong to the lactic acid bacteria group, which comprise fermentative Gram-positive bacteria and produce lactic acid as their main fermentation product. The genus *Lactobacillus*, with over 80 known species, belongs to the phylum *Firmicutes*, with a low DNA cytosine-guanine oligonucleotide (GC) content. Lactobacilli are naturally present in human and animal intestines and in fermented vegetables or dairy products. *L. rhamnosus* GG, which was isolated from human feces by Gorbach and Goldin, is the most studied probiotic (Salminen *et al.,* 2005). The high GC-content genera *Bifidobacterium* and *Propionibacterium* belong to the phylum *Actinobacteri* (Ben Amor *et al.,* 2007). Bifidobacteria are natural residents of the human intestine, but propionibacteria occur naturally in dairy products and traditionally serve as cheese-starters. Propionibacteria exhibit many beneficial properties in the human gut, among them their bifidogenic effect and inhibition of undesirable colonization. Propionibacteria as probiotics have been used in combinations, but little is known of their immune-modulating properties when used alone (Leverrier *et al.,* 2003).

2.2.2 General properties

Survival and adherence

To be effective, probiotic bacteria must survive the acidic conditions of the stomach and must be resistant to bile salts and digestive enzymes (Salminen *et al.,* 2005). Probiotic bacteria actively metabolize and synthesize proteins in order to adapt to the environment (Mater and Corthier, 2004). In animal models, they demonstrate protein synthesis during gastrointestinal transit (Saxelin *et al.,* 2005). The requirement of viability has been challenged, however, since even fragments of probiotics or heat-killed bacteria have stimulated immune responses. In mice, heat-killed *L. rhamnosus* GG has led to increased antigen-specific systemic IgG and mucosal IgA in response to ovalbumin immunization (He *et al.,* 2005). Nevertheless, live bacteria have stimulated higher levels of pro- and anti-inflammatory cytokines than have heat-killed preparations (Cross *et al.,* 2004).

Adherence of probiotic bacteria to intestinal cells may be vital to their colonization and to their clinical effects, because strains that adhere stay longer on the intestinal epithelium and maintain contact with immune cells (Salminen *et al.,* 2005). For example, in diarrhea, although one non-adhering lactobacillus strain, *L. bulgaricus*, is ineffective, the adhering *L. rhamnosus* GG strain is effective (Saavedra *et al.,* 1994). *Lactobacillus* species share a limited number of common genes which mediate their attachment to mucin and to human intestinal cells (Saxelin *et al.,* 2005). Because the intestinal environment affects probiotic gene expression, strains adhering similarly *in vitro* may adhere differently *in vivo*. That orally administered non-pathogenic *E. coli* O83 attaches to epithelial cells and colonizes better in breast-fed than in formula-fed infants indicates that feeding affects bacterial attachment (Lodinova-Zadnikova *et al.,* 1991). Although culturing probiotic strains in the feces indicates their survival during intestinal transit, little else may be concluded regarding their adherence by cultivation studies (Alander *et al.,* 1999).

Colonization

Colonization of probiotics in the gut is temporary and may depend on prior antimicrobial therapy (Agarwal *et al.,* 2003; Bennet *et al.,* 1992). Probiotic bacteria are detectable in the feces for weeks to months after administration, but no permanent changes in overall colonization patterns occur. Although the early months of life are critical to the formation of the commensal flora, even neonatal administration of probiotics fails to ensure permanent colonization (Gueimonde *et al.,* 2006). *L. rhamnosus* GG given to pregnant mothers was discovered in the feces of vaginally born infants; however, bacterial counts were low, and the discovery was temporary (Schultz *et al.,* 2004).

Strain-specificity and combinations

The effects of probiotics are strain-specific. *In vitro*, *Lactobacillus* or *Bifidobacterium* species modulate cytokine expression of dendritic cells differently, and they may act synergistically or inhibit other species of the same genus in mixtures (Christensen *et al.*, 2002; Maassen *et al.,* 2000; Vinderola *et al.,* 2002). Moreover, *Lactobacillus* strains with similar *in vitro* survival, growth, and adherence profiles also induce immune responses differently *in vivo* (Reid, 2005); the *in vivo* functionality of different strains may therefore not be deduced from *in vitro* experiments (Flinterman *et al.,* 2007; Vinderola *et al.,* 2004).

Probiotic products are recommended to be administered in combinations, as these may be functionally more efficient than individual strains (Rolfe, 2000). Introducing several strains together enhances their probability to colonize intestinal niches, and the strains may complement each other's health effects. In children receiving antibiotic therapy, probiotic combinations of several *Lactobacillus* and *Bifidobacterium* strains have more effectively prevented antibiotic-induced dysbiosis, *i.e.*, detrimental metabolic effects and clinical gastrointestinal symptoms, than have single strains (Timmerman *et al.,* 2004). *L. rhamnosus* GG, *L. rhamnosus* LC705, *P. freudenreichii* ssp. *shermanii* JS, and *Bifidobacterium breve* Bb99 adhere and inhibit pathogen adhesion more efficiently when administered together in combination than individually (Collado *et al.*, 2006, 2007). *L. rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* have also synergistically inhibited the growth of yeasts and molds *in vitro* (Suomalainen and Mäyrä-Mäkinen, 1999). In animal studies, mixtures of gut microbial species have more effectively stimulated the immune system than have single strains (Lanning *et al.*, 2000; Kelly *et al.,* 2005).

2.2.3 Mechanism of action

Metabolic activity

Fermentation products of lactic acid bacteria (acetate, propionate, butyrate) provide energy for the gastrointestinal mucosa and in distant organs (Tuohy *et al.,* 2005). Probiotic bacteria contribute to host energy resources also by influencing host genes involved in nutrient uptake and energy metabolism (Kelleher *et al.,* 2002; Walker *et al.,* 2006). The metabolism of lactic acid bacteria lowers gastrointestinal pH, which improves degradation of food antigens to less allergenic fragments (Untersmayr *et al.*, 2005). Low pH, together with lactic acid and other bactericide metabolites, inhibits the adhesion, growth, and activity of pathogenic bacteria (De Keersmaecker *et al.,* 2006).

Barrier function

Similarly to commensal bacteria, probiotics provide a protective barrier and offer colonization resistance against pathogens by competing for nutrients and binding sites (Saxelin *et al*., 2005). Microbiota form mucus and biofilms, and influence expression of host genes involved in the maintenance of the barrier function, thus impeding passage

of food antigens and sensitization against them (Walker, 2008; Saxelin *et al.*, 2005). Reinforcement of the barrier function of the relatively permeable gut epithelium of neonates by probiotics may be beneficial, because intestinal permeability plays a role in the pathogenesis of food allergy and eczema (Arvola *et al.*, 2004). Probiotic bacteria *in vitro* strengthen epithelial-cell tight junctions (Saxelin *et al.*, 2005). In children suffering from eczema, *L. rhamnosus* and *L. reuteri* together have reversed the increased gut permeability (Rosenfeldt *et al.*, 2004), and in cow's milk allergy and eczema, *L. rhamnosus* GG, but not the combination of four probiotic strains, has reduced intestinal permeability (Kuitunen *et al.*, 2007). In addition, in preterm infants, bifidobacteria have reduced intestinal permeability (Stratiki *et al.*, 2007).

Modulation of immune responses

Th1–type responses

Probiotic bacteria induce pro- and anti-inflammatory responses through TLRs on the dendritic cells which reside throughout the gut epithelium, and modulate immune responses strain-specifically (Hart *et al.*, 2004; Walker, 2008). Peptidoglycans and lipoteichoic acid in the lactobacilli cell wall are recognized by TLR2, and CpG-motifs of bifidobacterial DNA are recognized by intracellular TLR9 (Cario *et al.*, 2007; Saxelin *et al.*, 2005). Stimulated dendritic cells then orchestrate maturation of Th1, Th2, and T regulatory cell responses (Medzhitov *et al.*, 1997; Walker, 2008) (Figure 1). *L. casei shirota* in mice induces Th1 type pro-inflammatory responses and attenuates Th2-type cytokine secretion (Matsuzaki and Chin, 2000). A variety of *Lactobacillus* and *Bifidobacterium* strains stimulated human peripheral mononuclear cells to produce pro-inflammatory IL-6 and tumor necrosis factor-α (TNF-α) (Miettinen *et al.*, 1996). *Lactobacillus* strains induce secretion of TNF-α through TLR2 and the NFκB-pathway (Matsuguchi *et al.*, 2003). *L. rhamnosus* GG and pathogenic *Streptococcus pyogenes* both activate the NFκB-pathway, but the pro-inflammatory response to *L. rhamnosus* GG is much weaker than to *Str. pyogenes* (Miettinen *et al.*, 2000; Veckman *et al.*, 2004).

In infants with atopic heredity, probiotics have stimulated the maturation of the delayed Th1-type immunity (Prescott and Björkstén, 2007). In infants with eczema and cow's milk allergy, *L. rhamnosus* GG reverses their defective IFN-γ secretion of peripheral mononuclear cells (Pohjavuori *et al.*, 2004), and clinical improvement of eczema in infants using *L. fermentum* is associated with increased IFN-γ responses (Prescott *et al.*, 2005). Probiotics may stimulate the maturation of the immune system by inducing a weak physiological inflammation (Viljanen *et al.*, 2005b), which is similar to the inflammation maintained by the indigenous intestinal microbiota (O'Farrelly, 1998; Viljanen *et al.*, 2005a). In atopic eczema, *L. rhamnosus* GG elevated circulating inflammatory markers such as proinflammatory IL-6 and C-reactive protein (Viljanen *et al.*, 2005b).

Regulatory T–cell responses

Selective probiotics induce IL-10-producing regulatory T cells *in vitro* by modulating dendritic cell function through intercellular adhesion molecules (Smits *et al.*, 2005). *In vitro*, lactic acid bacteria including *L. rhamnosus* GG have stimulated human mononuclear cells to produce IL-10 (Miettinen *et al.*, 1996; Kopp *et al.*, 2008a), and

bifidobacteria (in a mixture of eight probiotic strains) have induced IL-10 secretion in dendritic cells (Martino *et al.*, 2008). In children suffering from eczema, *L. rhamnosus* GG alone and in a mixture of four probiotic strains has raised serum IL-10 (Pessi *et al.*, 2000; Viljanen *et al.*, 2005b). In healthy infants with atopic heredity, *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve*, and *P. freudenreichii* JS in combination induced a low-grade inflammation characterized by elevation of serum C-reactive protein, IL-10 and a pro-inflammatory response resembling those observable in helminth infections (Marschan *et al.*, 2008b).

Humoral responses

The anti-allergic effect of *L. rhamnosus* GG has been suggested to be due to reinforcement of an IgA-mediated defense. IgA on mucosal surfaces participates in antigen elimination and maintenance of mucosal barrier function (Brandtzaeg, 2002). Initiation of IgA production during the first weeks of life is highly dependant on the gut microbiota (Harris *et al.*, 2006), and several probiotic strains are capable of stimulating IgA secretion (Cross, 2002). *L. rhamnosus* GG stimulates both circulating and secretory IgA systems in allergic children (Viljanen *et al.*, 2005a), and in healthy bottle-fed (Rautava *et al.*, 2006) or breast-fed infants (Rinne *et al.*, 2005).

In acute rotavirus diarrhea, *L. rhamnosus* GG induces circulating rotavirus-specific and non-specific IgA-secreting cells (Majamaa *et al.*, 1995; Kaila *et al.*, 1992). In addition to IgA, certain probiotics have promoted both mucosal and systemic IgM and IgG antibody responses to orally administered vaccines (Isolauri *et al.*, 1995; Link-Amster *et al.*, 1994; He *et al.*, 2000). In infants receiving fermented formula compared to conventional formula for 4 months, intestinal poliovirus-specific IgA following parenteral poliovirus vaccine increased concomitantly with fecal bifidobacteria (Mullie *et al.*, 2004). *Lactobacillus* strains have also modulated allergen- and vaccine-specific immune responses (Taylor *et al.*, 2006; West *et al.*, 2008).

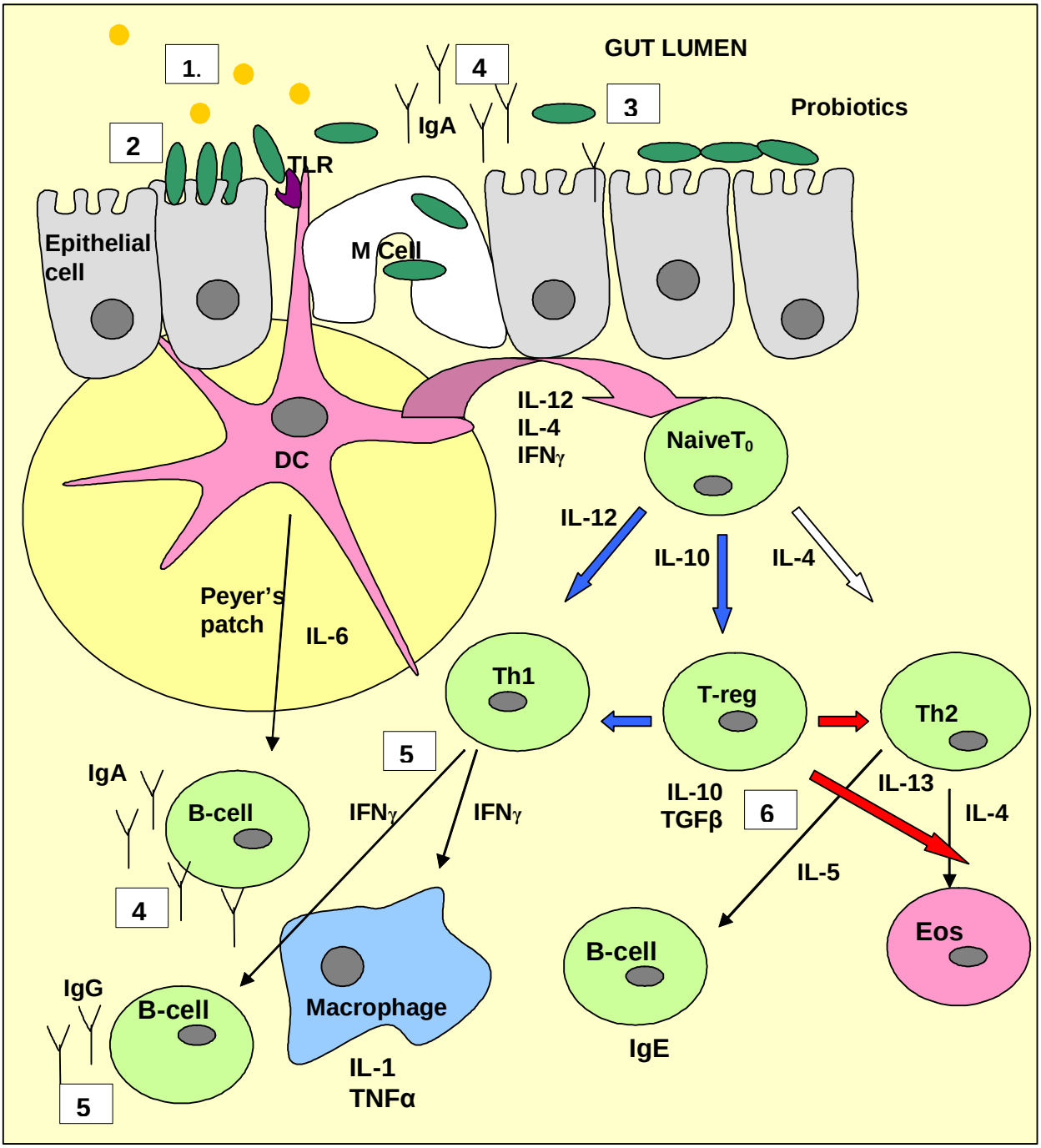


Figure 1 Mechanisms of probiotic action. 1. Metabolize facilitation of food degradation and bacteriosidic activity, 2. Competition with pathogens for receptor binding sites and substrates, 3. Increase in barrier function, 4. Increase in secretory and serum IgA, 5. Reinforcement of Th1 type immune responses, 6. Increase in regulatory activity to suppress Th2 type allergic responses TLR, Toll-like receptor, Th, T helper cell, Treg, regulatory T cell, DC, dendritic cell, MF, macrophage, IL, interleukin, Eos, eosinophilic leukocyte, IFN –γ, interferon- γ

