

Original article

The effects of selected probiotic strains on the development of eczema (the PandA study)

Background: Modification of the intestinal microbiota by administration of probiotic bacteria may be a potential approach to prevent allergic disease. We aimed to study primary prevention of allergic disease in high-risk children by pre- and postnatal supplementation of selected probiotic bacteria.

Methods: In a double-blind, randomized, placebo-controlled trial, a mixture of probiotic bacteria selected by *in-vitro* experiments (*Bifidobacterium bifidum*, *Bifidobacterium lactis*, and *Lactococcus lactis*; Ecologic® Panda) was prenatally administered to mothers of high-risk children (i.e. positive family history of allergic disease) and to their offspring for the first 12 months of life.

Results: Parental-reported eczema during the first 3 months of life was significantly lower in the intervention group compared with placebo, 6/50 vs 15/52 ($P = 0.035$). After 3 months, the incidence of eczema was similar in both groups. Cumulative incidence of parental-reported eczema at 1 and 2 years was 23/50 (intervention) vs 31/48 (placebo) and 27 (intervention) vs 34 (placebo), respectively. The number needed to treat was 5.9 at age 3 and 12 months and 6.7 at age 2 years. The intervention group was significantly more frequently colonized with higher numbers of *Lc. lactis*. Furthermore, at age 3 months, *in vitro* production of IL-5 (146 pg/ml vs 72 pg/ml; $P = 0.04$) was decreased in the probiotic-group compared with the placebo-group.

Conclusions: This particular combination of probiotic bacteria shows a preventive effect on the incidence of eczema in high-risk children, which seems to be sustained during the first 2 years of life. In addition to previous studies, the preventive effect appears to be established within the first 3 months of life.

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Key words: children; eczema; intestinal microbiota; multispecies probiotics; primary prevention; sensitization.

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Atopy and allergic diseases add considerably to childhood morbidity and the prevalence of allergic diseases has increased during the past few decades. This warrants the development of strategies to prevent allergic diseases. The hygiene hypothesis suggests that the increased prevalence of allergic diseases in children is associated with reduced exposure to microbial components early in life (1). Differences in the intestinal microbiota between allergic

and nonallergic children have been described, which precede the development of allergic disease suggesting a potential causal relationship (2, 3). Furthermore, reconstitution of the intestinal microbiota of germ-free rodents with *Bifidobacterium infantis* restored their development of the otherwise defective oral tolerance (4). Building on this hypothesis, primary and tertiary prevention of allergic diseases could be accomplished by administration of appropriate microbes.

To date, five clinical studies on primary prevention by probiotic bacteria of allergic diseases have been published with conflicting results regarding the effect on (atopic) eczema (5-9). No preventive effect on the development of other allergic diseases was reported so far. It has been questioned if, among other reasons, the use of different probiotic bacteria with assumingly different properties may have attributed to this disparity. Mechanisms by which probiotic bacteria could induce a beneficial effect, in

Abbreviations: *B.*, *Bifidobacterium*; BCSS, basic clinical scoring system; CFU, Colony forming units; DGGE, Denaturing gradient gel electrophoresis; FISH, Fluorescence *in situ* hybridization; IL, Interleukin; *Lc.*, *Lactococcus*; MCPC, Microbial community profiling and characterization; PBMC, Peripheral blood mononuclear cell; PHA, Phytohemagglutinin; qPCR, quantitative (real time) PCR; SCORAD, Scoring atopic dermatitis; SEM, Standard error of the mean; SPT, Skin prick test; TLR, Toll-like receptor; T-RF(LP), Terminal restriction fragment (length polymorphism).

this context prevention of allergic disease, remain to be elucidated. Probiotic bacteria may act at three different levels (10): intestinal microbiota modification, mucosal barrier fortification, and immunomodulation. Each probiotic strain has its unique immunomodulatory activity *in vitro*. This may hold true for their clinical application as well. We hypothesized that rationally selected strains might have more potential to target specific diseases such as allergic diseases. To study the possibilities of probiotic bacteria in primary prevention of allergic diseases in high-risk children, we selected three specific strains, *Bifidobacterium bifidum*, *Bifidobacterium lactis*, and *Lactococcus lactis*. The strains were selected from a large collection of 69 potentially probiotic strains (11) on the basis of their resistance to low gastric pH, pancreatic digestive enzymes and bile salts to allow their survival in the first part of the gastrointestinal tract, and general characteristics such as absence of production of D-lactate, reproducible growth and stable shelf-life. The selection of the specific bacteria was mainly based on their capacity to suppress *in vitro* production of Th2 cytokines and to stimulate IL-10 production, both by PBMCs (12, 13). The three selected strains (Ecologic® Panda) were administered prenatally to pregnant women and postnatally to their offspring at high risk to develop allergic diseases in a randomized double blind, placebo-controlled trial. In this study, we aimed to investigate the effect on the development of eczema during the first 2 years of life, and the initial effects on early microbial colonization and immune responses.

