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The role of infant gut microbiota modulation by perinatal maternal probiotic intervention in atopic eczema risk reduction

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Short title: Probiotics, gut microbiota modulation, and the risk of atopic eczema

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Abstract

Introduction: Probiotics have shown potential in reducing the occurrence of atopic eczema in high-risk infants. We aimed here to assess whether the preventive effect of maternal probiotic administration stems from compositional changes in early gut microbiota.

Methods: This study included 46 mother-infant pairs from an original randomized controlled trial assessing the impact of maternal probiotic intervention with either the combinations of *Lacticaseibacillus rhamnosus* LPR and *Bifidobacterium longum* BL999, or *Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999, or placebo beginning 2 months before expected delivery and ending 2 months after birth. All children were vaginally delivered, full term and breastfed. During the two-year follow-up period, the children were clinically evaluated by physicians for atopic eczema, and their gut microbiota was profiled at 1 and 6 months of age by 16S rRNA gene sequencing using an Illumina sequencing platform.

Results: Altogether, 19 of 46 children developed atopic eczema by the age of two years. At 1 and 6 months of age, gut microbial diversity was similar between children who developed atopic eczema and their healthy controls, but at the age of 6 months, children who developed atopic eczema manifested with significantly higher relative abundance of Clostridia. Probiotic intervention did not significantly influence microbial diversity, and the effects on microbial composition were not consistent with the changes associated with the development of atopic eczema.

Conclusion: The reduction of the risk of atopic eczema achieved by perinatal maternal probiotic intervention does not seem to require substantial gut microbiota modulation.

Original trial registration information: ClinicalTrials.gov identifier NCT00167700

Introduction

Specific probiotics have been documented to have clinical potential in reducing the risk of atopic eczema (AE) in genetically predisposed children [1]. Subsequently, the perinatal period has been shown to be a critical time for probiotics to exert this effect [2,3]. Today, the World Allergy Organization recommends considering probiotics for primary prevention of AE in pregnant and breastfeeding women at high risk for allergy in their children [4].

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [5]. Hence, the term "probiotic" underlines the importance of clinical efficacy. A dilemma in the scientific rationale for probiotic use may arise from defining substances advocated for microbiota modulation strategies by a health benefit, omitting the microbiota impact. While the effects of specific probiotics in the prevention of AE have mainly been attributed to restoration to normal of increased intestinal permeability, improvement of the intestine's immunological barrier functions, and reduced generation of proinflammatory cytokines [6,7], the present study addresses the question whether the effect of specific probiotics in primary prevention of AE is in part mediated by changes in the gut microbiota. The question is justified in concert with the disappearing microbiota hypothesis, according to which the increased prevalence of non-communicable diseases stems from the loss of ancestral microbial contacts and depletion of microbial diversity due to modern medical practices, improved sanitation, urban lifestyle, altered nutrition, and decreasing rates of breastfeeding [2,8]. Without corrections, imbalanced microbial communities are being transmitted from mothers to infants perpetuating the cascade effect from one generation to another [2,8].

With the gaps in current knowledge, we studied the gut microbiota composition of children whose mothers received probiotic intervention or placebo and children who developed AE with those who did not. We hypothesized that maternal probiotic intervention modulates infant gut microbiota composition, and the modifications are associated with a reduced risk of AE.

Methods

This study was based on a randomized, double-blind, placebo-controlled clinical trial, including 241 mothers with atopy and their infants, aiming to evaluate the impact of maternal probiotic intervention on the occurrence of AE in infants [9]. The mothers were determined as atopic by positive skin prick testing and by reported clinical history of AE, food allergy, allergic rhinoconjuctivitis, or asthma. When entering to the original study, all mothers were randomly assigned to receive a probiotic combination of either *Lacticaseibacillus rhamnosus* LPR (formerly *Lactobacillus rhamnosus*) and *Bifidobacterium longum* BL999 or a combination of *Lacticaseibacillus paracasei* ST11 (formerly *Lactobacillus paracasei*) and *Bifidobacterium longum* BL999 or placebo. The intervention was initiated 2 months before expected delivery and continued for the first 2 months during breastfeeding. The daily dose was 1×10^9 CFUs given as a powder in a sachet of 7 g/d and diluted in a glass of water. No probiotics were given directly to the infants. The children were followed up to 2 years of age.