2.2.4 Probiotics in general in pediatrics

Gastrointestinal disorders

The therapeutic effect of probiotics in children is well established in acute diarrhea. *L. rhamnosus* GG, *Str. thermophilus, L. reuteri*, and *L. acidophilus* have shortened the duration and reduced the severity of acute viral diarrhea (Kullen and Bettler, 2005; Guandalini *et al.,* 2000). In severe infectious diarrhea, however, probiotics may lack any effect (de Vrese and Marteau, 2007). The beneficial effect of probiotics in acute diarrhea depends on the dose, timing (early), and strain, with *L. rhamnosus* GG alone or in combinations showing the most consistent effect (Szajewska and Mrukowicz, 2001; van Niel *et al.,* 2002; Canani *et al.,* 2007). In the prevention of acute infectious diarrhea (Sazawal *et al.,* 2006) and antibiotic-associated diarrhea (Johnston *et al.,* 2006; Szajewska *et al.,* 2006) in children, certain probiotic strains have been effective. *L. rhamnosus* GG, *L. reuteri, L. casei*, or *Str. thermophilus* have successfully prevented infectious diarrhea in children attending day-care (Weizman *et al.,* 2005), in hospitalized children (Saavedra *et al.,* 1994), and in malnourished children with a high burden of gastrointestinal infections (de Vrese and Marteau, 2007). In Finland, with its low pressure of gastrointestinal infections, *L. rhamnosus* GG lacked any preventive effect among children attending day-care (Hatakka *et al.,* 2001).

Probiotics have showed promise in inflammatory bowel diseases and in necrotizing enterocolitis in preterm infants. They have also been beneficial in infantile colic (Rolfe, 2000; Savino *et al.,* 2007).

Respiratory tract infections

L. rhamnosus GG given in milk daily during 7 months reduced the number and severity of respiratory infections and days of absence of Finnish children in day-care (Hatakka *et al.,* 2001). A mixture containing *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve*, and *P. freudenreichii* ssp. *shermanii* JS given for 6 months to otitis-prone Finnish children lacked any preventive effect on the occurrence or recurrence of acute ear infection but led to increased nasopharyngeal carriage of *Moraxella* (Hatakka *et al.,* 2007). A formula containing *Str. thermophilus* plus *B. lactis* given to infants in day-care in the United States for 7 months lowered the frequency of antibiotic use (Saavedra *et al.,* 2004). Formulas substituted with *L. reuteri* or *B. lactis* during 3 months lacked any effect on respiratory illnesses in Israeli children in day-care (Weizman *et al.,* 2005), and *L. acidophilus* given to newborn Australian infants for 6 months lacked any effect on respiratory infections, but its use was related to increased antibiotic use (Taylor *et al.,* 2007).

Table 1. Probiotics in the treatment of eczema. SCORAD index: mild <25, moderate 25 to 50, severe >50 (SCORAD, 1993; Oranje *et al.*, 2007). *Lactobacillus rhamnosus* GG (LGG), hydrolyzed formula (HF), active treatment group (A), placebo (P)

Study (Country)	Placebo-controlled	N	Mean age (range)	SCORAD* at baseline	Sensitized %	Intervention (daily amount of probiotics in cfus)	Duration (weeks)	Change in SCORAD*
Majamaa *et al.* 1997 (Finland)	Yes	37	I ?(2 to 16) mo II 4 (0 to 8) mo	I Mild (23) II Moderate (26)	~ 30	AI: LGG $5x10^8$ /g of HF AII: LGG $4x10^{10}$ to nursing mothers P:HF	4	Reduced
Isolauri *et al.* 2000 (Finland)	Yes	27	5 mo	Mild (16)	?	AI: LGG $3x10^8$/g of HF AII: *B. lactis* Bb12 $1x10^9$ /g HF P:HF	8	Reduced
Rosenfeld *et al.* 2003 (Denmark)	Cross-over	43	5 (1 to 13) y	Moderate (38)	63	A: *L. rhamnosus* $20x10^{10}$ and *L. reuteri* $2x10^{10}$ powder P:Skimmed milk powder	6	Reduced in IgE-sensitized children
Viljanen *et al.* 2005 (Finland)	Yes	230	6 (1 to 12) mo	Moderate (33)	59	AI: LGG $10x10^9$ in powder, AII:LGG$10x10^9$, *L. rhamnosus* LC705 $10x10^9$, *B.breve* $4x10^8$, *P. freudenreichii* JS $4x10^9$ P:Microcrystalline cellulose	4	Reduced in IgE-sensitized infants
Weston *et al.* 2005 (Australia)	Yes	56	11 (6 to 18) mo	Moderate (42)	71	A: *L. fermentum* $2x10^9$ powder P: Maltodextran powder	8	Reduced
Brouwer *et al.*2006 (Netherlands)	Yes	42	4 (1 to 5) mo	Mild (24)	38	AI: LGG $3x10^8$ /g of HF AII: *L rhamnosus* $3x10^8$ /g of HF P:HF	12	No effect
Sistek *et al.* 2006 (New Zealand)	Yes	59	4 (1 to 10) y	Moderate (30)	100	A: LGG and *B lactis* $2x10^{10}$ powder P: Microcrystalline cellulose	12	Reduced in food IgE-sensitized infants
Fölster-Holst *et al.* 2006 (Germany)	Yes	53	4 (1 to 55) mo	Moderate (41)	38	A: LGG $10x10^9$ powder P: Microcrystalline cellulose	8	No effect
Grüber *et al.* 2007 (Germany)	Yes	103	7 (3 to 12) mo	Mild (24)	55	A: LGG > $10x10^9$ powder P: Microcrystalline cellulose	12	No effect

Table 2. Randomized placebo-controlled clinical trials on prevention of childhood allergies by probiotics. Active treatment group (A), placebo (P), colony-forming units (cfu)

Study (Country)	Number of patients	Treatment initiated	Follow up years	Recovery of probiotics in feces	Intervention amount of probiotics (cfu)	Outcome measures	Clinical effect of probiotics
Kalliomäki *et al.* 2001 (Finland)	High-risk A:77 P:82	Pregnant mothers and newborn babies	2, 4, 7	?	*L. rhamnosus* GG $1x10^{10}$ 2 to 4 weeks before delivery, 6 months after birth to lactating mothers, otherwise to bottle-fed infants	Eczema Sensitization	Reduced eczema at 2, 4, and 7 years No effect on sensitization
Taylor *et al.* 2007 (Australia)	High-risk A:89 P:88	Newborn babies aged <48hours	1	Lactobacilli 1 mo A:23% P:13% 6 mo A:22% P:36%	*L. acidophilus* $3x10^{9}$ daily for 6 months	Eczema Sensitization	No effect Increased sensitization
Abrahamsson *et al.* 2007 (Sweden)	High-risk A:95 P:93	Pregnant mothers and newborn babies	2	?	*L reuteri* $1x10^{8}$ from 36 gw to 12 months after birth	Eczema Sensitization	Reduced atopic eczema and sensitization
Kopp *et al.* 2008 (Germany)	High-risk A:50 P:44	Pregnant mothers and newborn babies	2	?	*L. rhamnosus* GG $5x10^{9}$ twice daily 4 to 6 weeks before delivery, 6 months after birth to lactating mothers, otherwise to bottle-fed infants	Eczema	No effect Increased current wheeze

2.2.5 Probiotics in the treatment of allergies

Airway allergies

Probiotic effects in airway allergies have been studied in adult patients. *L. rhamnosus* GG given to 18 Finnish birch-pollen-allergic adults for half a year, including the birch pollen season, had no effect on symptoms or need for medication (Helin *et al.*, 2002). Asian studies on seasonal allergic rhinitis have shown reductions in total and pollen-specific IgE with consumption of *L. gasseri* (Morita *et al.*, 2006), and improvement of symptoms with *L. acidophilus* (Ishida *et al.*, 2005a), *B. longum* (Xiao *et al.*, 2006), and *L. paracasei* (Wang *et al.*, 2004). Although yogurt containing *L. acidophilus* consumed during one month lacked any effect on asthma symptoms or lung function in atopic asthmatics, eosinophilia decreased, and IFN-γ tended to increase (Wheeler *et al.*, 1997). The studies on probiotics in airway allergies have been small, and no convincing evidence regarding any benefit exists, nor data on pediatric patients (Boyle and Tang, 2006).

Food allergy and eczema

L. rhamnosus GG in the treatment of eczema was first studied in 42 Finnish infants referred to a hospital for suspected cow's milk allergy in 1997 (Majamaa *et al.*, 1997). *L. rhamnosus* GG was given for one month to 11 breast-feeding mothers or directly to 15 infants receiving extensively hydrolyzed formula. In the control group, 16 infants received only extensively hydrolyzed formula. All infants were treated with emollients and topical corticosteroids, and cow's milk was eliminated from their diets. In the final analysis, 37 of 42 infants undergoing a positive cow's milk challenge after the intervention were included. Of these 37, the extent, intensity, and subjective symptoms of eczema, as measured by the SCORAD index (Oranje *et al.*, 2007; SCORAD, 1993), improved significantly in the 13 formula-fed infants receiving *L. rhamnosus* GG and in the 10 breast-fed infants whose mothers received *L. rhamnosus* GG. In the 14 control infants, the SCORAD index remained unchanged. However, at 2 months the eczema was comparably mild in both formula groups. Intestinal inflammation as measured by fecal TNF-α and α1-antitrypsin was attenuated significantly in the probiotic formula group compared to placebo (Majamaa and Isolauri, 1997). In another Finnish placebo-controlled trial, involving 27 infants suffering from eczema during exclusive breast-feeding, 9 were weaned onto extensively hydrolyzed formula, 9 infants onto the same formula with added *L. rhamnosus* GG, and 9 infants received the same formula with added *B. lactis* Bb12. After 2 months, in infants receiving the probiotic-containing formulas, severity of eczema fell significantly, whereas the placebo group showed no improvement; 6 months later, eczema had improved in all infants, with no difference between study groups (Isolauri *et al.*, 2000).

L. rhamnosus GG alone or in a mixture with *L. rhamnosus* LC705, *B. breve*, and *P. freudenreichii* JS given daily during one month to 230 Finnish infants referred to hospital for suspected cow's milk allergy provided no additional benefit over an elimination diet and intensive topical treatment. However, in the 136 IgE-sensitized

infants, *L. rhamnosus* GG alone, but not the mixture, reduced severity of the eczema (Viljanen *et al.,* 2005c). In two German studies, *L. rhamnosus* GG given for 3 months lacked any beneficial effect over placebo on mild-to-moderate eczema in 102 infants 3 to 12 months old (55% sensitized) (Grüber *et al.*, 2007) or when given for 2 months to 53 children aged 1 to 5 years, with 72% sensitized (Fölster-Holst *et al.,* 2006). In 42 Dutch infants aged 1 to 5 months, neither *L. rhamnosus* GG nor another *L. rhamnosus* strain given for 3 months in hydrolyzed formula was superior to hydrolyzed formula alone for eczema (Brouwer *et al.*, 2006). For 59 infants in New Zealand, the ameliorating effect of a 3-month supplementation of *L. rhamnosus* GG and *B. lactis* on eczema was confined to food-sensitized infants (Sistek *et al.*, 2006). *L. rhamnosus* and *L. reuteri* administered in relatively large quantities for 6 weeks in a cross-over manner to 43 Danish children (>60% sensitized) aged 1 to 13 years improved subjective symptoms of eczema, but this improvement was not shown in objective clinical symptom scores (Rosenfeldt *et al.*, 2003). *L. fermentum* given during 2 months to 53 Australian 6- to 18-month-old infants, 71% of whom were sensitized, ameliorated their moderate to severe eczema (Weston *et al.*, 2005) .

A meta-analysis performed on four treatment trials concludes that probiotics are unlikely to be beneficial in the treatment of eczema (Lee *et al.*, 2008). However, differences in probiotic preparations, in host genetics, and in environmental factors such as environmental microbial burden and diet may explain the varied results and make meta-analysis difficult to perform (Prescott and Björkstén, 2007). Table 1 summarizes randomized clinical trials on treatment of eczema.

2.2.6 Probiotics in the prevention of allergic diseases

In the first placebo-controlled randomized trial to prevent allergies in 132 genetically high-risk infants, *L. rhamnosus* GG was given first to pregnant mothers and thereafter to the lactating mothers or directly to non-breastfed babies during the 6 months after birth (Kalliomäki *et al.,* 2001b). At the age of 2 years, incidence of eczema halved in the *L. rhamnosus* GG group (23%) compared to the placebo group (46%) without any effect on sensitization against milk, egg, cat, or house dust mite as measured by serum antigen-specific IgE or against a panel of common food, pollen, and animal antigens in skin prick tests (18% *vs.* 14%). Although the beneficial effect on eczema was sustained at the 4- and 7-year follow-ups, neither respiratory allergies nor sensitization was reduced in the *L. rhamnosus* GG group (Kalliomäki *et al.,* 2001b, 2003, 2007). Recently, *L. rhamnosus* GG given to 105 pregnant mothers of high-risk infants for 4 to 6 weeks before delivery and to their infants during 6 months after birth compared to placebo neither reduced the incidence of eczema (28% *vs.* 27%) nor altered sensitization rates but was associated with an increased rate of recurrent wheezing episodes (26% *vs.* 9%) (Kopp *et al.*, 2008b).

L. acidophilus, not given during pregnancy but only to newborn infants of allergic mothers from age <48 hours to 6 months, failed to prevent allergies compared to placebo in the 12-month follow-up. Instead, the probiotic compared to placebo significantly elevated risk for IgE-associated eczema (26% *vs.* 14%) and sensitization against egg, cow's milk, peanut, house-dust mite, cat, grass, or mold as measured by skin prick tests (40% *vs.* 24%). Fecal colonization of bifidobacteria and coliforms assessed by a cultivation technique at ages one and 6 months did not significantly differ

between the study groups, but lactobacilli were more frequently detected in the probiotic than in the placebo group at one (23% *vs.* 13%) and 6 months (22% *vs.* 36%) (Taylor *et al.,* 2007). In a Swedish study, *L. reuteri* or placebo was given daily to pregnant mothers of high-risk families from 36 weeks of gestation until delivery and to their babies from birth until 12 months of age (Abrahamsson *et al.,* 2007). In the 188 infants completing the 2-year follow-up, the cumulative incidence of eczema was 36% in probiotic and 34% in the placebo group, and of atopic (IgE-associated) eczema was 17% *vs.* 28%, respectively. During the second year of life, atopic eczema was less common in the *L. reuteri* (8%) than in the placebo group (20%). The cumulative incidence of sensitization as measured by serum antigen-specific IgE against egg white and cow's milk or in a skin prick test against egg, milk, cat, birch, or timothy tended to be lower in the probiotic group (18% *vs.* 29%). The protective effect of *L. reuteri* was more pronounced in infants whose mothers were allergic; prenatal probiotic treatment of pregnant mothers was therefore considered noteworthy.

