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ORIGINAL ARTICLE

Clinical Mechanisms in Allergic Disease

Reduced Th22 cell proportion and prevention of atopic dermatitis in infants following maternal probiotic supplementation

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Summary

Background: In the randomized, controlled study Probiotics in the Prevention of Allergy among Children in Trondheim (ProPACT), maternal probiotic supplementation reduced the incidence of atopic dermatitis (AD) in the offspring. In the current study, we hypothesized that the effect was mediated by a shift in the T helper (Th) cells in the children.

Objective: To examine whether Th cell proportions were affected by maternal probiotic supplementation and thus could mediate the preventive effect of probiotics on AD.

Methods: A total of 415 pregnant women were randomized to ingest a combination of *Lactobacillus rhamnosus GG* (LGG), *Bifidobacterium animalis subsp. lactis Bb-12* (Bb-12) and *Lactobacillus acidophilus La-5* (La-5) or placebo, and their offspring were assessed for AD during the first 2 years of life. Peripheral blood collected at 3 months of age was analysed for regulatory T cells (n=140) and Th subsets (n=77) including Th1, Th2, Th9, Th17 and Th22.

Results: The proportion of Th22 cells was reduced in children in the probiotic group compared to the placebo group (median 0.038% vs 0.064%, P=.009). The difference between the probiotic and placebo groups was also observed in the children who did not develop AD during the 2-year follow-up. The proportion of Th22 cells was increased in children who developed AD compared to the children who did not develop AD (0.090% vs 0.044%, P<.001). Mediation analysis indicated that the preventive effect of probiotics was partially mediated through the reduction in Th22 cells.

Conclusion: Perinatal maternal probiotic supplementation with a combination of LGG, Bb-12 and La-5 reduced the proportion of Th22 cells in 3-month-old children. This may partially explain the preventive effect of probiotics on AD.

KEYWORDS
atopic dermatitis, probiotic, ProPACT, regulatory T cell, T cells, Th1, Th17, Th2, Th22

The senior authors Videm and Øien are contributed equally to the study.

1 | INTRODUCTION

Currently, there is no cure for atopic dermatitis (AD), one of the most prevalent chronic diseases in childhood, affecting more than 20% of children.[1] Thus, there is a growing interest in primary prevention of AD. Perinatal probiotics reduce the risk of AD, and the combination of prenatal and post-natal supplementation appears to be most effective.[2-4] In the Probiotics in the Prevention of Allergy among Children in Trondheim (ProPACT) study, we observed that probiotics given to mothers from 36 weeks gestation to 3 months post-natally reduced the cumulative incidence of AD in the offspring by 40% at 2 years,[4] with a trend towards an ongoing benefit until 6 years.[5] However, the mechanism for this preventive effect is unclear.

The pathophysiology of AD involves barrier defects, environmental factors and immune dysfunction.[6,7] The barrier defect is typically an early feature of AD which may be primary, but may also be secondary to the skin inflammation.[7-9]

T helper (Th) cells are thought to be particularly important in the inflammation of AD, determining the degree and direction of the immune response.[6] Regulatory T cells (Tregs) are essential for limiting the excessive response.[10] Th cells are CD4+ T cells that exert their action mainly by activating and recruiting other cell types, and reduced Th1/Th2 ratio has been considered important for atopic diseases.[11] This skewing towards a Th2 response and low numbers of Tregs early in life are risk factors for later development of AD.[12,13] More recently, other Th cell subsets like Th9, Th17 and Th22 have been shown to influence inflammation in AD.[11,14-17]

Early intestinal microbiota is essential for normal maturation of the immune system and T cell balance,[18-20] and unfavourable composition of the microbiota is associated with T cell inflammation in AD.[21,22] Probiotic bacteria have the ability to modify the Th balance through their effects on dendritic cells,[23-26] but there are conflicting results on the distinct effects of probiotics on T cell subsets in children. Some studies have reported a skewing towards a Th1 profile with increase in interferon gamma (IFNγ), while others have reported no effect on either the Th1/Th2 balance or Tregs.[27-30] The effect of probiotics on Th9, Th17 and Th22 cells has not been studied previously.