Methods

Participants

Families with a positive family history of allergic disease, i.e. atopic eczema, food allergy, asthma or allergic rhinitis in either the mother, or the father plus an older sibling with a history of allergic disease were recruited by an advertising campaign. Between March 2004 and July 2005, one hundred fifty-six pregnant women and their families were included at least 2 months before expected delivery. Children were excluded from the study if their mother received antibiotic treatment during the last 2 weeks of pregnancy, when the child was born before 37 weeks of gestation, if the children received antibiotic treatment in the first 2 weeks of life, if ingestion of the study product was difficult because of vomiting or feeding problems in general for longer than 3 weeks from birth, or if the children had other major medical problems.

Study design

In a double-blind, randomized, placebo-controlled clinical trial, the probiotic bacteria were prenatally administered to the pregnant mothers during the last 6 weeks of pregnancy and postnatally for 12 months to their offspring. The intervention group received once daily 3×10^9 colony forming units (CFU) (1×10^9 CFU of each strain: *B. bifidum* W23, *Bifidobacterium lactis* W52 (previously classified as *Bifidobacterium infantis*), and *Lc. lactis* W58) of freeze dried powder of the probiotic mixture (Ecologic® Panda, supplied by Winlove Bio Industries B.V., Amsterdam, the Netherlands).

The individual probiotic cultures carry the European Union qualified presumption of safety (QPS). The control group received placebo consisting of the carrier of the probiotic product, i.e. rice starch and maltodextran. Both supplements were dispensed as a stable powder in identical individually packed sachets containing 3 g of material. The contents of each sachet were mixed with at least 10 ml of water, breast milk or infants' formula, depending on the choice of feeding by the parents, and ingested as a suspension. Prior to the study, the stability and biological activity of the probiotic mixture in water, breast milk and infants' formula were confirmed by measuring pH and CFU after suspending the product. During the study, the viability of the probiotic product was tested every 6 months. Block randomization with a block size of 10 was used. Visits were scheduled at the age of 3 months, 1 and 2 years. After birth, parents received at least one phone call to check on compliance and offer assistance if necessary to study related questions. Parental written informed consent was obtained. The study was approved by the Medical Ethics Committee of the University Medical Centre Utrecht, the Netherlands.

Clinical outcome variables

Parents were asked to complete a weekly diary of health status of their child including presence and complaints of eczema, infectious or atopy related complaints, visits to the family doctor, feeding habits including adverse events or difficulties with feeding, medication use, and immunization status. Weekly completion of the diary was asked for to circumvent recall bias at the study visits. Furthermore, an adapted version of the British Medical Research Council questionnaire (14, 15) and the Dutch version of the European Community Respiratory Health Survey (16) was used to evaluate participants for symptoms indicative of food allergy, eczema, asthma, and allergic rhinitis at the age of 3, 12 and 24 months. In addition, parents were asked about their compliance and if they experienced problems with the daily administration of the study product. Parental-reported eczema was defined as eczema reported as complaint by the parents in the diaries. Physician-diagnosed eczema was defined as eczema reported by the parents and diagnosed as eczema by the family doctor or consulted physician. Eczema was defined as noninfectious dermatitis with typical features [redness, dryness, edema, oozing and itching (scratching)] and distribution (17). Atopic eczema was defined as eczema plus sensitization, i.e. detectable (>0.35 IU/ml) allergen specific IgE antibodies or a positive skin prick test (SPT). A complete physical examination was performed at every visit. The presence and severity of eczema at the time of the visit were assessed by the basic clinical scoring system (BCSS) (18) and SCORAD (19). SPTs were performed at 24 months. Histamine dihydrochloride (10 mg/ml) was used as a positive control and the solvent (glycerine) as a negative control. SPTs were performed with allergen extracts for egg white, cow's milk, peanut, hazelnut, cat, dog, house dust mite (*Dermatophagoides pteronyssinus*), birch, and grass (ALK-Abelló, Nieuwegein, the Netherlands). A wheal diameter at 15 min of ≥ 3 mm was considered positive. The physicians who were involved in the follow-up visits and physical examinations (LN, who performed all follow-up visits, and MH) were kept blinded with respect to group allocation until all children were seen at the age of 2 years (November 2007).

Determination of serum total IgE and specific IgE

Blood samples were collected at each follow-up visit to determine total IgE, a food panel (FP5, consisting of egg white, cow's milk,

codfish, wheat, peanut, and soybean), AlaTOP (multi allergen screening test containing several inhalant allergens), and specific IgE for egg white, cow's milk, peanut, house dust mite, and cat dander epithelium on the IMMULITE 2000 (Diagnostic Products Corporation, Los Angeles, CA, USA). For specific IgE tests and FP5, a positive result was defined as a concentration of ≥ 0.35 IU/ml. For AlaTOP, a result was classified as positive at ≥ 1.00 IU/ml.

Molecular analysis of fecal microbiota

Stool samples were collected according to a prescribed schedule: four stool samples from birth during the first 4 weeks of life starting with the first stool of the newborn and with 1-week intervals between each sample, and one sample at 3 months of age. At 12 months of age, a stool sample 1 week and 1 day preceding the end of the administration period, and two stool samples after (1 and 2 weeks respectively) the end of intervention. The stool samples collected 1 day preceding the end of the intervention period and 2 weeks after the end of intervention were used in the current analysis.