One retrospective observation concerns non-pathogenic *E. coli,* administered to 150 term and 77 pre-term infants in order to prevent colonization by pathogens and to stimulate immunity. Twenty years later, the infants colonized with the non-pathogenic *E. coli* compared to time-matched controls had fewer atopic diseases (Lodinova-Zadnikova *et al.,* 2003). Table 2 summarizes clinical trials on prevention of allergies by probiotics.

2.3 Prebiotics

2.3.1 Definition

The prebiotic concept was launched in 1995 by Gibson and Roberfroid. Prebiotics denote non-digestible, selectively fermented carbohydrate food ingredients beneficial to the host through stimulating the growth or activity of beneficial bacteria in the gut (Boehm and Stahl, 2007; Gibson and Roberfroid, 1995). Inulin is a naturally appearing prebiotic in vegetables; thus an adult diet including vegetables contains the recommended daily dose of prebiotics to maintain a balanced microflora. Fructo-oligosaccharides, which are among the most studied prebiotics along with inulin, are derived from natural sources through hydrolysis or manufactured enzymatically. Both inulin and fructo-oligosaccharides are polymers of D-fructose (Tuohy *et al.,* 2005). Prebiotic effects depend on solubility, distribution, and branching and length of the chains (Rossi *et al.,* 2005).

Human milk has been known for decades to promote bifidobacteria in the gut microflora of breast-fed infants. A carbohydrate assumed to be responsible for the bifidogenic effect was termed the "bifidus factor," which—when administered to bottle-fed infants—made their stools similar to those of breast-fed infants (Salmi and Huuhtanen, 1957). In human milk are plentiful galacto-oligosaccharides (7 to 12 g/L), which are today known to support the growth of bifidobacteria and beneficially modify stool consistency. The composition of human milk oligosaccharides is not reproducible by the food industry and is much more complex than the composition of bovine oligosaccharides. However, human and bovine milk oligosaccharides share an

important structure—β-glycosidically bound galactose—which protects the molecule against digestion in the small intestine (Boehm and Stahl, 2007).

2.3.2 Mechanism of action

Prebiotics are fermented by commensal bacteria, mainly by bifidobacteria, in the colon. More than direct immunological effects, prebiotics cause their effects through the metabolism of the bacteria they promote. The major products of prebiotic metabolism are short-chain fatty acids (acetate, propionate, butyrate) which may be anti-inflammatory (Tuohy *et al.,* 2005). Prebiotic fermentation results in increased hydrogen, carbon dioxide, and bacterial cell-mass (Cummings *et al.,* 2001). In bottle-fed infants, prebiotics facilitate the initial colonization by bifidobacteria (Haarman and Knol, 2005; Moro *et al.,* 2002) and result in a fermentation profile similar to that of breast-fed infants: increased short-chain fatty acids and lactic acid accompanied by lowered pH (Knol *et al.,* 2005). In bottle-fed infants receiving galacto- and fructo-oligosaccharides, fecal IgA tends to rise, which, however, fails to reach the level of breast-fed infants (Bakker-Zierikzee *et al.,* 2006). Stimulation of circulating and secretory IgA systems have also been reported with prebiotics (Bakker-Zierikzee *et al.,* 2006).

2.3.3 Prebiotics in allergies

In the treatment of eczema, prebiotics were effective in a French study comparing prebiotics and synbiotics, but the significance of either treatment is difficult to evaluate in the absence of a placebo group (Passeron *et al.,* 2006). A hydrolyzed formula containing fructo- and galacto-oligosaccharides 0.8g/100ml in the proportion 9:1, or hydrolyzed formula without prebiotics was given to 206 term infants with familial risk for atopy. The infants started to receive bottle-feeds during the first 2 weeks of life. At 6 months, infants receiving prebiotics had less eczema (10% *vs.* 23%) (Moro *et al.,* 2006) and had fewer infectious episodes than did infants receiving hydrolyzed formula alone (Arslanoglu *et al.,* 2007).

2.4 Synbiotics

Synbiotics combine efficacious probiotic strains with prebiotics to enhance the *in vivo* activity of the introduced probiotics and to stimulate beneficial microbial populations in the commensal flora (Collins and Gibson, 1999; Tuohy *et al.,* 2005). A synbiotic combining *L. casei* with dextran prevented development of eczema in mice and showed beneficial immunological effects in adult human patients suffering from pollen-induced rhinitis (Ogawa *et al.,* 2006). In a non-placebo-controlled trial, a synbiotic containing *L. rhamnosus* Lcr35 plus prebiotics was not superior to the prebiotic alone in the treatment of eczema (Passeron *et al.,* 2006).

2.5 Safety and regulations

Historical data support the safety of *Lactobacillus* and *Bifidobacterium*, which are natural residents of the human gut (Adams and Marteau, 1995; Reid, 2005). The major theoretical safety concerns of probiotic organisms are occurrence of bacteremia or endocarditis, transfer of antibiotic resistance, and detrimental metabolic effects such as production of D-lactate (Marteau, 2002; Snydman, 2008). As natural members of the indigenous gut flora, lactobacilli and bifidobacteria may cause opportunistic infections in immunocompromised patients. Although probiotic bacteria are not easily translocated in healthy subjects, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus* have been isolated from infection sites (Liong, 2008). *Lactobacillus* bacteremia or *Saccharomyces boulardii* fungemia may occur in elderly or critically ill patients with structural heart defects or indwelling catheters or in patients with short-bowel syndrome (Agostoni *et al.*, 2004; Reid, 2005; Snydman, 2008). Two cases of *L. rhamnosus* GG septicemia have been associated with the probiotic given to critically ill children after surgery to cure broad-spectrum antibiotic-induced diarrhea (Land *et al.*, 2005). *L. rhamnosus* GG has been safe in various populations including neonates, critically ill children, undernourished children, pregnant women, and immunocompromised HIV-infected adults (Salminen *et al.*, 2004a; Kullen and Bettler, 2005; Snydman, 2008). No *Lactobacillus* sepsis has occurred in the wealth of randomized clinical trials (Hammerman *et al.*, 2006), nor has increased consumption of *L. rhamnosus* GG, *L. acidophilus*, or *L. paracasei* augmented septicemias in population-based studies (Salminen *et al.*, 2002; Sullivan and Nord, 2006).

Natural antibiotic resistance of lactic acid bacteria to tetracycline, erythromycin, and chloramphenicol is encoded in antibiotic-resistance genes of plasmids, the transfer of which is rare (Snydman, 2008). The intrinsic vancomycin resistance of lactobacilli is not mediated by plasmids and therefore not horizontally transferable (Reid, 2005).

However, lactic acid bacteria residing in human intestine that is regularly exposed to antibiotics may acquire transferable antibiotic resistance (Mathur and Singh, 2005). Many lactobacilli and bifidobacteria are naturally susceptible to amoxicillin, which should be taken into account whenever it is used to prevent antibiotic-associated diarrhea (Charteris *et al.*, 1998). Bifidobacteria are naturally resistant to gentamicin, sulfamethoxazole, and polymyxin B. That few *Bifidobacterium* strains have been tetracycline-resistant warrants safety evaluation when selecting bifidobacteria for probiotic use (Masco *et al.*, 2006). Although no evidence exists of transferred antibiotic resistance associated with probiotic use, careful post-marketing surveillance is warranted (Klein *et al.*, 2000).

Probiotics should be selected from strains having neither detrimental metabolic effects nor production of D-lactate or toxins, nor deconjugation of bile salts or hemolytic activity (Marteau, 2002). Neither *L. rhamnosus* GG nor *L. rhamnosus* LC705 produces systemic-acidosis creating D-lactate. *L. rhamnosus* GG is non-toxic, and there is no lethal oral dose in mice (Snydman, 2008).

Some widely available probiotic products on the market in Europe have contained traces of β-lactoglobulin (Lee *et al.*, 2007). Such contamination with β-lactoglobulin has caused life-threatening allergic reactions in cow's milk-allergic individuals, emphasizing the importance of using products from guaranteed high-quality manufacturing processes (Moneret-Vautrin *et al.*, 2006).

L. rhamnosus GG has been well tolerated also in infants with low birth weight (Agarwal *et al.,* 2003). Healthy 3- to 24-month-old infants receiving a formula containing *S. thermophilus* and *B. lactis* for more than half a year compared to infants receiving regular formula grew normally, needed fewer antibiotics and less frequently suffered from infantile colic (Saavedra *et al.,* 2004). A synbiotic formula containing *B. longum* and galacto-and fructo-oligosaccharides was safe and well tolerated in neonates (Puccio *et al.,* 2007). In a randomized trial with 40 infants receiving regular formula or formula supplemented with *Str. thermophilus* and *L. helveticus* for the first 2 months of life, normal growth ensued in both groups. (Langhendries *et al.,* 1995; Agostoni *et al.,* 2004)

Prebiotics lack the traditional safety concerns of probiotics, because they are non-viable and present in regular food. High doses of prebiotics (in adults >20 g/day) have had laxative effects (Tuohy *et al.,* 2005) and because of production of gases as their fermentation products, unwanted flatulence is reported (Cummings *et al.,* 2001). Inulin and fructo-oligosaccharides have, in animal models, modified triglyceride and cholesterol levels (Pereira and Gibson, 2002). Galacto-oligosaccharides and polydextrose given to healthy neonates for 4 months were well tolerated, produced stool characteristics similar to those of breast-fed infants, and resulted in normal growth (Ziegler *et al.,* 2007).

The exact strain of each probiotic should be identified by internationally accepted methods (Reid, 2005), given a name in accordance with the International Code of Nomenclature, and filed in an internationally recognized culture registry (FAO/WHO, 2002). Probiotics are regulated mainly in the context of food additives, the health claims of which are more strictly regulated in the European Union than in the United States. In the European Union, probiotics are regulated within the context of the Novel Food Regulation EU 258/97. The European Food Safety Authority (EFSA) has proposed a Qualified Presumption of Safety status in food for *Lactobacillus* (including *L. rhamnosus*), *Bifidobacterium*, and *Propionibacterium* (particularly *P. freudenreichii* ssp. *shermanii*) species. In the US, certain probiotic strains have gained FDA approval as “Generally Regarded as Safe” (Liong, 2008). Any product marketed as for treatment or prevention of a disease should be registered as a drug and manufactured under the Good Manufacturing Practices standard (Reid, 2005).

3. Aims of the study

Our main objective was to discover whether a probiotic combination administered to pregnant mothers and to their newborn infants along with prebiotics during the 6 months after birth compared to placebo prevents childhood allergies from developing in infants with a hereditary risk for allergy. We aimed at discovering:

The effect of probiotics and prebiotics on the cumulative incidence of allergic diseases and sensitization at age 2 years

The long-term safety of probiotics and prebiotics and their impact on respiratory and gastrointestinal infections during the first 2 years of life

The effect of probiotics and prebiotics on vaccine-induced IgG antibody responses at 6 months against tetanus, diphtheria, and *Haemophilus influenzae* vaccines

Mucosal immune responses preceding the development of allergic diseases and sensitization, and the effect of probiotics and prebiotics on them

4. Material and methods

4.1 Study design

4.1.1 Participants

The randomized, double-blind, and placebo-controlled trial with two parallel groups was carried out at the Skin and Allergy Hospital of Helsinki University Central Hospital during the period November 2000 to March 2003. Pregnant mothers were recruited from antenatal clinics and through advertisements in local newspapers in the Helsinki suburban area with 900 000 inhabitants and with a birth rate of 13 000 per year. Mothers were eligible if one or both parents of the unborn child had a history of physician-diagnosed allergic disease (allergic rhinitis, eczema, or asthma), evaluated in telephone interviews by trained personnel. Pre-term infants (≤ 37 weeks of gestation) and infants with a major malformation were excluded. From multiple births only the first-born child was eligible (Figure 3).

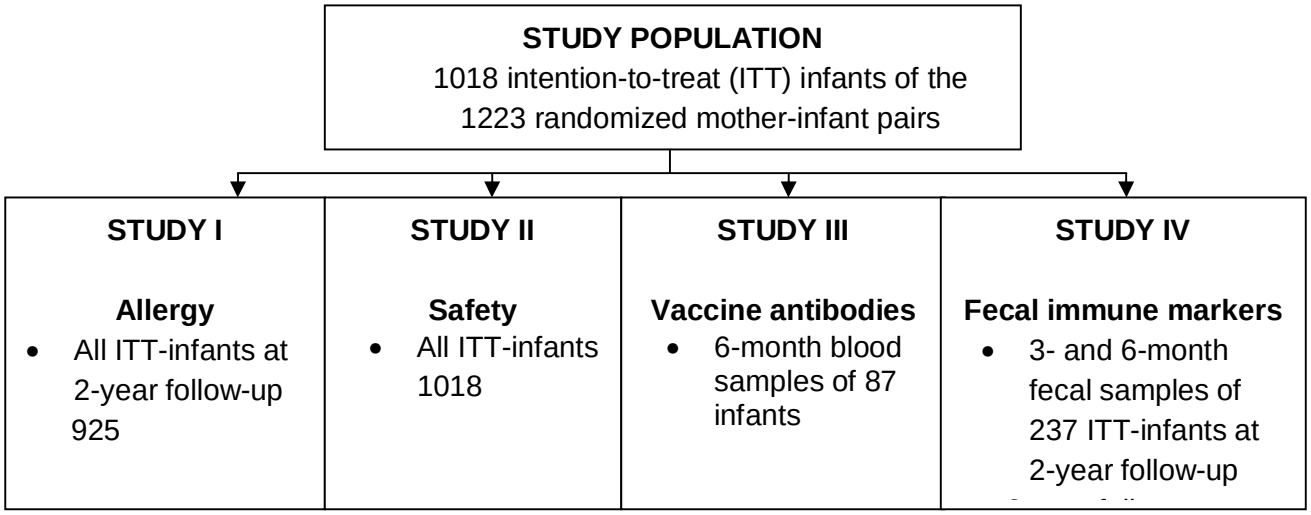


Figure 2 Study design: Populations in Studies I-IV

4.1.2 Intervention

From 36 weeks of gestation, mothers in the probiotic group took one capsule containing *Lactobacillus rhamnosus* GG (ATCC 53103) $5x10^9$ colony-forming units (cfu), *L. rhamnosus* LC705 (DSM 7061) $5x10^9$ cfu, *Bifidobacterium breve* Bb99 (DSM 13692) $2x10^8$ cfu, and *Propionibacterium freudenreichii* ssp. *shermanii* JS (DSM 7076) $2x10^9$ cfu twice daily. Selection of the strains was based on their availability and on current knowledge as to the safety and efficacy of individual strains or combinations. The supplier regularly checked the viability of the bacteria in the capsules, and the total amount of $\geq 10^9$ cfu viable bacteria throughout storage was guaranteed.