Given the importance of T cell subsets in the pathogenesis of AD, we hypothesized that the risk reduction for AD observed in the ProPACT study may be due to an increase of regulatory and decrease of inflammatory Th cells in the children.[6]

Our aim of this study was to examine whether the Th cells were affected by maternal probiotic supplementation and whether the preventive effect of probiotics on AD was mediated through an effect on Th cells.

2 | METHODS

2.1 | Study population

Study subjects were recruited from the double-blinded randomized, placebo-controlled ProPACT study.[4] Briefly, 415 pregnant women from an unselected population were computer-randomized to ingest probiotic milk corresponding to a daily dose of $5x10^{10}$ colony-forming units (CFU) *Lactobacillus rhamnosus* GG (LGG), 5×10^{10} CFU *Bifidobacterium animalis* subsp. lactis Bb-12 (Bb-12) and 5×10^{9} CFU *Lactobacillus acidophilus La-5* (La-5), or placebo from 36 weeks of gestation to 3 months post-natally while breastfeeding.

Information regarding demographics and risk factors for allergy-related diseases was obtained from questionnaires completed during pregnancy, at the ages of 6 weeks, 1 year and 2 years. AD was defined according to the U.K. working party's diagnostic criteria for AD.[31] Children were offered and recommended an examination by a trained nurse if they had an itchy rash for more than 4 weeks. A paediatrician examined all children at 2 years. The severity was assessed with the Nottingham Eczema Severity Score (NESS).[32] Blood samples were collected from the children at 3 months postpartum, marking the conclusion of the maternal probiotic supplementation period. Peripheral blood mononuclear cells (PBMC) were separated within 8 hours using density centrifugation with Lymphoprep (Fresenius Kabi, Oslo, Norway) and stored in freezing media containing 20% DMSO in liquid nitrogen.

Children with complete questionnaires, who attended the clinical follow-up at 2 years and had available PBMC with adequate cell count for analysis, were eligible for inclusion in this study. Samples from 140 children, 68 from the probiotic group and 72 from the placebo group, were included (Figure 1). All participating mothers signed a written consent form. The study was approved by the Regional Committee for Medical Research Ethics in Central Norway (097-03) and registered at ClinicalTrials.gov (NCT00159523).

2.2 | Cell analyses

Thawed cells were rested overnight at 10^5-10^6 cells/mL in RPMI-1640 medium supplemented with L-glutamine and 10% fetal calf serum in 24-well flat-bottomed plates in a 37°C humified atmosphere containing 5% CO_2. Samples with cell count$>1.5\times10^6$ were split into aliquots, where one aliquot was stained unstimulated for Treg markers using CD3 FITC/CD4 e-fluor450/CD25 APC, CD127 PerCP-Cy5.5/FoxP3 PE/fixable viability dye 506 (FVD 506). The second aliquot was stained for intracellular cytokines after stimulation for 5 hours with 50 ng/mL phorbol 12-myristate 13-acetate, 950 ng/mL ionomycin with brefeldin A and monensin (eBioscience, San Diego, CA, USA) using CD3 FITC/CD4 e-fluor450/IFNγ PE-Cy7/IL-4 PE/IL-9 PerCP-efluor710/IL-17A APC-efluor780/IL-22 APC-efluor660/FVD 506. Samples with cell count $<1.5\times10^6$ were only stained for Treg. Treg staining was performed on all 140 samples whilst stimulation and subsequent cytokine staining for detection of the other Th subsets could be performed in 77 samples. All antibodies were purchased from eBioscience.

Samples were analysed on a FACSCantoII flow cytometer, and gating was performed with FACSDiva software v6.1.3 (BD Bioscience, San Jose, Ca). A minimum of 100 000 events were collected. The Th cells were gated as CD3+ and CD4+. Further characterization was as follows: Treg (FoxP3+/CD25hi+/CD127$^{lo/neg}$), Th1 (IFNγ+), Th2