Microbial community profiling and characterization. This qualitative analysis is based on Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis, and was performed by the Dr. van Haeringen Laboratories (Wageningen, the Netherlands).

DNA isolation for quantitative PCR and Denaturing gradient gel electrophoresis analysis. DNA was extracted from 200 mg of fecal samples taken from eight randomly chosen infants per group at seven different time points using the FAST DNA SPIN Kit (Q-biogene, Carlsbad, CA, USA). The DNA was eluted in 100 μ l of autoclaved water and stored at -20°C .

qPCR. The number of *Lc. lactis* and bifidobacteria were determined in reactions of 25 μ l with primers LcoLacF and LcoLacR (20), and Bif-recA-F and Bif-recA-R (21) respectively, both targeting a specific fragment of a single copy gene (*recA*). As *recA* occurs as a single copy gene in bacterial genomes, gene copy numbers are equivalent to cell numbers, assuming one genome per bacterial cell. See additional Methods information in the Data S1.

Bifidobacterium-specific PCR amplification and Denaturing gradient gel electrophoresis. DNA isolated from infant feces at week 2, week 3, and week 12 was used as a template to perform the *Bifidobacterium* genus-specific PCR using 16S rRNA gene targeting primers, Bif164-f and Bif662-GC-r (22). DGGE analysis of PCR amplicons was essentially performed as described previously (22) using the DCode System (Bio-Rad), only that a gradient of 45–50% denaturant was used.

Whole blood cultures and cytokine analysis

Blood samples were collected at the age of 3 months. The blood was diluted 1:10 with RPMI 1640 containing L-glutamine (2 mM) and penicillin (100 U/ml)/streptomycin (100 μ g/ml) (all from Invitrogen Life Technologies, Carlsbad, CA, USA). Whole blood cultures were carried out in quadruplicate. Cells were cultured in medium only or stimulated with a combination of anti-CD2 monoclonal antibody (mAb) (CLB-T11.2/1 and CLB-T11.1/1, 1 μ g/ml) and anti-CD28 mAb (CLB-CD28/1, 4 μ g/ml) (both obtained from Sanquin, Amsterdam, the Netherlands), or phytohemagglutinin (PHA; Murex Biotech Ltd, Kent, UK) at a final concentration of 25 μ g/ml. Culture supernatants were collected at 48 and 72 h. Cytokines were detected in supernatants by multiplex assay (Luminex) as previously

described in detail (23). ^3H thymidine incorporation was measured to determine lymphocyte proliferation.

Statistical analysis

To detect a 50% reduction in the frequency of (atopic) eczema (based on the data published by Kalliomäki et al. (9)) at the 5% significance level with 80% power, 49 participants per group were required. A larger number was recruited to allow for an estimated 20% withdrawal rate. For baseline characteristics, chi-square test was used to compare frequencies, and the independent samples *t*-test was used to compare continuous data. To study the effects of intervention, odds ratios with 95% CI were calculated by multiple logistic regression, correcting for potential confounders such as maternal atopy, birth weight, breastfeeding and gender. Cytokine data were analyzed as continuous data. The cytokine data were normalized by logarithmic transformation and analyzed by the independent samples *t*-test to determine differences between groups. qPCR data of the intestinal microbiota were analyzed by ANOVA/repeated measurements with Bonferroni *post hoc* test to correct for multiple comparisons. Differences were considered significant at 2-tailed $P < 0.05$. All calculations were performed with spss 14.0 for windows (Chicago, IL, USA).

Results

Baseline characteristics and participants

A total of 156 participants were included in the study of whom 102 completed the 3 months of follow-up. Ninety-eight of 156 participants were followed up at 12 and 24 months of age (see Fig. 1). Thirty-five participants met the exclusion criteria after the mother was included. Participation was discontinued on parental request in 23 cases (see Fig. 1). Motivational problems resulted in almost 15% of dropouts. Another reason for dropout was maternal (pregnancy related) health problems, which made further participation for these parents too demanding. The rate of dropouts was similar in both groups, as were the reasons for discontinuation. Baseline characteristics were similar in both groups (see Table 1). The total number of pets at home was significantly higher in the placebo group compared with the intervention group (see Table 1). Baseline characteristics of the dropouts did not differ from the participants who completed the study, which potentially rules out bias.

Incidence of eczema and sensitization

Eczema was reported by the parents in the weekly diaries during the first 3 months of life in 15/52 (29%) children in the placebo group and 6/50 (12%) in the intervention group, odds ratio (OR) 0.322 (95% CI 0.108–0.960), $P = 0.035$ (see Fig. 2A). Not all parents consulted their family physician to evaluate their child's eczema. In 11/52 (21%) children in the placebo group and 3/50 (6%) children in the intervention group, eczema was diagnosed by the consulted physician, OR 0.217 (95% CI 0.053–0.891), $P = 0.021$ (see Fig. 2A). The incidence of