Each newborn infant received one opened capsule containing the same probiotics mixed with 20 drops of sugar syrup (22% glucose, 20% fructose, and 27% saccharose), containing 0.8g of galacto-oligosaccharides of bovine origin in liquid form once daily for 6 months after birth. In the placebo group, mothers and their infants took capsules containing microcrystalline cellulose, and the infants received sugar syrup without galacto-oligosaccharides, all scheduled exactly as in the probiotic group.

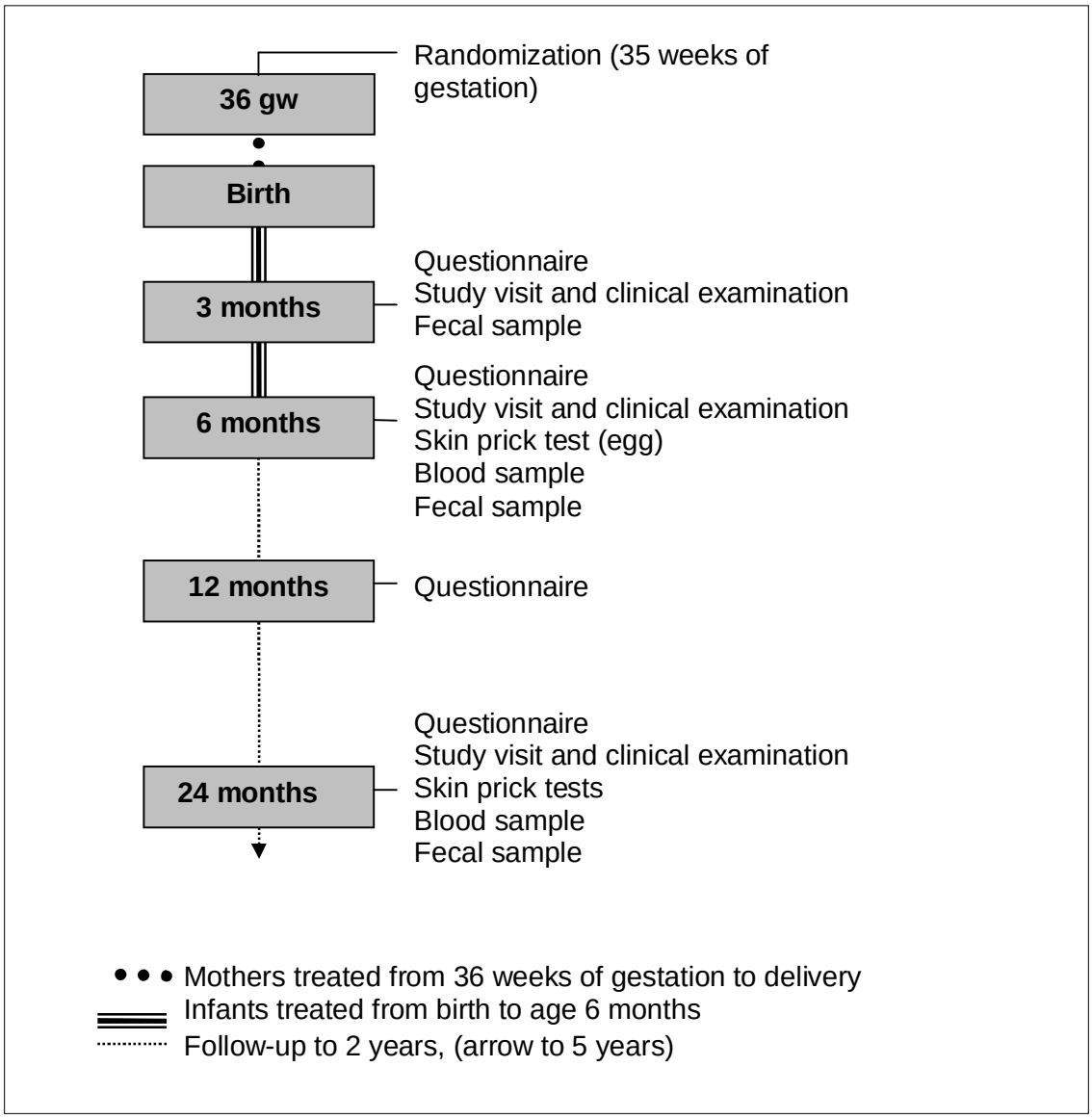


Figure 3 Study protocol. In the probiotic group, mothers received a mixture of *Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii* JS twice daily from 36 weeks of gestation up to the delivery. Their infants received the same mixture of probiotics and galacto-oligosaccharides. In the placebo group, mothers and their infants received capsules containing microcrystalline cellulose, and the infants received sugar syrup.

Parents received illustrated instructions to mix the probiotic powder with liquid (water, breast milk, or formula) in a teaspoon and were instructed to feed the mixture with a spoon. The products (supplied by Valio, Helsinki, Finland), which looked, smelled, and tasted identical, were delivered in numbered packages directly to the mothers for their use over a 4-week period, and for the infants' use during the first 3 months. The rest was supplied at the 3-month visit. The packages containing the capsules were stored frozen until opened, and then refrigerated. The parents were instructed to bring the remaining products to the study visits, and not to feed other probiotic preparations to the infants during the intervention. Compliance with the treatment was evaluated at the 3- and 6-month visits by asking the amount of doses not given and by counting the returned capsules. The mothers were encouraged to breast-feed, but if breast-feeding was insufficient, we recommended normal adapted cow's milk-based formula. The use of hypo-allergenic formula was restricted to infants allergic to cow's milk.

4.1.3 Vaccination

Vaccinations were given according to the Finnish national vaccination schedule. According to this schedule the infants received a DTwP vaccine (National Public Health Institute, Helsinki, Finland) consisting of 19 flocculating units of purified diphtheria toxoid, 5 flocculating units of purified tetanus toxoid, and 5×10^9 inactivated *Bordetella pertussis* cells at ages 3, 4, and 5 months. At 4 months, they received a *Haemophilus influenzae* type b (Hib) conjugate vaccine, consisting of 10 µg Hib capsular polysaccharide polyribosylribitol phosphate (PRP) covalently conjugated to tetanus toxoid (PRP-T) (Hiberix®; GlaxoSmithKline, Rixensart, Belgium), or to *Corynebacterium diphtheria* toxin CRM197 (HbOC) (HibTITER®; Wyeth-Lederle, Vaccines & Pediatrics, Philadelphia, PA, USA). During the study, the vaccine distributed to well-baby clinics by the National Public Health Institute of Finland changed from the HbOC to the PRP-T.

4.1.4 Study visits and questionnaires

Study visits occurred when the infants were 3, 6, and 24 months of age. The study pediatrician, blinded to group assignment, recorded the infant's history of symptoms related to allergic diseases, and clinically examined the infants. We advised the parents to contact the study nurses or the pediatrician in the event of adverse reactions during the intervention or if their child exhibited symptoms related to allergies such as eczema, urticaria, vomiting, excessive crying, wheeze, or persistent or exercise-induced cough between the study visits. Infants with suspected allergies were clinically evaluated. Parents were advised to bring the child's primary health-care chart to the study visits for recording vaccination dates and anthropometric measurements.

The parents completed non-validated questionnaires when their child was 3, 6, 12, and 24 months old. The questionnaires covered the respective periods 0 to 3, 3 to 6, 6 to 12, and 12 to 24 months. The first questionnaire covered pregnancy, birth, neonatal morbidity, feeding and feeding-related behaviors, and symptoms of infantile colic, parental education, and household size. In all questionnaires we inquired about the

number of infections and antibiotic courses, use of commercially available probiotic products, symptoms of allergic diseases, attendance at day-care, parental smoking, and household pets. The questionnaires were delivered by mail except the 3- to 6-month questionnaire, which was provided in person at the 3-month visit. The questionnaires were returned during the study visits (at 3, 6, and 24 months) or by mail (12 months) (Figure 3).

4.1.5 Diagnostic criteria of allergic diseases

Food allergy was defined as an adverse reaction (urticaria, eczema, gastrointestinal symptoms) in an open food challenge after improvement during a 2-week elimination diet (Bruijnzeel-Koomen *et al.*, 1995). Eczema was defined as an itchy skin condition and ≥3 of the following symptoms: familial history of atopic disease, dry skin during the last year, history of eczema, and visible eczema involving typical sites (Williams *et al.*, 1994). Severity Scoring of Atopic Dermatitis (SCORAD) was assessed at the study visits (SCORAD, 1993). Asthma was defined as ≥2 physician-diagnosed wheezing episodes, accompanied by persistent cough or exercise-induced symptoms (NAEP, 2002). Allergic rhinitis was defined as antigen-specific sensitization with a history of ≥2 symptoms of nasal discharge, blockage, sneeze/itch, recurrently during antigen contact (ARIA, 1994). Children with any positive skin prick test or any serum antigen-specific IgE >0.7 kU/l (Vanto *et al.*, 1999) at 2 years were sensitized. Any allergic disease in a sensitized infant was considered atopic (Johansson *et al.*, 2004).

4.1.6 Diagnostic tests and laboratory analysis

Skin prick tests

Skin prick tests were performed at the 6-month and 24-month visits on the volar surface of the forearm with separate sterile lancets (ALK-Abellò, Hørsholm, Denmark). We used histamine dihydrochloride (10 mg/ml) as a positive control and the solvent (glycerin) as the negative control (ALK-Abellò). The allergen extracts tested included egg white (1000 I.C. /ml; Stallergenes, Antony, France) at age 6 months and egg white, fish (1000 I.C. /ml; Stallergenes), cat, dog, birch, timothy (10 HEP; ALK-Abellò), cow's milk, and wheat grains (diluted in 0.9% sodium chloride) at 2 years. The wheal size was calculated as the mean of the longest diameter and its orthogonal diameter at 15 minutes. A wheal sized ≥3mm greater than the negative control we considered positive (EAACI, 1993).

Blood samples

A blood sample was drawn with parental permission at 6 months and at 2 years. Local anesthetic cream (EMLA®, Astrazeneca, Södertälje, Sweden) was applied to the puncture site prior to blood sampling. Centrifuged serum samples were stored at -40ºC.

At 2 years, serum allergen-specific IgE antibody concentrations against cow's milk, egg white, birch, timothy, dog, and cat were measured by immunoassay (ImmunoCAP® system, Pharmacia diagnostics, Uppsala, Sweden) with a detection limit of 0.01 kU/L. A concentration of >0.7 kU/l was considered positive (Vanto *et al.*, 1999). Serum total IgE was measured by the same immunoassay with a detection limit of 2 kU/l.

At 6 months, we measured serum toxin-neutralizing IgG antibodies against diphtheria and tetanus by a double-antigen ELISA with a detection limit of 0.007 IU/ml (Kristiansen *et al.*, 1997). All samples drawn ≥ 14 days after the third DTwP dose were eligible. We measured serum anti-capsular polysaccharide Hib IgG antibodies by enzyme immunoassay with a detection limit of 0.3 µg/ml. Samples that were drawn ≥20 days after the first and not later than 2 days after the second Hib vaccine dose were eligible. Oligosaccharides derived from Hib polysaccharides conjugated to human serum albumin (HbOHA, NIBSC, Potters Bar, UK) served as coating antigens, and serum pool lot 1983, received from the Centre for Biological Evaluation & Research (CBER), Bethesda, MD, USA, served as a reference (Mäkelä *et al.*, 2003).

Fecal analyses

For the fecal studies, parents took samples from their infants' diapers at home and froze (-18°C) the samples within 15 minutes and brought the frozen samples in screw-capped containers to the hospital, where they were stored at -40°C until analyzed.

To assess probiotic bacteria in the feces (I), fecal samples were collected from a randomly selected sample of 131 infants at birth, and at 3, 6, and 24 months. Concentrations of lactic acid bacteria were analyzed on MRS agar (LabM, International Diagnostics Group, Lancashire, UK), and of bifidobacteria on Raffinose-bifidobacterium agar (Hartemink *et al.*, 1996). Concentrations of the two *L. rhamnosus* strains were determined on MRS-vancomycin agar and the concentration of *P. freudenreichii* JS on modified YEL agar (Suomalainen and Mäyrä-Mäkinen, 1999). The detection limit was 103 cfu/g feces. The two *Lactobacillus* strains and *P. freudenreichii* JS strain were identified by random amplified polymorphic DNA (RAPD). The polymerase chain reaction was performed with the Dynazyme polymerase kit (Finnzymes, Espoo, Finland), and random primers 5'AGTCAGCCAC3' and 5'ACGCGCCCT3' were used for *L. rhamnosus* GG and LC705, and 5'CGAGCCGTC3' and 5'AGTCAGCCAC3' for *P. freudenreichii* JS. Primers were used at 3 µM and deoxynucleotides at 200 µM concentration. Initial denaturizing occurred at 94^{o}C for 2 min, and a further 40 cycles at 94^{o}C 15 s, at 37^{o}C for 30 s (for *L. rhamnosus* GG and LC705); at 30^{o}C for 30 s (for strain JS), and at 72^{o}C for 2 min. The RAPD bands were separated in 1.5% agarose by gel electrophoresis (Tynkkynen *et al.*, 1999).

To assess fecal immune markers (IV), samples taken from 237 infants at 3 and 6 months were homogenized with phosphate-buffered saline (PBS; w/v ratio 1:4) on a shaker for 30 min at +4°C and then centrifuged for 15 min at 10 000 g at +4°C. The supernatants were stored at -70°C for subsequent analysis. Fecal IgA concentrations were measured by enzyme-linked immunosorbent assay (see Viljanen *et al.*, 2005a). Microtiter plates were coated with anti-IgA antibodies diluted to 1:1000 in 50 mM NaHCO3 pH 9.5, 100 µl/well, and held overnight at +4°C. The coated plates were

washed in PBS and saturated with 2% bovine serum albumin PBS for 1 hour at room temperature before washing in PBS. Diluted (1% BSA-0.05% Tween 20-PBS) fecal extracts were incubated on the plates overnight and then washed 3 times (0.05% Tween-PBS). Anti-human IgA conjugated to alkaline phosphatase was added, incubated for 1 hour, and plates were made as described earlier (Savilahti *et al.*, 1993). The detection limit was 5µg IgA/L. Fecal α1-AT was determined by a single radial immunodiffusion method. The antiserum (Orion Diagnostica, Espoo, Finland) was diluted to 1:37.5 in the agar. Diffusion was allowed to occur for 1 wk at +4°C. The detection limit was 80 µg/g. TNF-α was measured by the Quantikine HS TNF-α Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA) with a detection limit of 5 pg/g. Fecal calprotectin was measured with an enzyme immunoassay (Phical Test; Valpro AS, Oslo, produced by NovaTec Immunodiagnostica, Dietzenbach, GmBH, Germany) with a detection limit of 9.75 µg/g. All samples were analyzed in duplicate.

4.2 Statistical analyses

4.2.1 Sample size calculation

The sample-size calculation of the main study was based on an expected 40% cumulative incidence of allergic diseases at the end of the 5-year follow-up. To detect a 10% absolute reduction (Odds ratio, OR, 0.64) by probiotics at 5% significance level and with 90% power, the estimated size of each group allowing a 20% drop-out was 597. The sample size in Studies III and IV was based on the number of available samples.