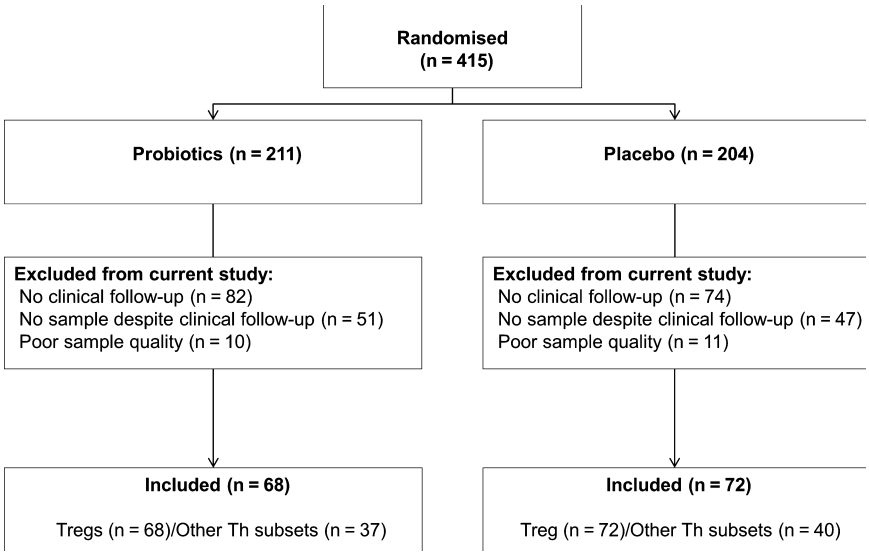

FIGURE 1 Inclusion of participants

(IL-4+/IFNγ^{neg}), Th9 (IL-9+), Th17 (IL-17+), Th22 (IL-22+/IL-17^{neg}). Fluorescence-minus-one (FMO) (samples stained with all antibodies except the one of interest) and internal cell populations were used as controls. Positive events were defined as positive above the level of controls. Proportions of T cell subsets are given as percentages of the total Th cell population. Detailed gating strategy is shown in Figure S1.

2.3 | Statistics

STATA IC13.1 (StataCorp, College Station, TX, USA) was used for statistical analysis. Data are given as percentages with 95% confidence interval (CI) assuming a binominal distribution for categorical data and mean with standard deviation or median with interquartile range (IQR) for continuous data as appropriate. Differences between groups were analysed with the chi-square test for categorical data and t-test or Wilcoxon-Mann-Whitney test for continuous data. Trends were tested with Cuzick's nonparametric test for trends across ordered groups. Regression analysis was performed after Box-Cox transformation to normality. Two-sided *P*-values <.05 were considered statistically significant.

We performed mediation analysis to estimate to what extent the effect of the probiotics on AD could be attributed to an effect on the Th cell subsets. Mediation, in statistical terms, is when some or all of the effects of the exposure on the outcome are caused via a third variable, the mediator.[33] If this is the case, the total effect can be partitioned into two parts: the direct effect and the indirect effect. The direct effect is the effect of the exposures not going through the mediator, and the indirect effect is the effect going through the mediator. Recent methods of mediation analysis use a counterfactual framework and allow for the natural variation of the level of the mediator between the subjects.[34] The natural direct effect (NDE) is then defined as the effect of the exposure on the outcome if the mediator is fixed to the level it would be in the absence of the exposure. The natural indirect effect (NIE) is defined as the effect on the outcome if the exposure is fixed to present, and the mediator is changed from the level it would take in the presence of exposure, to the level it takes in the absence of the exposure. The total effect is then a combination of the NDE and the NIE. In this model, Total effect=NIE*NDE.

We performed causal mediation analysis using the user-written "paramed" command in Stata[35] as this allows for categorical outcomes. This command uses a counterfactual framework combining the results of two parametric regression models: a linear regression of Th subset proportion (the mediator) conditional on probiotics (the exposure) and a log-linear regression model of AD (the outcome) conditional on probiotics and the Th subset proportion. Due to the high prevalence of AD, we chose a log-linear model, as this relaxes the assumption of a rare outcome. This model gave the effect in risk ratio (RR).[36] Causal mediation analyses were performed only for the Th subsets that were significantly associated with probiotics. The hypothetical model is illustrated by a directed acyclic graph (Figure 2).