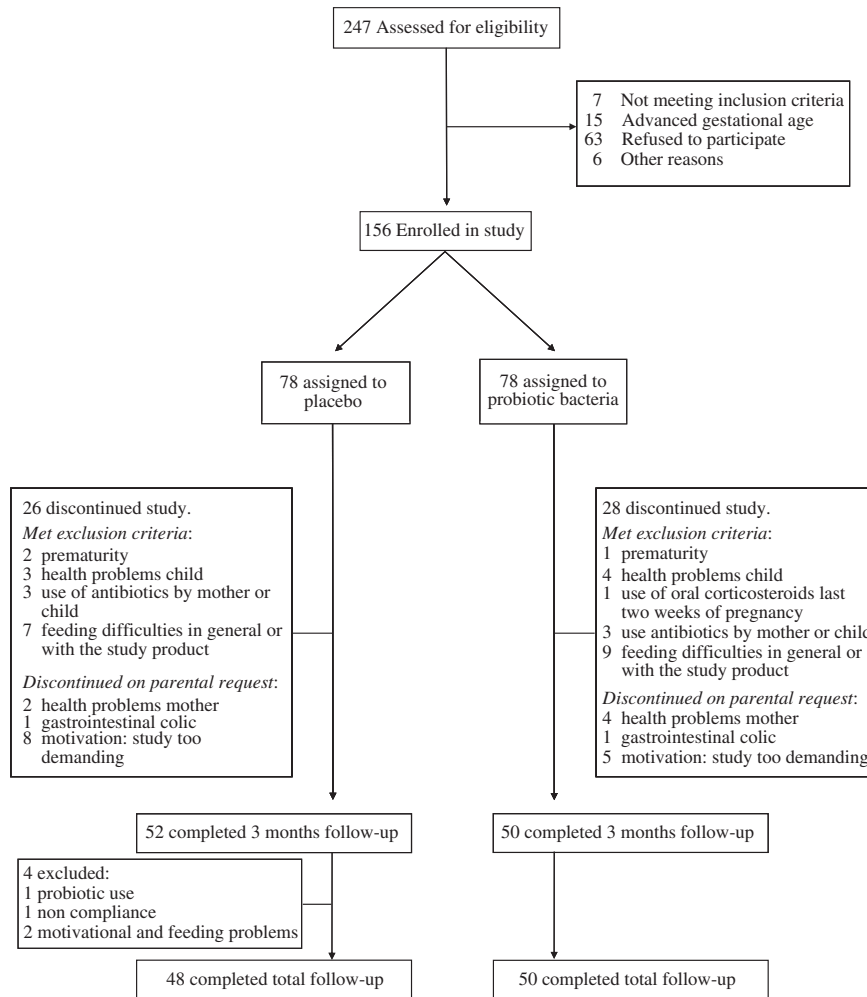


Figure 1. Screening, enrollment and outcomes.

eczema increased during the second half of the first year of life. Between the age of 3–12 months and 12–24 months, the incidence of eczema was similar in both groups. Cumulative incidence of parental reported eczema at 1 and 2 years was 23/50 (intervention) vs 30/48 (placebo), and 27 (intervention) vs 33 (placebo), respectively (Fig. 2B). At the age of 3 months, relative risk reduction was 58%, which decreased to 26% and 22% at 1 year [OR 0.495 (95% CI 0.221–1.105) $P = 0.086$] and 2 years of age [OR 0.518 (95% CI 0.227–1.180) $P = 0.117$] respectively. To indicate the clinical significance of the observed effects on the development of eczema, the number needed to treat was calculated, which was 5.9 at the age of 3 and 12 months and 6.7 at the age of 2 years. The severity of eczema was similar in both groups and consisted of mild to moderate eczema in the majority of cases: mean (\pm SD) SCORAD score at 3 months was 21.0 (\pm 10.5) vs 20.4 (\pm 10.9), at 1 year 19.6 (\pm 8.5) vs 15.8 (\pm 6.2) ($P = 0.289$), and at 2 year 18.7 (\pm 14.4) vs 26 (\pm 13.8) ($P = 0.270$),

respectively, for the placebo vs intervention group. No difference was observed in the cumulative incidence of atopic eczema. During the 2-year follow-up period, 8/48 (17%) of the placebo group vs 10/50 (20%) ($P = 0.876$ by χ^2 test) of the intervention group suffered from eczema and were sensitized. The diagnosis of asthma or allergic rhinitis is difficult at this age, but no differences were observed in respiratory symptoms such as wheezing, coughing and rhinitis. Total IgE was measured in 89%, 94% and 92% of participants at the age of 3, 12 and 24 months respectively. No differences were observed either in median total IgE between groups at different ages or in the number of participants with increased total IgE at the different ages (Table 2). In the intervention group, a trend was observed towards more frequent sensitization, especially to food allergens, compared with the placebo group at the age of 2 years and during the whole study period (see Table 2). However, this did not result in more food allergic participants in the probiotics group.