4.2.2 Randomization and blinding

The statistician performed computer-generated block randomization of the probiotic and placebo groups and sent a blinded randomization list (groups A and B) to the supplier (Valio Ltd). At 35 weeks of gestation, the supplier mailed the products for the mothers for their use during pregnancy and for the infants' use during 3 months. The supplier brought the products for the infants' use over 3 to 6 months to the hospital for the infant's 3-month visit. The randomization code was kept in a sealed envelope, retained by the database consultant, who recoded the letters A and B before each analysis. All comparisons between treatment groups were double-blinded. The database consultant revealed the randomization key to the statistician only after data management of the analyses. The clinicians and other study personnel had no access to the randomization codes before completing the 5-year follow-up.

4.2.3 Outcome measures

In Study I, the primary outcome measure was the cumulative incidence of any allergic disease or any atopic (IgE-associated) disease at the age of 2 years; the secondary

outcome measures were eczema and IgE-sensitization. Study II presents the long-term safety data of the intervention from birth to the age of 2 years. The outcome measures were neonatal morbidity, reasons for termination of the intervention, adverse events, growth (length, weight, and head circumference), incidence of infections, and use of antibiotics. In Study III, the outcome measures were mean, detectable, and protective antibody concentrations against diphtheria, tetanus, and Hib at the age of 6 months. In Study IV, the primary outcome was the association of fecal immune markers at 3 and 6 months with allergic diseases and IgE-sensitization at 2 years. Fecal immune markers served as dependent variables in relation to probiotic treatment and as independent variables in relation to allergic diseases and IgE-sensitization.

4.2.4 Statistical analyses

In Study I, logistic regression analysis served to compare the cumulative incidences of allergic diseases between the probiotic and the placebo group at the end of the 2-year follow-up. The results are given as ORs with 95% confidence intervals (CI). Potential confounders were first explored by the Breslow-Day test, and stratified analyses were then performed. Multivariable logistic regression was used to adjust the outcomes for possible confounders (gender, delivery, duration of breast-feeding, use of antibiotics, and use of probiotics after intervention). Fecal bacterial counts in the treatment groups were compared by Mann-Whitney U-test and colonization differences by the Chi-square test. The results are given as risk ratios and 95% confidence intervals.

To compare data with skewed distributions between the probiotic and placebo groups, we used the independent samples t-test on the logarithmically transformed data: serum IgE concentrations (I), the number of infections and antibiotic courses (II), vaccine antibody concentrations (III), and fecal immune markers (IV). The results appear as the probiotic: placebo ratios with 95% confidence intervals. For undetectable concentrations of vaccine antibodies (III) and of fecal markers (IV), we applied the detection limit divided by 2.

In Study II, the Chi-square test was used to compare categorized or dichotomized conditions between groups. Weight, height, and head circumference were converted to SD scores by Pediator® software using the data on Finnish children as the reference and then analyzed with the t-test for independent samples.

In Study III, univariate logistic regression served to compare the study groups with respect to the prevalence of detectable and protective Hib antibody concentrations. Potential confounding factors were tested by the Mantel-Haenszel method and Breslow-Day test, and then the group comparisons were adjusted by stepwise logistic regression. In the adjusted group comparisons, in the beginning, the study group was forced to the model with probability for variables to enter $F \leq 0.05$ and to be removed $F \geq 0.10$.

The nested case-control study design was applied in Study IV, where infants with an allergic disease or sensitization (cases) were compared with healthy controls (no allergic diseases and not sensitized). The immune marker concentrations (at 3 and 6 months) were divided into tertiles, and then their association with allergic diseases was analyzed by univariate logistic regression. The possible confounding variables (probiotic treatment, gender, duration of breastfeeding) were included as categorical covariates. The results are given as odds ratios with 95% confidence intervals. We used ANOVA for repeated measures to analyze time-effect and interaction, and analysis of

variance (2-ANOVA), where antibiotic treatment during the intervention was included as a categorical covariate. Correlations between fecal IgA and inflammatory markers were assessed separately for the 3- and 6-month measurements and for both measurements concomitantly by the correlation method according to Bland & Altman (1995).

Data were analyzed by SPSS, version 13.0 (I), 14.0 (II), 12.0 (III), or 15.0 (IV) (SPSS Inc, Chicago, IL, USA).

4.3 Ethics

Each mother signed a written informed consent which ensured that she could reject any part of the study when desired without consequences for her child's care. The ethics committee of the Hospital for Children and Adolescents of Helsinki University Central Hospital approved the study protocol. Financial support was provided by Valio Research and Development through the independent Helsinki University Hospital Research Institute, but without financial benefit to the researches.

Probiotics, which are generally considered safe, are being consumed in increasing amounts also during pregnancy and childhood in developed coutries. Prebiotics were administered in amounts too low to cause abdominal discomfort. The study products fulfilled the quality requirements for probiotic bacteria, and all infants were carefully monitored by the study group. The study pediatrician was available for consultations on the child's health and symptoms related to allergies during the whole follow-up period.

5. Results

5.1 Participants (I-IV)

Of the 1223 randomized mothers, 156 (13%) discontinued during pregnancy before the birth of the child. Of the newborn infants, 63 (including 14 B-twins) were ineligible (Figure 4). Of the 45 premature infants, 30 were born before initiation of the treatment, and 8 infants in the probiotic and 7 in the placebo group were born prematurely to mothers who had already begun the intervention. The intention-to-treat probiotic group included 506 infants, and the intention-to-treat placebo group included 512. Compliance for the intention-to-treat infants was similar in both our treatment groups with a mean 161 (±50) treatment days for the probiotic and 162 (±48) for the placebo group. The 80% compliance level (≥144 treatment days) was 88% in both groups. Seven infants in the probiotic and 13 on the placebo consumed commercially available probiotics during the intervention—against the protocol. No commercially available regular infant formula containing pro- or prebiotics was available. Of the intention-to-treat infants, a total of 939 (92%) infants completed the 6-month and 925 (91%) the 2-year follow-ups. All analyses were based on the intention-to-treat population. For infants' baseline characteristics for Studies I to IV, see Table 3.

Table 3. Baseline characteristics of infants in Studies I-IV

		Study I		*Study II*		*Study III*		*Study IV*	
	Total	*Probiotic*	*Placebo*	*Probiotic*	*Placebo*	*Probiotic*	*Placebo*	*Probiotic*	*Placebo*
Number of infants	925	461	464	506	512	47	40	120	117
Males (%)	49	50	49	50	49	55	58	52	43
Birth weight g (±SD)	3 593 ±479	3 595 ±477	3 591 ±482	3 595 ±483	3 593 ±484	3 580 ±417	3 566 ±471	3 584 ±499	3 676 ±500
Cesarean section (%)	17	17	17	17	17	11	15	19	22
Maternal smoking after pregnancy (%)	14	16	13	16	13	15	12	13	8
Partially breast-fed ≥6 months (%)	69	71	68	71	68	67	55	72	72
Duration of breast-feeding months (±SD)	8.4	8.6±5.4	8.2±4.9	8.6±5.4	8.2±5.0	8.3±5.4	8.0±5.2	8.8±5.2	8.9±4.9
Initiation of solid foods months (±SD)	4.0	4.0±0.8	4.0±0.7	4.0±0.8	4.0±0.7	4.0±0.8	4.0±0.7	3.9±0.7	4.0±0.7
Attending day care center <2 years (%)	50	50	51	50	51	57	54	52	50
First-born child in the family (%)	55	58	52	58	52	47	62	65	56
Maternal allergy (%)	81	80	81	81	81	74	80	78	79
Both parents allergic (%)	38	38	38	38	38	42	48	42	44
Mother's age years (±SD)	31.1	30.8	31.5	30.6	31.5	30.6	31.2	31.2	31.0

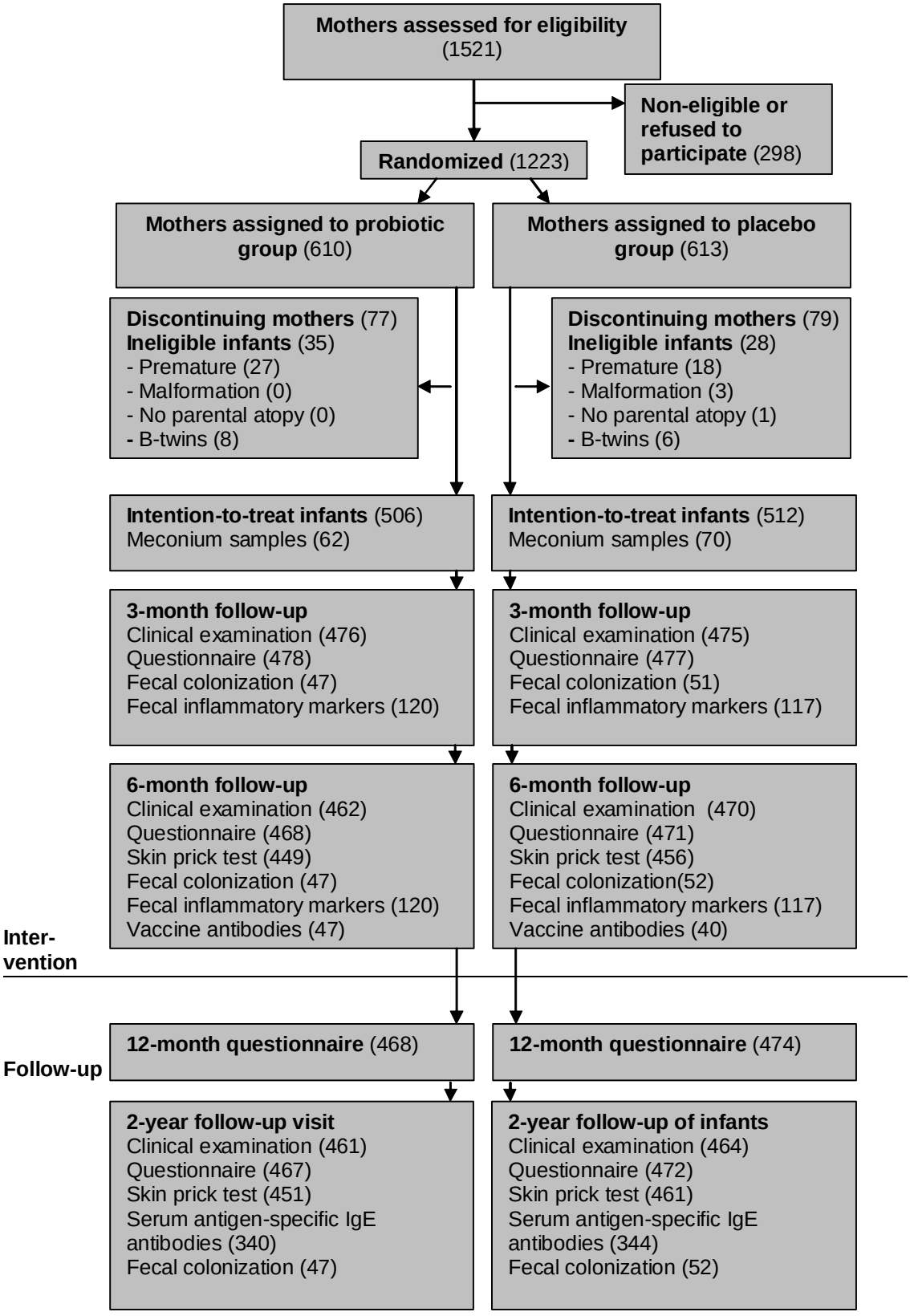


Figure 4 Flow of participants

5.2 Allergic diseases and sensitization (I)

5.2.1 Main outcome

The cumulative incidence of any allergic disease (food allergy, eczema, asthma or rhinitis) at age 2 years was 33%. The cumulative incidence of eczema was 29%, food allergy 8%, asthma 3%, and rhinitis 1%. An open food-challenge was performed on 13% of all infants whose food-related symptoms disappeared during a 2-week elimination diet.

Probiotic treatment compared to placebo had no significant effect on the appearance of any allergic disease by age 2. However, probiotic treatment compared to placebo tended to reduce atopic (IgE-associated) diseases (P=0.052). The outcomes were adjusted by means of multivariate logistic regression for sex, delivery by cesarean section, any breast-feeding for at least 6 months, and the use of antibiotics during the intervention, and regular (daily or ≥2 days a week) consumption of probiotics. Compared to placebo, the adjusted risk for any allergic disease by probiotics was OR 0.82 (95% CI 0.61 to 1.08; P=0.159) and for any atopic (IgE-associated) disease 0.65 (0.45 to 0.94; P=0.022). The relative risk reduction for any atopic disease by probiotics was 29% (adjusted 35%).

Of infants suffering from any allergic disease, 88% had eczema. The cumulative incidence of eczema was lower in the probiotic than in the placebo group, 26% *vs.* 32% (P=0.035). Atopic (IgE-associated) eczema occurred in 92% of children suffering from any atopic disease. The cumulative incidence of atopic eczema was significantly lower in the probiotic than in the placebo group, 12% *vs.* 18% (P=0.025). Compared to placebo, the adjusted risk for eczema with probiotics was 0.69 (0.52 to 0.93; P=0.015) and for atopic eczema 0.61 (0.42 to 0.90; P=0.012). The relative risk reduction for eczema was 26% (adjusted 31%) and for atopic eczema 34% (adjusted 39%). The number needed to treat was 16 for eczema and 19 for atopic eczema.

Between the probiotic and placebo groups, sensitization rates did not differ significantly: 28% *vs.* 31%. The adjusted OR was 0.82 (0.61 to 1.10; P=0.184) (Table 4).

Table 4. Main clinical outcomes (unadjusted) at the age of 2

Outcome	*Probiotic N=461* (%)	*Placebo N=464* (%)	*OR*	*95% CI*	*P*
Allergic disease	31.5	35.1	0.85	0.64 to 1.12	0.236
Atopic disease	14.0	18.8	0.71	0.50 to 1.00	0.052
Eczema	26.0	32.3	0.74	0.55 to 0.98	**0.035**
Atopic eczema	12.4	17.7	0.66	0.46 to 0.95	**0.025**
Sensitization	28.0	31.2	0.86	0.65 to 1.14	0.289
Food allergy	7.6	8.0	0.95	0.59 to 1.53	*0.902
Asthma	3.0	3.4	0.89	0.42 to 1.82	*0.853
Allergic rhinitis	1.3	1.5	0.86	0.23 to 2.58	*1.000

*Fisher's exact test

5.2.2 Other clinical parameters and sub-group outcomes

Sensitization rates in the two study groups as measured by skin prick test to egg white at 6 months of age were comparable. Serum total-IgE concentrations did not differ significantly between probiotic and placebo groups, but were significantly higher among boys than girls, geometric mean (95% CI) 33.6 (28.9 to 39.1) *vs.* 20.8 (17.8 to 24.4) kU/, OR 0.62 (0.50 to 0.77; $P<0.001$). Mean (95% CI) SCORAD index among infants with diagnosed eczema by age 2 years was 20.1 (17.5-22.6) in the probiotic and 20.5 (18.2-22.7) in the placebo group. Among boys, the relative risk reduction for atopic eczema was 41% (Table 5). In infants receiving exclusively breast-milk ≥4 months, some allergic disease occurred in 58 of 182 (32%) in the probiotic and 75 of 180 (42%) in the placebo group, OR 0.65 (0.43 to 1.01; $P=0.053$), and some atopic disease occurred in 22 of 181 (12%) *vs.* 38 of 179 (21%), OR 0.51 (0.29 to 0.91; $P=0.021$), respectively.