Potential confounders of the relationship between Th subset proportions and AD development included atopy in the family, maternal atopy, pets or the use of antibiotics the first year. None of these were included as covariates in the mediation analysis as they were not statistically significantly associated with the proportion of Th22 cells in separate analyses (Table S1).

3 | RESULTS

There were no significant differences in baseline characteristics of the pregnant women and their children between the two study groups (Table 1). Consistent with the findings of the main study, there was a statistically significant reduction in the risk of AD following probiotic supplementation (Table 1). The distribution of baseline characteristics and outcome for the participants included in this study were similar to the participants of the original ProPACT study

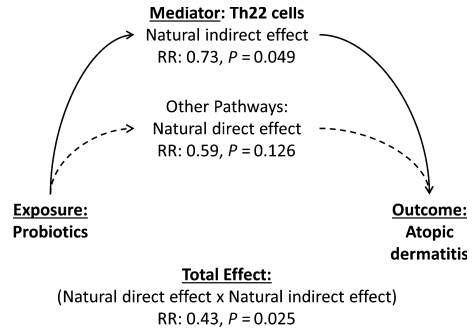

FIGURE 2 The Th22 cells as potential mediator of the preventive effect of probiotics on AD. A directed acyclic graph illustrating the causal model where the effect of probiotics on AD partly goes via the effect on Th subsets. Mediation analysis was conducted to determine the extent to which Th22 mediated the preventive effect. The estimates are given as risk ratios (RRs). The natural indirect effect estimates the effect through the mediator Th22 (solid line). The natural direct effect estimates the remaining effect of probiotics on AD going through other pathways (dashed line). The total effect is the product of the natural indirect and the natural direct effect

(Figure S2). During the two-year follow-up, 44 (31.4%) children fulfilled the diagnostic criteria for AD, of which 16 had debut of AD before 3 months of age. The severity of AD was mild to moderate, with a median NESS score of 8 (IQR 6-9). Fifteen of these children had transient AD and no longer fulfilled the diagnostic criteria for AD at 2 years. Median age of debut for AD was 5 months (IQR 2-15) for the probiotic group and 6 months (IQR 3-12) for the placebo group.

The proportion of Th cell subsets as percentages of the total Th cell population in the probiotic and placebo groups are shown in Table 2. Th9 cells were not detectable in any samples, and Th22 cells were not detectable in 1 of 77 samples. The median proportion of Th1 cells in boys was statistically significantly higher than in girls (1.0% (95% CI 0.65-1.3) vs 0.76% (95% CI 0.55-0.95), *P*=.039). For the other Th subsets, no gender differences were observed.

TABLE 1 Characteristics of the study groups

Characteristics	Probiotic (n=68)	Placebo (n=72)
Male gender, % (95% CI)	51.5 (39.4-63.3)	40.3 (29.4-52.2)
Born > 2 weeks before term, % (95% CI)	5.4 (1.7-15.8)	6.0 (2.2-15.2)
Antibiotics first year, % (95% CI)	19.4 (11.4-30.9)	21.1 (13.0-32.4)
Maternal smoking, % (95% CI)	3.0 (0.1-11.5)	2.8 (0.7-10.8)
Maternal atopy, % (95% CI)	38.2 (27.3-50.6)	50.7 (39.0-62.3)
Atopy in the family, % (95% CI)	63.2 (50.9-74.0)	70.4 (58.5-80.1)
Siblings, % (95% CI)	45.6 (33.9-57.8)	37.5 (26.9-49.4)
Pets in the house, % (95% CI)	20.6 (12.4-32.2)	26.4 (17.3-38.0)
Birthweight, g (SD)	3692 (448)	3595 (492)
Maternal age, years (SD)	31 (3)	30 (4)
Allergy-related disease at 2 years, % (95% CI)		
Atopic dermatitis	23.5 (14.8-35.3)	38.9 (28.1-50.8)
Allergic Rhinoconjunctivitis	1.5 (0.2-10.2)	0
Asthma	5.9 (2.2-15.0)	11.1 (5.6-21.0)

Data are given as percentages with 95% confidence interval (CI) for frequencies and mean with standard deviation for continuous data.