Table 1. Baseline characteristics

	Placebo (n = 52)	Probiotics (n = 50)
Family characteristics		
Maternal atopic disease	44 (85%)	41 (84%)
Age of mother at the time of birth	31.4 (30.4–32.5)	32.3 (31.1–33.5)
Older siblings		
None	18 (35%)	17 (34%)
One or more	34 (65%)	33 (66%)
Pets at home*		
Cat(s)	11 (21%)	8 (16%)
Dog(s)	8 (15%)	3 (6%)
Bird(s)	2 (4%)	0 (0%)
Other	9 (17%)	2 (4%)
Birth characteristics		
Male gender	23 (44%)	18 (36%)
Mode of delivery		
Vaginal	46 (89%)	46 (92%)
Caesarean section	6 (11%)	4 (8%)
Birth weight (g)	3658 (3520–3796)	3558 (3412–3705)
Gestational age (weeks)	39.7 (39.3–40.1)	39.7 (39.2–40.2)
Breastfeeding during the first year of life		
Mean duration of breastfeeding months	7.2 (6.1–8.3)	7.0 (5.9–8.2)
Duration intake study products mother (weeks)	6.0 (5.6–6.4)	6.0 (5.6–6.4)

* $P = 0.021$.

Data represent absolute numbers (percentage) or mean (95% CI).

Composition of the gastrointestinal microbiota

In a primary analysis, with randomly chosen fecal samples of 38/48 participants in the placebo group and 35/50 in the intervention group, the development of the intestinal microbiota was evaluated by MCPC, which is a qualitative analysis based T-RFLP analysis. Participants in the probiotic group were significantly more frequently colonized with higher numbers of *Lc. lactis* compared with the placebo group during the first 3 months of life (data not shown). No differences were observed in the first 4 weeks of life in the number of children colonized by bifidobacteria. At the age of 3 months, all children in the probiotic group and 85% of the placebo group were colonized with bifidobacteria (data not shown). Subsequently, to confirm and quantify these findings, the number of *Lc. lactis* and *Bifidobacterium* spp. was quantified using qPCR in fecal samples of eight randomly chosen participants in both groups. *Lc. lactis* was present in all fecal samples from the intervention group, but only in 2/8 from the placebo group. The number of *Lc. lactis* was significantly higher in the intervention group (see Fig. 3A). *Bifidobacterium* spp. were present in all of the individuals in high numbers (see Fig. 3B). To study qualitative differences in the composition of *Bifidobacterium* spp., fecal samples were analyzed by DGGE. *B. bifidum* was present in all the samples analyzed from the intervention group (8/8) but only in half of the placebo group (4/8). Fecal colonization with *B. lactis* was more difficult to detect (Fig. 4).

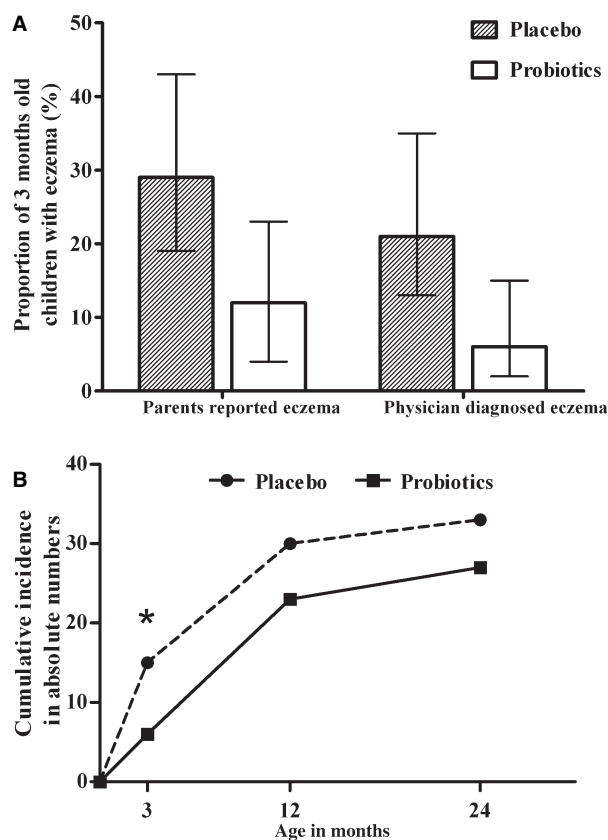


Figure 2. (A) Preventive effect of probiotic supplementation on eczema at age 3 months. Columns represent the percentage of affected children and bars are 95% CI. Univariate statistical analysis was performed with chi-square test and multivariate analysis by multiple logistic regression analysis. * $P < 0.05$. (B) Cumulative incidence in absolute numbers of parental reported eczema during the first 2 years of life. Univariate statistical analysis was performed with chi-square test and multivariate analysis by multiple logistic regression analysis. * $P < 0.05$.