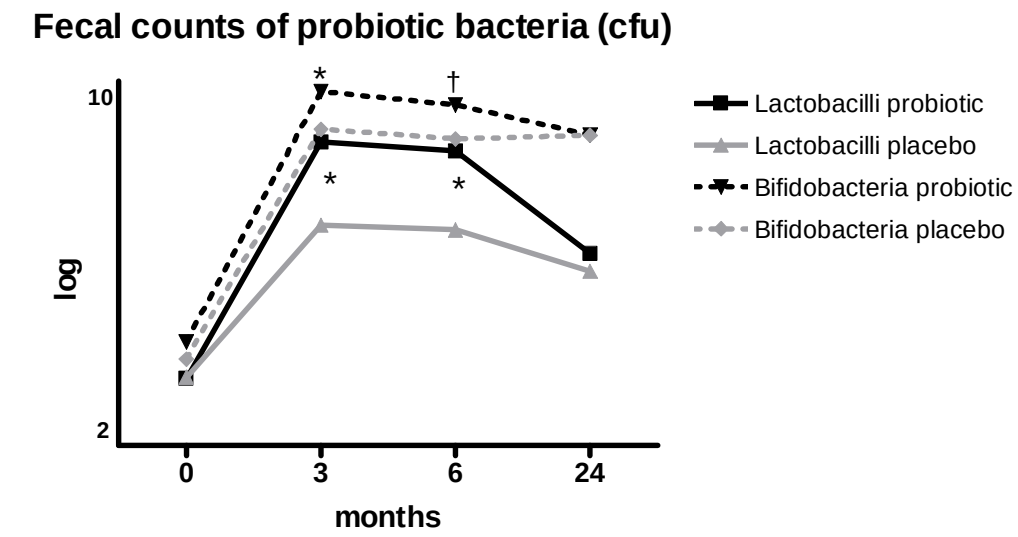
Table 5. Other clinical parameters and sub-group outcomes at age of 2 (years)

Outcome	*Probiotic N=461*	*Placebo N=464*	*OR*	*95% CI*	*P*
Egg SPT-positive 6 months (%)	12.7	13.6	0.92	0.63 to 1.36	0.556
Total IgE (kU/L)*	25.7 (22.2-29.8)	27.2 (23.1-32.0)	0.95	0.76 to 1.18	0.626
Total IgE (kU/L) in boys*	31.3 (25.2-39.0)	36.1 (29.3-44.4)	0.87	0.67 to 1.17	0.360
Sensitized boys (%)†	29.8 [67/225]	35.0 [79/226)	0.79	0.53 to1.17	0.268
Atopic eczema in boys (%)†	12.2 [28/229]	19.0 [43/226]	0.59	0.35 to 0.99	**0.046**

*Geometric mean (95%CI), † Sample size in brackets

5.3 Recovery of probiotic bacteria in the feces (I)

Meconium samples contained bifidobacteria in both the probiotic (18%) and the placebo (11%) group but contained no lactobacilli or propionibacteria. During the 6-month intervention, geometric mean amounts of fecal lactobacilli, bifidobacteria, *L. rhamnosus* GG, *L. rhamnosus* LC705, and *Propionibacterium freudenreichii* JS *shermanii* were significantly higher in the probiotic than in the placebo group (repeated measurements ANOVA, $P<0.001$ for each strain). At 2 years, however, fecal recovery of the studied probiotics in the two study groups was comparable (Figures 5 and 6).



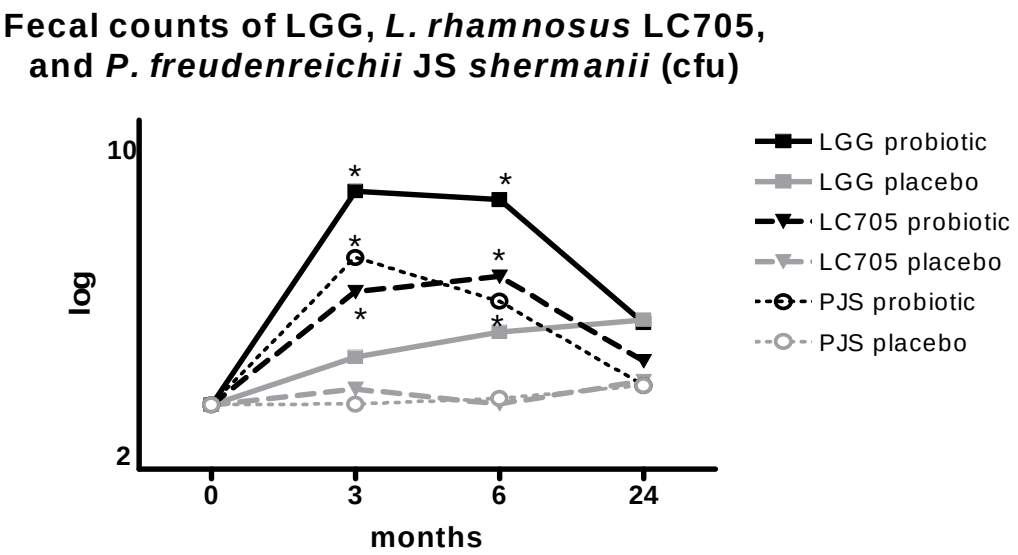


Figure 5 Recovery of probiotic bacteria in the feces. Total log10 counts of fecal a. Lactobacilli and bifidobacteria b. *L. rhamnosus* GG (LGG), *L. rhamnosus* LC705 (LC705), and *Propionibacterium freudenreichii* JS *shermanii* (PJS) in probiotic (black) and placebo (gray) groups. Significances between probiotic and placebo groups **P*<0.001, †*P*=0.002 . The number of samples in the probiotic group was 61 at birth and 47 at 3, 6, and 24 months, and in the placebo group 70 at birth, 51 at 3 months, and 52 at 6 and 24 months.

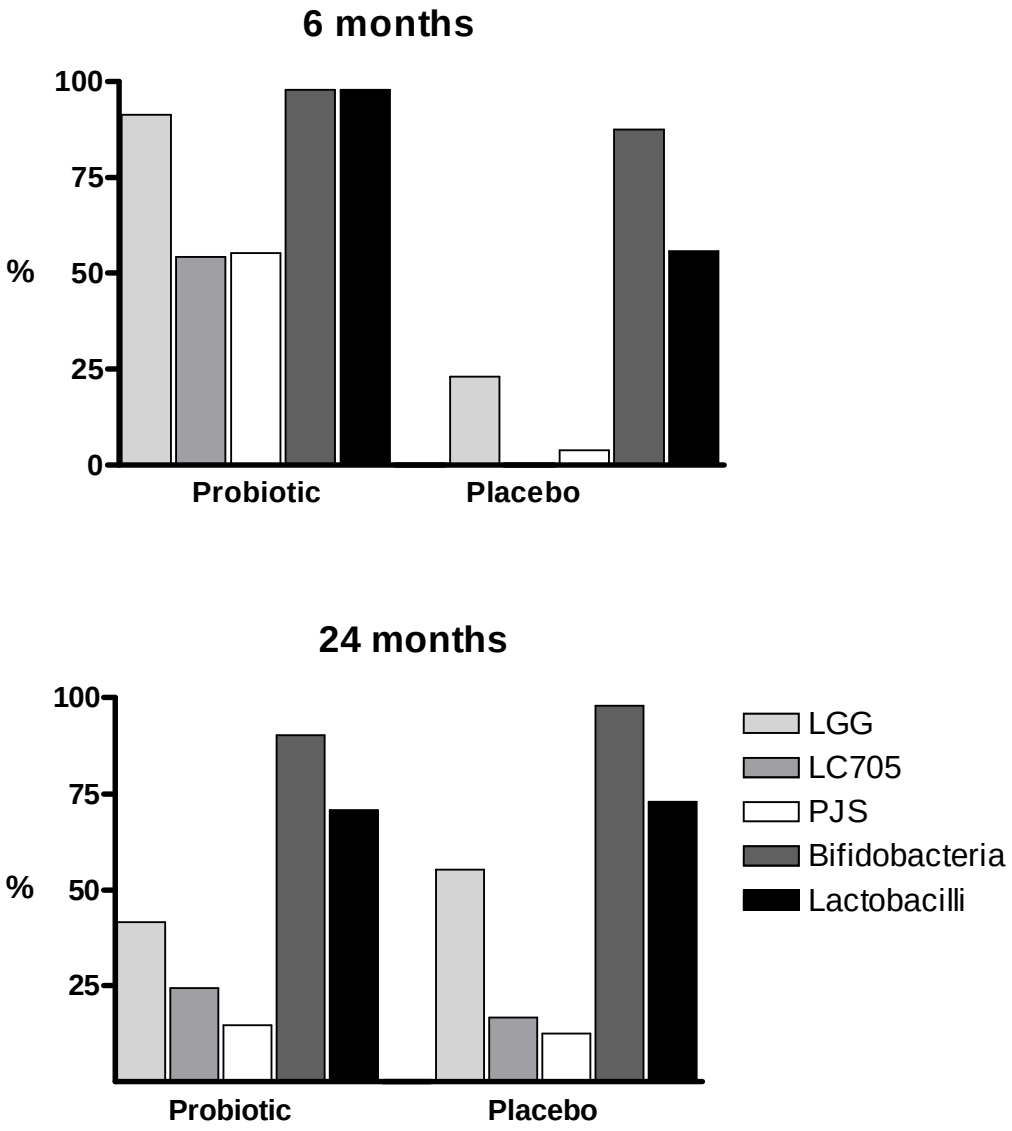


Figure 6 Prevalence of the probiotic bacteria, *L. rhamnosus* GG (LGG), *L. rhamnosus* LC705 (LC705), and *Propionibacterium freudenreichii* JS *shermanii* (PJS), in fecal samples at 6 and 24 months. Significances between probiotic and placebo groups were P=0.036 for all bifidobacteria and P<0.001 for the other bacteria. At 2 years, no significant differences occurred. The number of samples was 47 in the probiotic and 52 in the placebo group.

5.4 Safety (II)

5.4.1 Neonatal morbidity

Between the probiotic and placebo groups, neonatal morbidity, according to parental reports, was comparable (Figure 7) in terms of antibiotic treatment due to suspected or verified infection, jaundice requiring phototherapy, hypoglycemia requiring glucose infusion, or oxygen supplementation after birth.

5.4.2 Infantile colic and defecation

In both the probiotic and the placebo group, infantile colic (crying ≥4 hours per day, on ≥3 days a week) occurred in 4%. Crying ≥4 hours per day once or twice weekly occurred in 10%. Defecation of ≥3 times daily was less frequent in the probiotic group (18% *vs.* 29%; $P<0.001$).

5.4.3 Tolerance and adverse events

In the probiotic and placebo groups, feeding-related symptoms occurred similarly: abdominal discomfort (6.9 *vs.*7.2%), excessive vomiting (1.4% *vs.* 2.3%), and excessive crying (2.6 *vs.* 1.8%). Of all the families, 3.2% (3.2% *vs.* 3.3%) considered the treatment protocol difficult to follow, and for 0.6% (0.4% probiotic, 0.8% placebo) infants they discontinued the treatment due to major difficulties in swallowing of the powder. One infant who experienced a choking event associated with ingestion of the powder recovered completely. In both study groups, symptoms causing discontinuation of the intervention were equally frequent. Any reason for hospitalization after discharge from the maternity hospital up to 2 years of age was unlikely to be related the intervention (Figure 8).

5.4.4 Growth

Infants in both the probiotic and the placebo group grew normally. The mean (±SD) length at 6 months was 68.4±2.4 cm in both groups, and at 2 years 88.4±3.2 cm in the probiotic *vs.* 88.6±3.1 cm in the placebo group. In these respective groups, the mean (±SD) weight at 6 months was 8.16±0.98 *vs.* 8.09±0.95 kg and at 2 years 12.8±1.5 *vs.* 12.8±1.4 kg. Head circumference mean (±SD) was 35.3±1.4 in the probiotic and 35.2±1.5 in the placebo group at birth, 43.9±1.3 in both of the groups at 6 months, and 49.4±1.5 *vs.* 49.5±1.7 at 2 years. Figures for length, weight, and head circumference between the probiotic and placebo groups at every measurement were comparable.

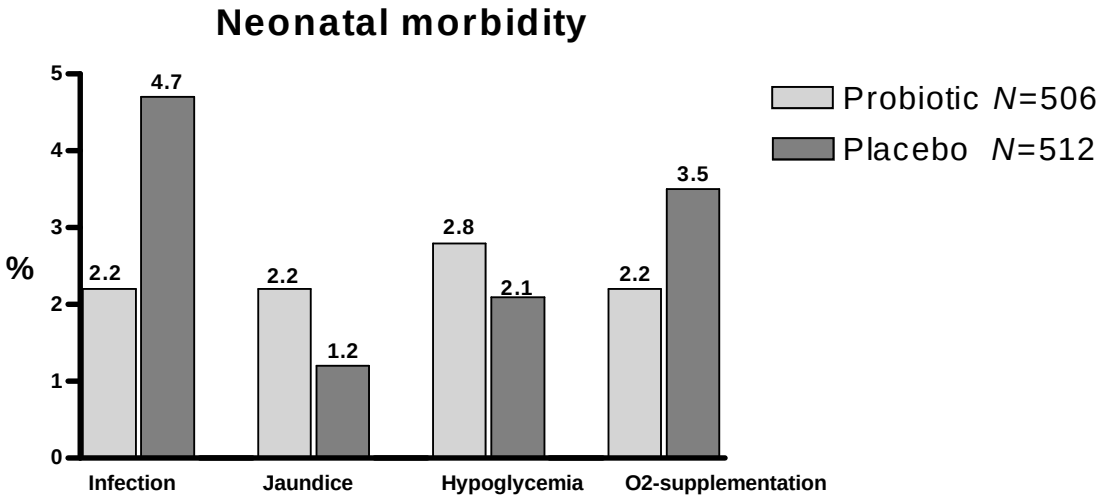


Figure 7 Neonatal morbidity according to parental reports among intention-to-treat infants Infection: received antibiotics for verified or suspected neonatal septicemia, jaundice: received phototherapy, hypoglycemia: received glucose infusion

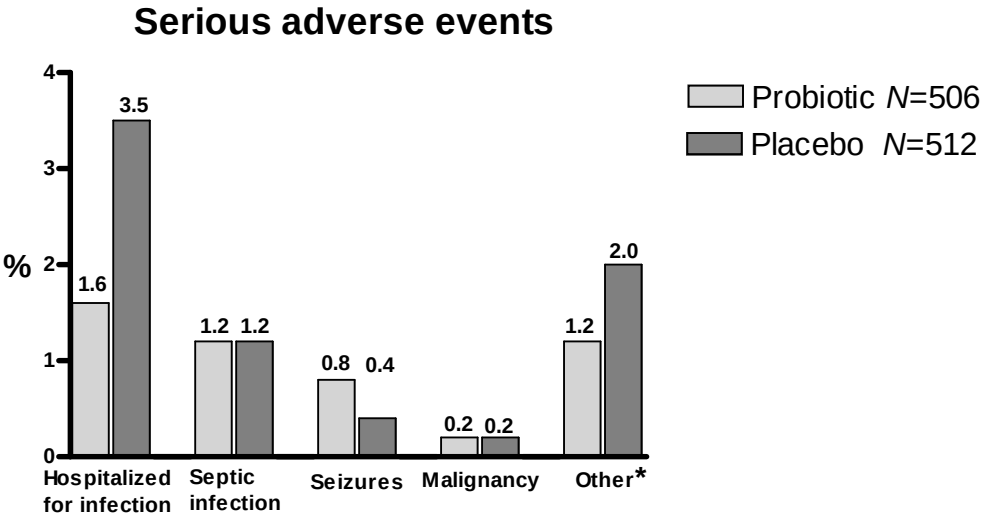


Figure 8 Serious adverse events according to parental reports during the first 2 years of life. *Other diagnosis included surgery (aortic coarctation, patent ductus arteriosus, ventricular septal defect, testis retention, cleft palate, ductus thyreoglossus), neonatal hepatitis, meconium plug obstruction, growth hormone deficiency, celiac disease, and gastroesophageal reflux

5.4.5 Common infections and antibiotic use

During the intervention (0 to 6 months), the proportion of infants in the probiotic and placebo groups suffering from any respiratory infection, middle ear infection, or

gastroenteritis was comparable (Table 6a). During the follow-up period between 6 and 24 months, the number of infants suffering from any respiratory infection was significantly lower in the probiotic group (P=0.023) (Table 6a). During the follow-up period between 6 and 24 months of age, significantly fewer episodes of respiratory infections in the probiotic than in the placebo group (P=0.009), and probiotics tended to reduce the total number of middle ear infections (P=0.068) (Table 6b). From birth to age 2, 99.5% of all children were reported to have suffered at least once from middle ear or other respiratory infection.