The proportion of Th22 cells was significantly lower in the probiotic group compared to the placebo group (median 0.038 vs 0.064, *P*=.009) (Table 2). This difference between the probiotic and the placebo group was also observed in the subgroup of children who did not develop AD (median 0.034 vs 0.060, *P*=.018), but not for the children who developed AD (median 0.096 vs 0.090, *P*=.64) (Figure 3).

When considering the effect of the proportion of Th22 cells on subsequent development of AD in the first 2 years of life, independent of probiotic supplementation, we found an increased proportion of Th22 cells among children who developed AD compared to those who did not (0.090 vs 0.044, *P*<.001). The Th22 proportion also increased with increasing severity of AD as measured by the NESS score (*P*<.001) and decreased with later debut age (*P*<.001) (Figure 4).

There were no significant differences in the proportions of other Th subsets or the Th1/Th2 ratio between the probiotics and placebo groups (Table 2), nor were the other Th subsets associated with the incidence, severity or age of debut of AD.

For the subgroup of participants with all the Th subsets analysed (n=77), the RR for developing AD by 2 years of age was 0.43 (95% CI: 0.21-0.90, *P*=.025) in the probiotic compared to the placebo group. This can be considered the total effect of probiotics on the cumulative incidence of AD in this group. Causal mediation analysis suggested that there was a significant effect of probiotics on AD mediated by changes in the proportion of Th22 cells (NIE RR: 0.73, 95% CI: 0.54-1.00, *P*=.049); see Figure 2.

4 | DISCUSSION

In the present study, maternal probiotic supplementation led to a statistically significant lower proportion of Th22 cells in three-

TABLE 2 T cell subpopulations in children by intervention group

	Probiotic n=68 (37)[a] Median (IQR)	Placebo n=72 (40)[a] Median (IQR)	*P*-value
Tregs	3.3 (2.5-4.4)	3.6 (2.7-4.5)	.25
Th1	0.8 (0.60-1.2)	0.81 (0.54-1.2)	.68
Th2	0.23 (0.14-0.33)	0.20 (0.14-0.28)	.51
Th9	<0.01	<0.01	
Th17	0.21 (0.079-0.300)	0.15 (0.068-0.21)	.25
Th22	0.038 (0.028-0.059)	0.064 (0.045-0.092)	**.009**
Th1/Th2 Ratio	4.5 (2.9-6.2)	4.4 (2.9-7.4)	.68

Proportions of T cell subsets are given as percentages of total Th cell population. Groups were compared using the Wilcoxon–Mann–Whitney *U*-test. Significant *P*-values in bold.

[a]Treg staining was performed on all samples (n=140). Cytokine staining for detection of the other Th subsets was performed on samples with adequate cell counts (n=77).

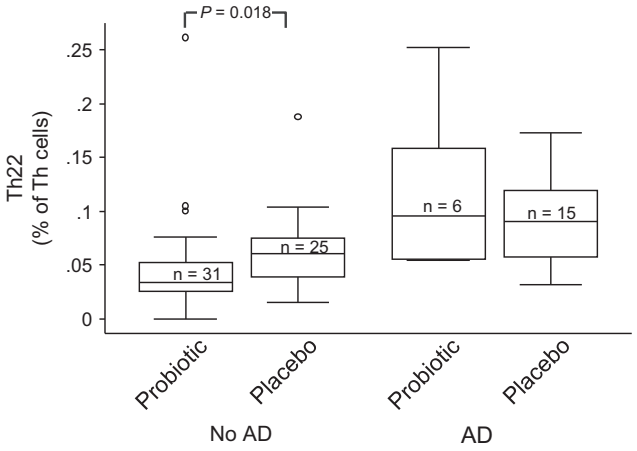

FIGURE 3 The proportion of Th22 by intervention group and presence of AD. The proportion of Th22 cells was significantly lower in the probiotic group in the subgroup of children without AD (*P*=.018), but not in the children with AD (*P*=.64)

month-old children. Mediation analysis suggested that the reduction in Th22 cells was a partial mediator of the preventive effect by probiotics on AD. There were no statistically significant effects of maternal probiotic supplementation on Treg, Th1, Th2, Th9 and Th17 cell proportions. To our knowledge, this is the first study to show that maternal probiotic supplementation affects the Th22 subsets in the children and that this effect may explain some of the preventive effect of probiotics on AD.