Cytokine production in whole blood cultures at 3 months of age

In 46/48 participants of the placebo group and 43/50 in the intervention group, whole blood cultures were performed and levels of cytokines at 48 and 72 h of culture were measured (Fig. 5). Despite large individual variability, IL-5 was significantly reduced ($P = 0.04$) in the intervention group at 72 h of cell cultures stimulated with anti CD2/CD28 mAbs (Fig. 5B). In the placebo group, mean \pm SEM levels of IL-5 were 145.7 ± 49.2 (pg/ml) vs 72.2 ± 21.8 (pg/ml) in the probiotics group. For IL-13, a trend was observed towards decreased production in the intervention group at 48 and 72 h of cell cultures stimulated by anti CD2 + CD28 mAbs, 169.7 ± 63.9 (pg/ml) vs 79.7 ± 22.8 (pg/ml) placebo vs intervention group ($P = 0.08$) and 442.1 ± 100.4 (pg/ml) vs 299.2 ± 84.7 (pg/ml) placebo vs intervention group ($P = 0.06$) respectively (Fig. 5A and B). In a subgroup analysis, the mean production of IL-5 and

Table 2. Sensitization: total IgE, allergen-specific IgE antibodies and skin-prick test reactions

	Probiotic	Placebo	P-value
Total IgE (IU/ml)*			
3 months	4.4 ± 1.3	3.8 ± 0.8	0.665
12 months	18.5 ± 4.5	22.2 ± 4.2	0.545
24 months	35.1 ± 7.7	54.6 ± 13.1	0.210
One or more sIgE >0.35 IU/ml			
3 months	3/43 (6.9%)	1/51 (2%)	0.230
12 months	9/47 (19.1%)	4/45 (8.9%)	0.158
Food allergens	8/47	4/45	0.247
Inhalation allergens	1/47	1/45	0.975
24 months	9/44 (20%)	4/46 (9%)	0.113
Food allergens	8/44 (18%)	3/46 (6.5%)	0.091
Inhalation allergens	2/44 (5%)	3/46 (7%)	0.66
Positive SPT at 24 months	4/46 (9%)	4/47 (8.5%)	0.975
Food allergens	2/46 (4%)	3/47 (6%)	0.66
Inhalation allergens	3/46 (7%)	1/47 (6%)	0.29
Sensitization**			
At 24 months	10/50 (20%)	7/48 (14.6%)	0.451
Ever (0–24 months)	17/50 (34%)	9/48 (18.8%)	0.087
Ever for food allergens (0–24 months)	15/50 (30%)	7/48 (15%)	0.067

*Data indicate mean ± SEM.

**Either positive SPT or/and sIgE >0.35 IU/ml.

IL-13 was consistently highest in the participants of the placebo group who developed eczema during the first 3 months of life, i.e. 2–3 fold increased compared with the participants in the placebo group who did not develop eczema. In the probiotics group, mean production of IL-5 and IL-13 was not different between the participants who did or did not develop eczema in the first 3 months of life, but was in general lower compared with the placebo group with no eczema. No differences of *in vitro* IL-10 production were found in CD2/CD28 stimulated cultures (see Fig. 5A and B) or in PHA stimulated cultures (data not shown). The *in vitro* lymphocyte proliferative response to either anti CD2/CD28 or PHA did not differ between the groups (data not shown).

Discussion

With perinatal administration of Ecologic® Panda, we aimed at influencing early colonization with probiotic bacteria to provide immunoregulatory signals, which would result in the primary prevention of allergic disease such as eczema. In this study, we have shown that administration of selected strains of probiotic bacteria prevented the development of eczema during the first 2 years of life in high-risk children. The children who received probiotic bacteria were more frequently colonized with higher numbers of *Lc. lactis*, and reduction of eczema at 3 months of age was paralleled by a decreased *in vitro* production of IL-5 and to a lesser extent IL-13.

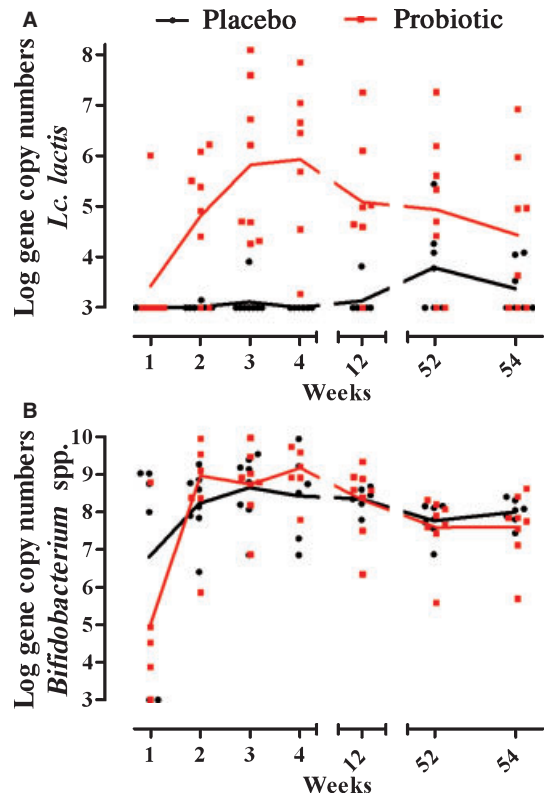


Figure 3. Real-time PCR quantification of *Lactococcus lactis* (A) and *Bifidobacterium* spp. (B). qPCR in stool samples of eight randomly chosen participants per placebo and intervention group at weeks 1, 2, 3, 4, 12, 52 (1 day before end of ingestion period) and 54 (2 weeks after end of ingestion period) using *recA* as target gene. Results are depicted as log gene copy numbers per gram feces. Each symbol represents data from an individual participant. Statistical analysis was performed with two-way ANOVA/repeated measurements. The number of *Lc. lactis* is significantly ($P < 0.0001$) higher in the probiotic group during the first year of life. Bonferroni post-tests indicated significant differences at week 2 ($P < 0.01$), week 3 and 4 ($P < 0.001$), and week 12 ($P < 0.05$).