During the intervention (0 to 6 months), fewer infants received antibiotics in the probiotic (23%) than in the placebo group (28%) (P=0.049). However, after the intervention, most infants received antibiotics, with no significant differences between the probiotic (80%) and the placebo (83%) group (Table 6, Figure 9).

Table 6 a. Occurrence (at least once) of infections and antibiotic courses during the intervention (0 to 6 months) and after the intervention (6 to 24 months)

	0 to 6 months			***6 to 24 months***		
	Probiotic N=468 (%)	*Placebo N=471 (%)*	*P*	*Probiotic N=468 (%)*	*Placebo N=471 (%)*	*P*
Ear infection	15	19	0.179	72	76	0.204
Respiratory infection	66	68	0.678	93	97	**0.023**
Gastroenteritis	13	14	0.664	74	71	0.345
Antibiotic use	23	28	**0.049**	80	83	0.249

Table 6 b. Number of infections and antibiotic courses after the intervention from 6 months to age 2 years

	Probiotic N=468	*Placebo N=471*			
	Geometric mean	*Geometric mean*	*Ratio*	*95% CI*	*P*
Ear infection	1.7	1.9	0.89	0.78 to 1.01	0.068
Respiratory infection	3.7	4.2	0.87	0.79 to 0.97	**0.009**
Gastroenteritis	1.3	1.2	1.02	0.92 to 1.12	0.736
Antibiotic use	2.2	2.4	0.92	0.81 to 1.05	0.206

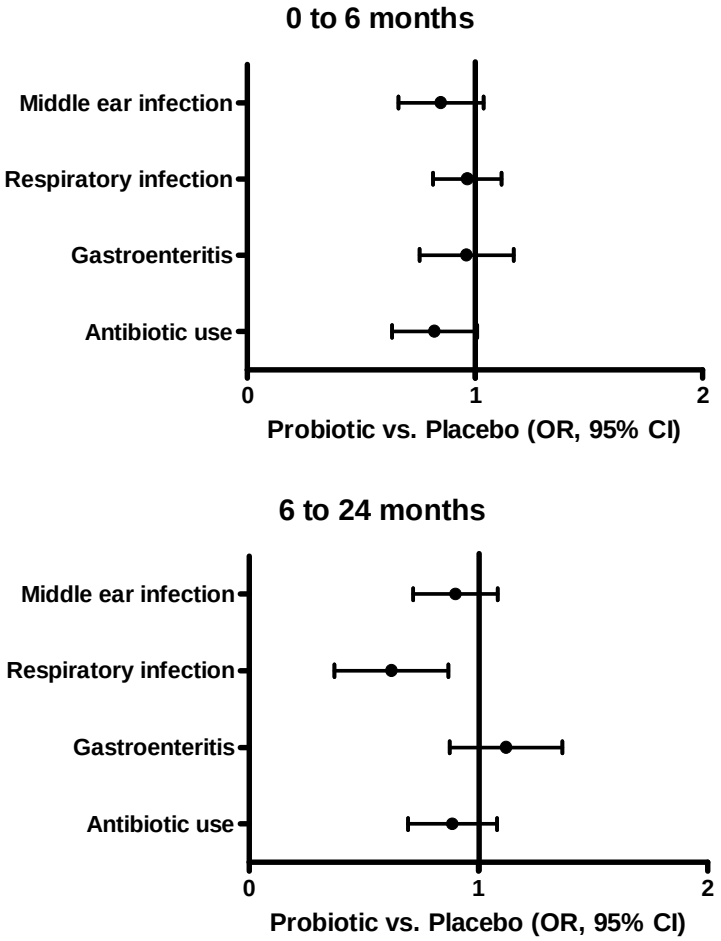


Figure 9 Risk for occurrence (≥ once) of infections and antibiotic courses during intervention (0-6 months) and during follow-up (6-24 months)

5.5 Vaccine antibody responses (III)

The probiotic treatment had no deleterious effect on vaccine antibody responses. In the probiotic group, the geometric mean Hib IgG concentration tended to be higher, but the difference was non-significant. Anti-Hib antibodies were detectable in 21 of 32 (66%) in the probiotic group, and in 14 of 29 (48%) in the placebo group (P=0.174). Protective anti-Hib IgG concentrations (≥1 µg/ml) occurred more frequently in the probiotic group, 16 of 32 (50%), than in the placebo group, 6 of 29 (21%), OR 3.83 (1.23 to 11.92; P=0.020). None of the 7 infants (3 probiotic, 4 placebo) vaccinated with the HbOC conjugate showed an anti-Hib response. Hib antibodies in PRP-T-vaccinated infants exceeded 1 µg/ml in 16 of 29 (55%) in the probiotic *vs.* 6 of 25 (24%) in the placebo group, OR 3.90 (1.20 to 12.61; P=0.023). Differences in the geometric mean concentrations of anti-diphtheria IgG and of anti-tetanus IgG between the study groups were non-significant. In all infants, diphtheria and tetanus IgG titers exceeded the protective level of 0.01 IU/ml (Table 7).

Table 7. Serum antibody concentrations at 6 months of age for infants vaccinated against tetanus, diphtheria, and *Haemophilus influenzae* type b(Hib)

	Probiotic N=47	*Placebo N=40*			
	Geometric mean (95% CI)	*Geometric mean (95%CI)*	*†Ratio*	*95% CI*	*P*
Diphtheria IU/ml	0.38 (0.14 to 0.78) [37]	0.47 (0.19 to 1.40) [37]	0.80	0.45 to 1.43	0.449
Tetanus IU/ml	1.01 (0.47 to 1.49) [37]	0.81 (0.56 to 1.39) [37]	1.23	0.82 to 1.86	0.310
Hib µg/ml*	0.75 (0.15 to 2.71) [32]	0.40 (0.15 to 0.92) [29]	1.87	0.96 to 3.64	0.064

*Sample size in brackets, † ratio probiotic:placebo

5.6 Mucosal immune markers (IV)

5.6.1 Fecal immune markers in predicting allergies

In Study IV, of the 124 infants with an allergic disease or sensitization (cases), 117 were sensitized and 99 manifested an allergic disease. The control group comprised the 113 healthy non-sensitized infants. The geometric mean fecal IgA or fecal inflammatory markers were comparable between the cases and controls (data not shown).

Having a high fecal IgA concentration at age 6 months tended to reduce the risk for cumulative incidence of any allergic disease by age 2. Furthermore, the high fecal IgA reduced risk for any atopic (IgE-associated) disease (*P* for trend=0.044) (Table 8). Increased fecal IgA (T2 compared to T1) reduced risk for eczema, OR 0.44 (0.22 to 0.91; *P*=0.026) and atopic (IgE-associated) eczema, OR 0.47 (0.23 to 0.99; *P*=0.039) at 2 years of age.

Fecal IgA concentrations correlated with concentrations of all the fecal inflammation markers; IgA *vs.* α1-AT (*r*=0.68; *P*<0.001), IgA *vs.* TNF-α (*r*=0.30; P<0.001) and IgA *vs.* calprotectin (*r*=0.66; *P*<0.001).

TNF-α and α1-AT in allergic and healthy infants (data not shown) were comparable. Having high fecal calprotectin at the age of 6 months seemed to associate with reduced risk for atopic diseases at age 2 years (T3 compared to T2), OR 0.49 (0.25 to 0.98; *P*=0.044).

5.6.2 Effects of probiotics on fecal immune markers

Probiotic treatment, compared to placebo, tended to raise geometric mean fecal IgA and TNF-α, and significantly raised α1-AT (*P*=0.001) at the age of 3 months, but these differences disappeared with age (Figure 10). Antibiotic treatment was a confounding factor; the probiotic effect on IgA, TNF-α, and α1-AT was apparent only among infants without any antibiotic use during the intervention. When adjusted for antibiotic use, fecal calprotectin at the age of 6 months was significantly higher in the probiotic group (*P*=0.045). In all infants, the immune markers decreased from 3 to 6 months of age

(P<0.001), with a more pronounced reduction of the initially higher α1-AT (P=0.055) and TNF-α (P=0.034) in the probiotic group (Figure 10).

Table 8. Association of fecal IgA and calprotectin concentrations at 6 months with risk for allergic diseases and sensitization by age. The lowest tertile group (T1) is the reference group in all logistic regression analyses.

Fecal marker		*Allergic disease or sensitization N=124*				*Allergic disease N= 99*				*Atopic (IgE-associated) disease N=90*			
		%	*OR*	*95% CI*	*(P)*	*%*	*OR*	*95% CI*	*(P)*	*%*	*OR*	*95% CI*	*(P)*
IgA	T1	61	1.00		(0.294)	59	1.00		(0.059)	58	1.00		**(0.044)**
	T2	50	0.65	0.34 to 1.25		41	**0.46**	**0.23 to 0.92**		39	**0.44**	**0.21 to 0.90**	
	T3	49	0.63	0.33 to 1.22		43	0.51	0.26 to 1.02		40	**0.48**	**0.24 to 0.96**	
Calprotectin	T1	57	1.00		(0.376)	54	1.00		(0.272)	52	1.00		(0.170)
	T2	54	0.87	0.46 to 1.64		48	0.82	0.42 to 1.59		47	0.86	0.44 to 1.71	
	T3	46	0.65	0.34 to 1.21		39	0.58	0.29 to 1.13		34	0.52	0.26 to 1.05	

Tertiles: IgA ug/ml 3 months <1420.68, 1420.68-2196.57, >2196.57, 6 mo<711.85, 711.85-1235.13, >1235.13 Calprotectin ug/g 3 mo <123.76, 123.76-259.34, >259.34, 6 mo <20.46, 20.46-51.00. Global *P*-value for trend is adjusted for probiotic intervention

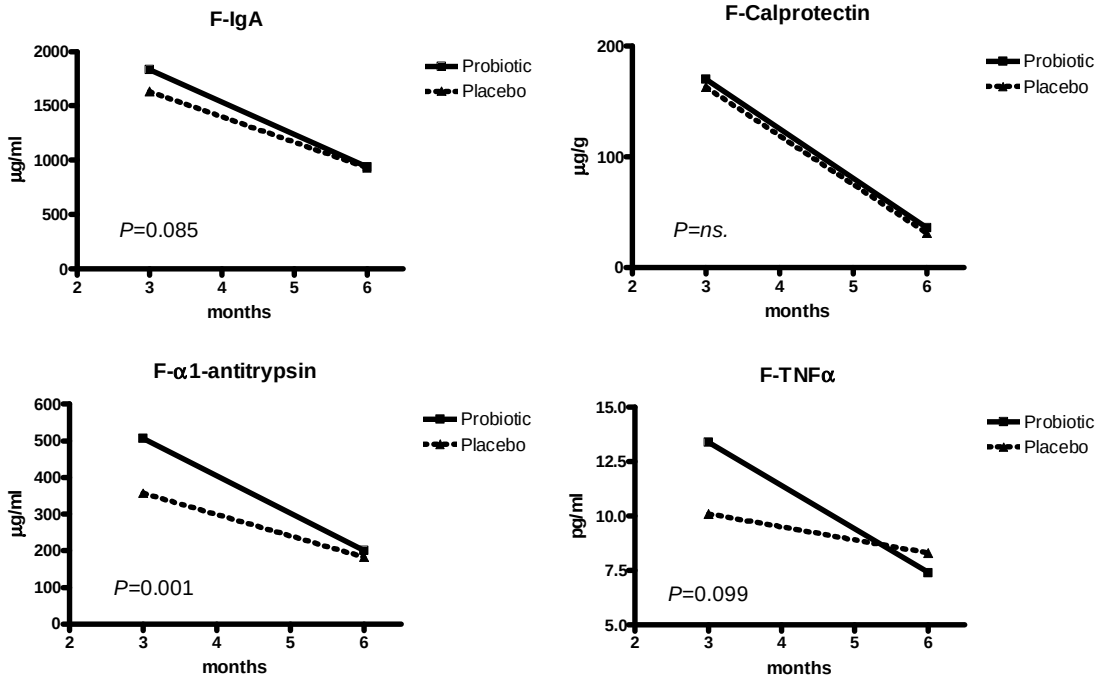


Figure 10 Fecal concentrations of IgA, calprotectin, α1-antitrypsin, and tumor necrosis factor-α (TNF- α) at 3 and 6 months of age in infants receiving pro- and prebiotics (n= 120) or placebo (n= 117)

6. Discussion

6.1 Methodological considerations

6.1.1 Intervention

The results of this study apply to urbanized high-risk infants in a high-income country. Selection of high-risk families was based on telephone interviews by trained personnel, because allergy testing during pregnancy is unreliable and not recommended. The unexpectedly high (17%) drop-out rate before initiating the infant's intervention was due to the refusal of mothers to participate or to the ineligibility of their newborn infants (premature, major malformations). We considered an intervention during pregnancy crucial to the probiotic effect because bacteria in the mother's intestine colonize the gut of a newborn infant during vaginal delivery. An additional study visit at 35 gestational weeks before randomization might have helped us to avoid randomizing mothers not sufficiently motivated and thus to lower the initial withdrawal. Although some parents considered the study protocol difficult to follow, the 8% drop-out rate thereafter, during the 2-year follow-up, was low. High compliance rates and high rates of discovery of supplemented bacteria in the infants' feces indicate the success of the intervention and good adherence to treatment. High follow-up rates may also speak for our trained personnel, who were ready to counsel and support the families throughout the study. The sample-size calculation allowed a 20% drop-out and accordingly, the sample size in Studies I and II was adequate.

Our intervention included a combination of probiotics and prebiotic galacto-oligosaccharides. Both probiotics and prebiotics had been studied separately before, but to the best of out knowledge, a similar intervention using their combination had not been performed previously. By choosing a combination we aimed to broadly stimulate the mucosal immune system. However, evidence accumulated during the study of strain-specificity and of probiotic interactions in the mixtures. Although individual strains in the mixture proved immunologically active, and their combinations had been synergistic *in vitro* (Collado *et al.*, 2007; Rolfe, 2000), clinical studies on their effectiveness in the treatment of eczema appeared only after initiation of this study (Viljanen *et al.,* 2005c). We used prebiotics in order to enhance the growth and activity of probiotics in the gut and aimed at synergistic action. In view of the content of oligosaccharides in human breast milk (>0.8g/100ml) or of the 0.8g/100ml used in a prebiotic allergy-prevention trial with formula-fed infants, however, our total daily amount of 0.8g was low (Moro *et al.*, 2006). We chose a low dose to avoid undesirable gastrointestinal symptoms caused by large quantities of prebiotics, but the significance of such a low dose in a population of breast-fed infants can be argued (Tuohy *et al.*, 2005).