Several studies have shown that probiotic intake during pregnancy and early infancy may act as a primary preventive measure against AD.[2-4] This preventive effect may be mediated through an early colonization of beneficial bacteria like LGG. The early gut microbiota is known to be important for shaping the immune system and establishing the Th cell balance.[18] Our group has previously shown that LGG bacteria ingested by mothers colonized their gut and that the LGG was transferred to the gut microbiota of the children.[37]

Previous animal and in vitro studies suggest that immunomodulatory effects of probiotics are related to an increase of Tregs.[23] However, similar to previous clinical studies on the effects of probiotics in children, we did not find that maternal probiotics affected the Tregs.[29,30] Likewise, effects on the Th1 and Th2 subsets have been hypothesized as possible mechanisms for the beneficial effects of probiotics on AD.[23-26] Significant effects on the Th1 and Th2 cell proportions were not found in this study. Some previous intervention studies, but not all, have found that probiotics increase the Th1 cytokines.[27-30,38] These conflicting results may be due to differences in dose, duration or strain of probiotics as well as timing of blood sampling.

We found a significantly lower proportion of Th22 cells in the probiotic group. Furthermore, mediation analysis suggested that this effect may partly explain the preventive effect of probiotics on AD. Taken together, our findings challenge the view that the mechanism of the preventive effect of maternal probiotics on AD is mediated by the Tregs or the Th1/Th2 axis. Instead, our results suggest that a reduction in Th22 cell proportion may play a role in the preventive effect of probiotics on AD.

Although this study cannot explain precisely how a reduced Th22 proportion might contribute to the preventive effect of maternal probiotics on AD, this finding is consistent with recent research that has highlighted the importance of Th22 in the development of AD.[7] Th22 cells, with their main cytokine IL-22, potentially have both protective and harmful effects on the skin and mucosa depending on the context. The IL-22 receptor is mainly expressed in non-hematopoietic cells, including epithelial cells. IL-22 improves the epithelial barriers by inducing keratinocyte proliferation, increased mucus production and antimicrobial peptides.[39,40] On the other

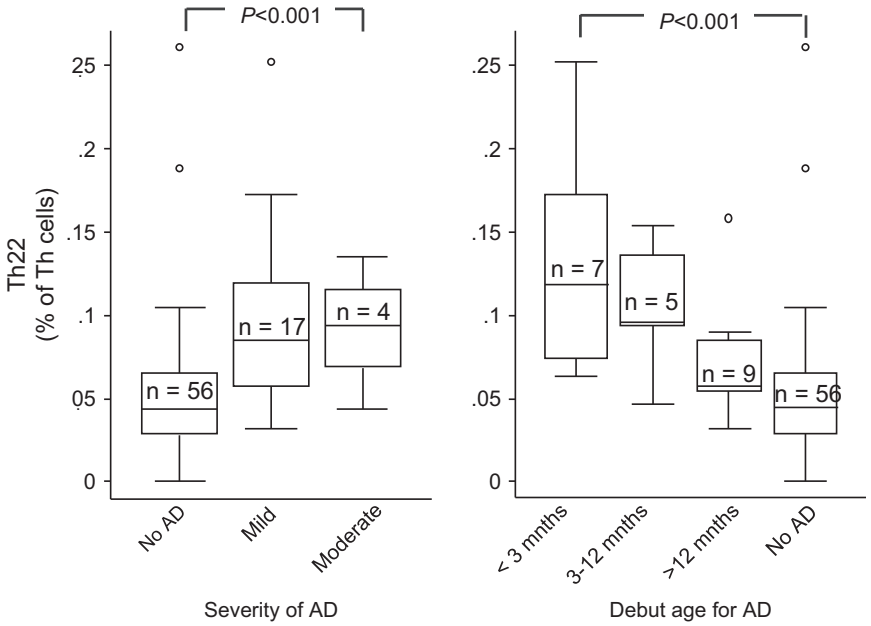

FIGURE 4 The proportion of Th22 by the severity and age of debut of AD. The proportion of Th22 increased with increasing severity of the AD as measured with NESS score (*P*<.001) and decreased with age of debut (*P*<.001)