Thus far, five clinical trials on primary prevention of allergic disease by perinatal administration of probiotic bacteria have been published with inconsistent results (5–9). Two studies showed a preventive effect of probiotic supplementation on the development of eczema at the age of 2 years, with a relative risk reduction of 50% (9) and 26% (5). In a third study, the cumulative incidence of eczema was similar in intervention and placebo group, but less atopic eczema was observed in the intervention group (8). In two studies, supplementation of probiotic bacteria failed to reduce the risk of atopic dermatitis and resulted in increased sensitization to allergens in one study (6, 7). A major reason for these discrepant clinical results may be the use of different probiotic strains or preparations. In this respect, however, it is interesting that the results of Kopp et al. (7) are in sharp contrast with the study of Kalliomaki et al., (9) although an

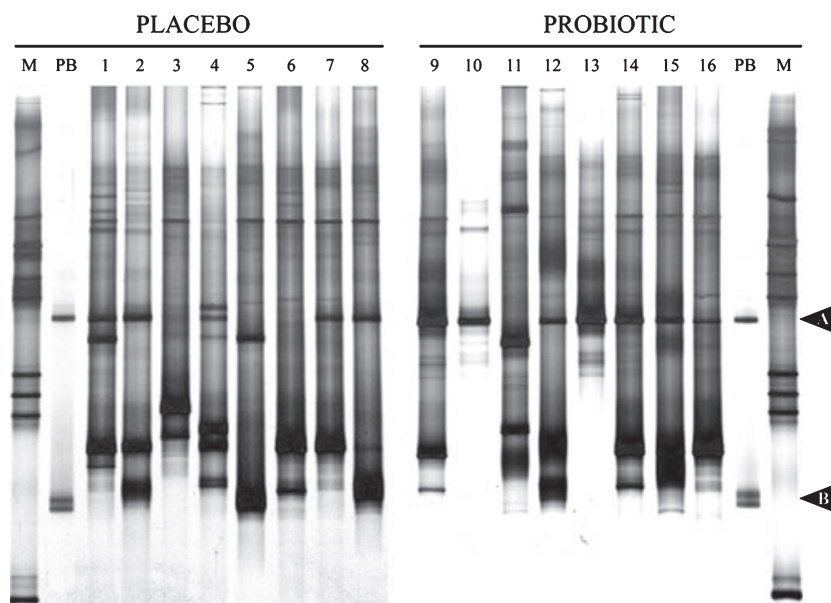


Figure 4. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified bifidobacterial 16S rRNA gene fragments from eight randomly chosen participants per placebo group (numbered 1–8) and intervention group (9–16) after 3 weeks of supplementation. The mobility of the PCR products obtained in DGGE was compared with the profile of the probiotic mixture Ecologic® Panda obtained with the same primer set (lanes indicated by PB). Fragment A: *Bifidobacterium bifidum*; fragment B: *Bifidobacterium lactis*.

identical probiotic strain is used (LGG) in an also otherwise comparable study design. In contrast to most other studies, we have aimed to develop a target-specific combination of probiotic bacteria (12, 13).

Our study indicates that the beneficial effects of probiotic bacteria are established within the first 3 months of life. We observed a relative risk reduction of 58% of parental reported eczema at 3 months of age. This effect seemed to be sustained until the age of 2 years, although relative risk reduction decreased with age. At the age of 2 years, we found a similar relative risk reduction as described by Kukkonen et al. (5). In high-risk children, the earliest signs of eczema can develop during the first 3 months of life, but the highest incidence rate occurs in the second half-year of life (24). In children older than 3 months, a similar incidence was observed in both groups. However, from a clinical perspective, reducing the burden of eczema certainly in the first year of life is very valuable. We speculate that prenatal administration of probiotic bacteria to the mother might be essential in this process. Probiotic bacteria were administered during the last 6 weeks of pregnancy to the mother as this may cause temporary changes in the composition of the maternal intestinal microbiota and consequently may have influenced early colonization in their offspring. The vaginal and intestinal microbiota of the mother are one of the first colonizers of the neonatal gut (25). Very little is known about the immunological effects of maternal probiotic supplementation on the fetus. Modulation of fetal immune responses may be a mechanism of action, although findings from recent

studies are not consistent (26, 27). In line with previous observations (6), children in the probiotics group tended to be more frequently sensitized to food allergens, especially hen's egg, compared with the placebo group. Although this did not result in more food allergic participants in the probiotics group, sensitization to hen's egg at the age of 12 months has previously been found to be predictive of sensitization to inhalant allergens at a later age (28, 29). However eczema, which was more prevalent in the placebo group, has been shown to be a predictor for the development of sensitization as well (30). No differences were observed in respiratory symptoms indicative of asthma or allergic rhinitis, although diagnosis is difficult at this age. As the participants of this study are all high-risk children who may develop allergy or atopic manifestations other than eczema later in childhood, all participants will be followed-up at the age of 5–6 years old. Specifically asthma (including spirometry), allergic rhinitis and again allergic sensitization for food and inhalant allergens will be addressed.