We used opened capsules containing probiotics in powder in order to guarantee the viability of the bacteria. Although parents were given illustrated instructions to mix the powder with an adequate amount of liquid, administration of the powder caused a choking event in one infant. Therefore, if probiotics are administered as a powder, parents should receive thorough instructions to mix it with sufficient amounts of liquid.

The 6-month duration of our intervention was based on a previous study with the hypothesis of a relatively short post-natal time-window in shaping of the immune system towards non-allergy (Garn and Renz, 2007). However, the immune system is tuned towards allergy also later in life, for example, allergic diseases and sensitization occur increasingly in people moving from low to high allergy-prevalence countries or from rural to urban environments (Viinanen *et al.*, 2007). Thus, in the light of the current evidence, the duration chosen may have been insufficient.

6.1.2 Diagnostic criteria of allergic diseases

Diagnostic criteria of our clinical outcome measures were based on internationally accepted guidelines or consensus reports and followed the recommendations of the European Academy of Allergy and Clinical Immunology for diagnostic criteria for dietary allergy-prevention trials (Muraro *et al.*, 2004). We used the UK Working Party's diagnostic criteria for eczema, the most extensively validated criteria available (Brenninkmeijer *et al.*, 2008). Although their sensitivity may be lower than are other criteria frequently used, their specificity is high, and their application in a clinical trial is—straightforward (Williams *et al.*, 1994). The primary outcome measure chosen was the cumulative incidence of any allergic disease. Although allergies are a heterogeneous group of hypersensitivity-type inflammatory diseases with various backgrounds, probiotics may, according to the current hypothesis, promote immune homeostasis through several pathways (Freitas *et al.*, 2003). As the pathogenesis and prognosis of allergic diseases associated with IgE-sensitization differ from those of non-IgE-associated diseases, defining our secondary outcome measures according to sensitization was reasonable.

Our cumulative incidences were higher than in unselected cohorts, but did not exceed the highest reported frequencies of high-risk populations. However, information is scant as to the incidence of allergic diseases and as to sensitization in high-risk populations with diagnoses based on doctors' diagnoses instead of on parental reports. Eczema is the most common manifestation of allergies in early life. The 32% cumulative incidence of eczema in our placebo group was higher than the 16% cumulative prevalence of eczema reported for unselected Finnish children at age 5 (Lehtonen *et al.*, 2003), and in line with the 36% for eczema observed in high-risk Swedish children at age 2 (Abrahamsson *et al.*, 2007). The 8% incidence of food allergy agrees with previous reports within high-risk populations (Sampson, 2003). Our 3% incidence of recurrent wheezing with persistent symptoms is in line with the 3% incidence of frequent wheezing seen in high-risk infants before age 3 years (Ly *et al.*, 2006). Allergic rhinitis occurs late in the atopic march, and clear recurrent symptoms by age 2 years are unlikely to appear. That incidences other than of eczema were still low at the age of 2 years did not allow comparisons. The 30% sensitization rate at 2 years in this study was clearly higher than previously reported for unselected birth-cohorts (Høst and Halken, 2000), indicating successful selection of a high-risk population.

6.1.3 Questionnaires

In Study II, use of diaries in addition to structured questionnaires, interviews, and parental reports would have added value to our tolerance and safety analyses. Diaries would have been useful in evaluating the probiotic effects on duration and severity of childhood infections. However, diaries were not included in hopes of enhanced compliance during the long follow-up period, but the frequent questionnaires, visits, and recommendations to contact the study personnel were considered adequate.

6.1.4 Blood and fecal samples

Results of Studies III and IV should be interpreted in light of the fact that sample sizes were based on the samples available. The small sample size in Study III limits its value. In addition to the changed Hib vaccine, we encountered such problems as a delay in obtaining ethics-committee permission, parental refusal of blood sampling, and some failure to follow vaccination schedules. The number of eligible samples was therefore small, and the results must be interpreted with caution. Selection of fecal samples in Study IV was based on the presence or absence of allergic diseases or sensitization and therefore allowed study of fecal immune markers in predicting allergies, but not primarily the probiotic effect on them.

6.2 Effects of the intervention

6.2.1 Clinical effects

The hypothesis that probiotics and prebiotics will prevent any allergic disease when given to pregnant mothers and to their high-risk children for the long-term after birth was not confirmed. However, probiotics did prevent atopic (IgE-associated) diseases and also eczema, especially when IgE-associated. The majority of infants suffering from any allergic disease had eczema. Eczema appears early in the atopic march, in the progressive sequence of allergic diseases leading to airway allergies. IgE-associated eczema is a strong positive predictive factor for further allergic diseases (Kusel *et al.*, 2005). Whether prevention of IgE-associated eczema by probiotics predicts fewer allergic diseases later in life cannot be concluded based on our 2-year follow-up.

Our treatment effect was not as strong as in the first prevention trial, in which *L. rhamnosus* GG alone halved the incidence of eczema from 46% to 23% (Kalliomäki *et al.*, 2001b). Compared to that finding, the 32% cumulative incidence of eczema in our placebo group was lower, and the relative risk reduction was 26%. In the present study, we administered the products directly to the newborn infants regardless of mode of feeding. In the study performed by Kalliomäki *et al.* (2001b), probiotics were given to infants only if not breast-fed at all; otherwise the capsules were given to the lactating mothers. Another difference between the two studies is the probiotic product. Although our mixture contained similar amounts of the same viable *L. rhamnosus* GG strain, other probiotics in our mixture may have modified its effect. In one study where this same mixture of probiotics was compared to *L. rhamnosus* GG or placebo, the mixture

lacked any clinical effect on eczema (Viljanen *et al.,* 2005c). This mixture, when compared to *L. rhamnosus* GG, induced different cytokine profiles and stimulated the immune system in a different manner (Pohjavuori *et al.,* 2004; Marschan *et al.,* 2008a).

A recently published German study had a relatively small sample size and used the same *L. rhamnosus* GG strain as in the Kalliomäki group trial but from another supplier in smaller amounts ($1x10^{10}$ *vs.* $5x10^{9}$). It reported similar incidences of eczema in the probiotic and in the placebo group (28% *vs.* 27%). Although that treatment was initiated during pregnancy, after delivery, probiotics were given during 6 months for the most part to lactating mothers instead of direct administration to infants (Kopp *et al.,* 2008b). The authors saw *in vitro* immunological effects (enhanced IL-10 and IFN-γ release of mononuclear cells) from their intervention, but no *in vivo* effects on cytokine patterns in the mothers (Kopp *et al.,* 2008a).

In studies showing a positive effect, probiotics have been administered during pregnancy (Abrahamsson *et al.,* 2007; Kalliomäki *et al.,* 2001b). Taylor *et al.* (2007) used *L. acidophilus* in high-risk infants, but initiated this intervention after birth. Colonization of the mother's intestine before birth has great importance for the infant's colonization, at least in infants born vaginally.

Our treatment affected sensitization neither at 6 months nor at 2 years, which is consistent with the first prevention trial (Kalliomäki *et al.,* 2001b). Except for the Swedish study reporting trends toward decreased sensitization at 2 years in infants receiving *L. rhamnosus* for 12 months from birth, probiotics have not prevented sensitization (Abrahamsson *et al.,* 2007; Kalliomäki *et al.,* 2001b; Taylor *et al.,* 2007; Kopp *et al.,* 2008b). IgE-sensitization has been considered a key event in the pathogenesis of allergic reactions, and its prevention is therefore considered primary (Asher *et al.,* 2004). Although early sensitization predicts appearance of allergic airway diseases (Nickel *et al.,* 1997), some sensitized infants remain asymptomatic; hence the role of IgE in the establishment of allergic diseases remains unclear. The mechanisms of allergy are more complicated than previously assumed: complex networks of pro- and anti-inflammatory responses modified by genetic and environmental factors (Freitas *et al.,* 2003). Probiotics may not affect sensitization per se, but they regulate the path from sensitization to clinical disease and raise the proportion of asymptomatic infants.

Long-term follow-up in the Kalliomäki group study showed that the cumulative risk for developing eczema remained reduced in the *L. rhamnosus* GG group. Children in the active treatment group had more allergic rhinitis and asthma, but that difference was non-significant. This does not support the view that probiotics administered during early life would prevent progress of the "atopic march" (Kalliomäki *et al.,* 2003, 2007).

Our results should be interpreted in the light of the fact that over 70% of the study infants received breast milk during the 6-month intervention. Although no significant difference between the study groups occurred, breast-feeding was assessed as a confounding factor in all the main analyses. Galacto-oligosaccharides and IgA are plentiful in human milk, and both of them may modulate the risk for allergies and childhood infections (Brandtzaeg, 2003).

6.2.2 Detection of probiotics in the feces

Infants receiving probiotics showed significantly more frequently and more abundantly lactobacilli, bifidobacteria, and all the supplemented bacterial strain in their feces, but

differences in bacterial recovery were temporary. It can be speculated as to why probiotics fail to colonize permanently (Alander *et al.,* 1999; Kullen and Bettler, 2005). Inherited features of the host may affect colonization patterns more than environmental factors do, and certain probiotics have induced immunological responses against themselves and thereby contributed to the strains' disappearance (Walker, 2008). Nevertheless, compared to the Australian study (Taylor *et al.,* 2007), our initial probiotic colonization, as assessed by the modern PCR 16S rDNA technique, was much more frequent and was higher. Neither Kalliomäki's trial nor the two other published trials reported bacterial colonization of their infants (Kalliomäki *et al.,* 2001b; Abrahamsson *et al.,* 2007; Kopp *et al.,* 2008b). Although fecal samples are not representative of the whole mucosa-associated microbiota, the inverse association in our study between the abundance of certain species of the indigenous gut microbiota and development of atopic diseases supports the idea of probiotics at least partly compensating for the delayed immune maturation responsible for establishment of allergies in genetically prone infants (Wold, 1998).

6.2.3 Safety and infections

Our study indicates that the use of live probiotic bacteria and prebiotic nutrients, even with long-term administration to newborn infants, carries no risks. Results of Studies II and III indicate that probiotics improve maturation of the immune system among infants with atopic heredity. Probiotics improved these infants' resistance to respiratory infections: During their first 6 months, they received antibiotics less frequently than did infants on placebo, and thereafter, to the age of 2 years, they suffered from fewer respiratory infections. Probiotics also improved their IgG antibody response to the first dose of Hib polysaccharide vaccine. Our placebo group responded poorly to their first PRP-T dose (Käyhty *et al.,* 1991), which, in infants with a genetic predisposition to allergic disorders, may be due to delayed maturation of the immune system (Martinez and Holt, 1999). The immune-enhancing effects observed are in line with current knowledge of probiotics' and prebiotics' causing increased resistance to common childhood infections (Hatakka *et al.,* 2001; Weizman *et al.,* 2005; Arslanoglu *et al.,* 2007).

6.2.4 Fecal IgA and inflammatory markers

Having high fecal IgA at 6 months of age protected against development of atopic diseases. Again, early immunological differences were evident between the IgE-sensitized—the true atopics—and healthy controls. By elimination of food antigens in the intestinal mucosa, IgA reduces antigen load and may thus inhibit manifestation of symptomatic diseases in such IgE-sensitized infants. Fecal IgA and inflammatory markers correlated positively. Induction of a subtle physiological inflammation in the intestinal mucosa may be critical to stimulation of the immune system and to induction of IgA production (Viljanen *et al.,* 2005b).

High fecal calprotectin at age 6 months inversely associated with atopic diseases. Calprotectin is a marker of intestinal inflammation (Kolho *et al.,* 2006; Sipponen *et al.,* 2008), and its concentrations are much higher and more variable in newborn infants

than in older children or adults (Rugtveit and Fagerhol, 2002). It has been speculated that the high fecal calprotectin concentrations during the first weeks or months of life reflect increased granulocyte migration into the gut lumen in response to the initial establishment of gut microbiota (Rugtveit and Fagerhol, 2002). Commensal bacteria that colonize the sterile newborn gut induce inflammation, which is balanced by inhibitory mechanisms and results in development of tolerance. The initially high levels of inflammation markers and their fading over time may be a physiological response to the establishment of commensal bacteria and to the presentation of other, and new oral antigens (O'Farrelly, 1998).

Probiotic intervention was associated with high fecal α1-AT concentrations; however, these concentrations were 100-fold lower than reported for infants with manifest food allergy or eczema (Majamaa *et al.*, 1996). In infants suffering from eczema, both *L. rhamnosus* GG and the same mixture of probiotics as in this trial have induced inflammation. However, *L. rhamnosus* GG seemed to induce more Th1-type responses, and this mixture promoted regulatory type responses (Viljanen *et al.*, 2005b). In the blood samples of infants in the same study at age 6 months, the proinflammatory response associated with probiotics resembled chronic helminth infection-associated induction of regulatory mechanisms (Marschan *et al.*, 2008b). We suggest that similarly to commensal bacteria, a subtle inflammation is the mechanism of probiotic bacteria to support the development of tolerance.

7. Summary

A mixture of probiotics including *Lactobacillus rhamnosus* GG (ATCC 53103), *Lactobacillus rhamnosus* LC705 (DSM 7061), *Bifidobacterium breve* Bb99 (DSM 13692), and *Propionibacterium freudenreichii* ssp. *shermanii* JS, along with galacto-oligosaccharides, was able to prevent eczema, especially atopic eczema. This combination eventually failed, however, to prevent the development of any allergic disease or IgE-sensitization by the age of 2 years. Moreover, probiotics with prebiotics prevented eczema, especially atopic (IgE-associated) eczema. The effect was not as strong as in the first prevention trial using *L. rhamnosus* GG alone, but was in line with findings in recent trials. Whether the positive effect endures and extends to airway allergies later in life will be explored in follow-up on these same infants.

Probiotic bacteria were recovered in the feces of infants during the supplementation, but this recovery was temporary.

Probiotic treatment during pregnancy and immediately after birth over the long term was safe and resulted in normal growth. It seemed slightly to enhance resistance to common respiratory infections in infants genetically prone to atopy. Probiotics did not interfere with immune responses to vaccines but improved systemic antigen-specific IgG antibody responses to *Haemophilus influenzae* type b polysaccharide vaccine. These findings need confirmation.

High intestinal IgA in early life predicted fewer atopic (IgE-associated) diseases up to age 2 years, and correlated positively with intestinal inflammation markers. We suggest that a low-grade inflammation induces production of mucosal IgA. Probiotics may induce a low-grade non-symptomatic intestinal inflammation, which may be among the mechanisms of probiotic bacteria in stimulating the maturation of the immune system.

8. Clinical implications

Although promising, current evidence as to the efficacy of probiotics and prebiotics in primary prevention of allergies is conflicting, and their possible preventive effect on atopic eczema needs confirmation in long-term follow-ups. Probiotic bacteria seem to beneficially stimulate the maturation of the immune system of atopy-prone infants. Parents may choose to administer probiotics to their newborn infants, because selected probiotic bacteria carry no risks.

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