hand, there is evidence that IL-22 destabilizes the skin barrier in inflamed skin by inducing keratinocyte hyperplasia and suppressing the production of proteins crucial for epidermal integrity, such as keratin and filaggrin.[41,42] Furthermore, IL-22 amplifies the inflammatory response by inducing keratinocyte release of proinflammatory proteins and chemokines.[40,43] Thus, a lower Th22 cell proportion may block an early vicious cycle of inflammation and barrier defects and thereby prevent the development of symptomatic AD.

As some children with AD had high levels of Th22 despite being in the probiotic group, one can speculate whether the preventive effect of probiotics was restricted to specific populations. Our group has recently reported that the effect of probiotics in the prevention of AD can be dependent on the intrinsic microbiota.[44] High levels of *Bifidobacterium dentium* were associated with lack of the probiotic effect. Polymorphisms of pattern recognition receptors may also account for a differential effect of the probiotic bacteria.[45] LGG has been shown to modulate innate signalling pathways in a dose-dependent way[25]; thus, the effect may be related to the abundance of LGG. However, due to the sample size of our study, we could not perform a reliable subgroup analysis addressing this question. Future studies including cellular models, animal models and larger randomized studies would be needed to pursue these questions.

The major strength of this study is the prospective, randomized, controlled design within a general population with a clear clinical effect of the intervention. This offers the unique possibility to investigate mechanisms of the preventive effects of probiotics on AD. Other strengths include using well-recognized criteria for AD, with clinical examinations of all the children.

There are some limitations to our study. A high proportion of the children were excluded from our study because of incomplete clinical data or missing samples. However, comparing baseline characteristics and outcome, the participants included in this study are comparable the original study population.[4] Furthermore, the randomized controlled design reduced the chance of any systematic bias between the placebo and probiotic groups; thus, the results should be valid.

Valid conclusions from mediation analysis require a correctly specified model as well as some strong assumptions. Firstly, it is assumed that the exposure (probiotic supplementation) preceded the mediator (Th22), which in turn preceded the outcome (AD). Considering the complex interaction between the epithelium and the Th subset, the reduction in Th22 cells may arguably reflect reduced skin inflammation that caused a reduction in both Th22 cell proportions and AD cases. However, the Th cell proportion was examined at 3 months of age, before most of the children developed AD. Furthermore, even in the subgroup of children that did not develop AD, lower Th22 cell proportions were found in the probiotic group compared to the control group. This supports the hypothesis that low Th22 cells was a preceding event and may be a mechanism for the protective effect of probiotics on AD.

Other assumptions of the mediation analysis revolve around the absence of unmeasured confounding between combinations of the exposure, mediator and outcome.[36] The randomized design meets the condition for mediation analysis that there are no unmeasured confounders of the relationship between the exposure and the outcome, or between the exposure and the mediator. However, additional assumptions not ensured by the randomization are that there should be no unmeasured confounders of the relationship between the mediator and the outcome, and no mediator–outcome confounder that are affected by the exposure. Heritable and environmental risk factors of AD may be potential confounders of the mediator–outcome relationship, as they potentially may also affect the Th cell proportions. Due to the small sample size, it was not feasible to include additional covariates in the mediation model. That said, the presence of atopy in the mother or family, pets in the house or the use of antibiotics the first year was not significantly associated with the proportion of Th22 cells in single variable analyses. It therefore seems reasonable that the exclusion of these potential confounders has not significantly impacted the results. However, possible associations may have been discovered in a larger sample, and not including these as confounders could have biased our results.

5 | CONCLUSION

Perinatal maternal probiotic supplementation with a combination of LGG, Bb-12 and La-5 reduced the proportion of Th22 cells, but did not affect the proportion of Tregs, Th1, Th2, Th17 cells or the Th1/Th2 ratio in the offspring. Furthermore, the study suggests that the prevention of AD in offspring following maternal probiotic supplementation may partially be due to this reduction in Th22 cell proportion. Larger studies, as well as studies on basal biological mechanisms, are needed to confirm the results.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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