Few studies have investigated the influence of probiotics on the intestinal microbiota of infants. In our study, administration of the probiotic mixture resulted in higher and more frequent colonization of *Lc. lactis* and more frequent colonization of *B. bifidum*. This indicates that the oral administration of the probiotic mixture was successful and compliance was good. The presence of *Lc. lactis* in a minority of participants in the placebo group could be explained by the fact that 30% of the lactating women have this micro-organism in their breast milk (31) and most of the babies included in the study

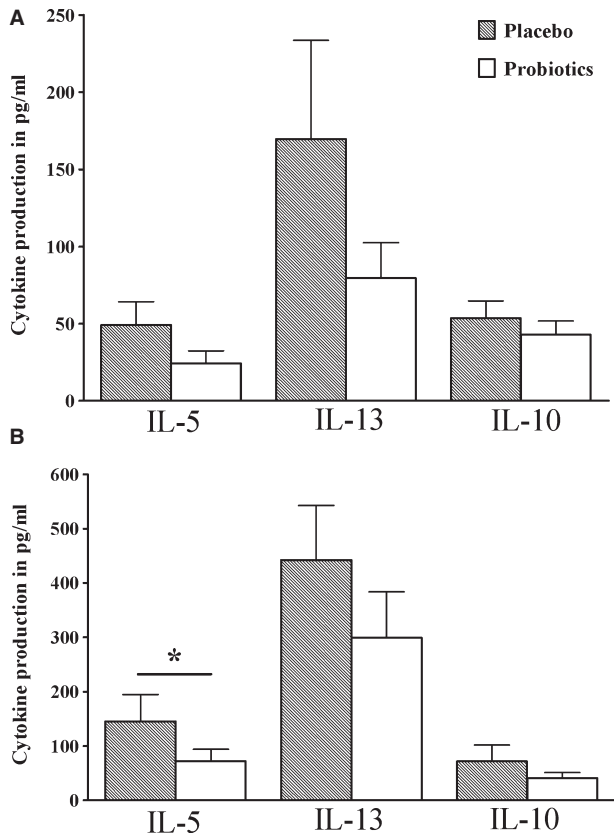


Figure 5. Production of cytokines in whole blood cultures at age 3 months. Whole blood cultures were left unstimulated (medium) or stimulated with anti CD2/anti CD28 monoclonal antibody, or Phytohemagglutinin (PHA). Supernatants were collected at 48 h and 72 h and cytokines were measured by a multiplex assay (Luminex). (A) Anti CD2/CD28 48 h; (B) Anti CD2/CD28 72 h. Data represent mean ± SEM. **P* < 0.05.

were breastfed. The apparent drop in mean carriage rate of *Lc. lactis* at 52 weeks may be caused by the expansion of the colonic anaerobic microbiota, which will dominate in the fecal samples. Perinatal administration of probiotic bacteria did not lead to an increase in carriage and total counts of bifidobacteria in the intestinal microbiota. The supplied number of bifidobacteria was apparently not high enough to detect an elevation in the endogenous (high) numbers of bifidobacteria. Species-specific analysis, however, revealed that endogenous *Bifidobacterium* species have been replaced by *B. bifidum* as this microbe was more frequently located in the intestinal microbiota of the intervention group. In comparable intervention studies, supplemented strains were more frequently found in the intervention group at the end of the administration period, but no permanent colonization occurred (5, 32). Furthermore, LGG administration in the first few months of life did not significantly interfere with the variation or quantity of the gut microbiota as measured by FISH analysis (33). Whether in our study permanent colonization will ensue and whether probiotic supplementation

will result in modulation of the gut microbiota are the subject of our current research.

The results of this study suggest that primary prevention of eczema by perinatal administration of probiotic bacteria indeed involves modulation of the early colonization of the intestinal microbiota, which may result in modulating the development and maturation of the infants' immune system. Modulation of the immune response via interaction with intestinal dendritic cells with subsequent effects on T-cell differentiation and induction of regulatory T cells has been suggested (34). Furthermore, recognition of commensal bacteria by TLRs on intestinal epithelial cells and cells of the mucosal immune system is essential for intestinal (immune) homeostasis (35, 36). Probiotic signaling through TLRs may contribute to maintaining mucosal and intestinal homeostasis and thereby preventing eczema.

In our study, we had a rather large number of dropouts, although this is not uncommon in this type of studies. Previous published studies also suffered from almost 25% of dropouts (5, 6). Almost 40% of the dropouts were based on parental request, mainly because of motivational problems, i.e. the study was too demanding. Trials may be prone to bias associated with postrandomization exclusion. However, we ensured that randomization was successful, i.e. placebo and intervention group were equal with respect to potential risk factors and confounders.

In conclusion, this intervention study shows a preventive effect of early administration of selected probiotic bacteria on the incidence of eczema, but not atopic eczema in high-risk children. This preventive effect seems to be established within the first 3 months of life together with significant changes in the intestinal microbiota and decreased IL-5 production.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Molecular analysis of fecal microbiota.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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