From the original study population, 48 children who were full term, vaginally delivered, did not receive any antibiotic treatments for the first week after birth, were breastfed for at least 1 month, committed to the 2 years of follow up and gave fecal samples at the ages of 1 and 6 months, were selected for the secondary microbial analyses [10]. As 8 fecal samples were removed due to insufficient analytical quality, two children were left without any samples and were removed from the analyses, leaving the final study population of 46 children. Of the 46 subjects, 20 children had been included in the placebo group and 26 children in the maternal probiotic intervention groups. From the infants whose mothers had received probiotic intervention, 16 children were exposed to maternal intervention by the combination of *Lacticaseibacillus rhamnosus* LPR and *Bifidobacterium longum* BL999, and 10 children were exposed to the combination of *Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999. Altogether, 22 subjects had been exposed to maternal intrapartum antibiotic treatment and 24 children had not.

The study physicians examined all children during scheduled control visits at 1, 3, 6, 12 and 24 months of age. The diagnosis of AE was based on the criteria introduced by Hanifin consisting of pruritus, typical morphology, and age-related distribution and chronicity [11]. AE was defined as relapsing if two or more AE episodes occurred, and chronically persistent if the episodes lasted longer than a month without remissions. The prospectively collected study records included maternal medical data, information regarding the duration of gestation, mode of delivery, use of intrapartum antibiotics, duration of breastfeeding, and children's health data. JMP Pro 16.2.0 was used in statistical analyses when differences in clinical characteristics of the study population were compared between the placebo group and probiotic intervention groups and between children who developed AE and children who did not. A two-sample t-

test was utilized for continuous variables and with a Fisher's Exact Test for categorial variables. P-values <0.05 were considered statistically significant.

The fecal samples for microbial analyses were collected at the ages of 1 and 6 months and stored at -80 °C for subsequent processing. Microbial DNA extraction and sequencing by Illumina MiSeq sequencing platform have been described previously [10]. Briefly, microbial DNA was extracted from stool samples using an automated InviMag® Stool DNA Kit (Stratec Molecular, Berlin, Germany) and a KingFisher magnetic particle processor (Thermo Fisher Scientific Oy, Vantaa, Finland) with some necessary modifications [12]. Then, a 2 x 300bp paired-end run Illumina MiSeq platform (FISABIO sequencing service, Valencia, Spain) was used for amplification of primers targeting the V3-V4 region of 16S rRNA gene [13]. The quality of sequences was assessed by Prinseq lite program (min_ length:50; trim_qual_right:20; trim_qual_type:mean; trim_qual_ window:20) [14]. Taxonomic assignment of the referenced ASVs was carried out using QIIME 2 version 2023.5 and the SILVA database version 138.1. The microbial differences between groups based on time of the sample collection, probiotic intervention, and AE development were analyzed using MicrobiomeAnalyst 2.0 [15] with data normalized by cumulative sum scaling (CSS). Analysis of variance (ANOVA) was used to analyze the differences between diversity indexes, richness, and microbial taxa among the groups. For the multivariate analysis of bacterial taxa, permutational analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) were conducted. Finally, DESeq2 method was used to identify differentially abundant taxa [16].

Results

In total, 19 of 46 children developed AE during the first two years of life. In line with our previously published primary outcome of the original study [9], the development of AE was significantly more common in the children whose mothers received placebo as compared to the probiotic interventions (p = 0.0008), (Table 1). No additional significant differences in clinical characteristics were observed between the children in the probiotic intervention groups and the children in the placebo group.

Gut microbiota composition in children developing atopic eczema

First, we analyzed microbiota differences between children who developed AE and those who did not, irrespective of maternal probiotic intervention. The gut microbiota alpha diversity and beta diversity were similar between children developing and not developing AE at the ages of 1 and 6 months (Fig. 1). In the analyses of microbial taxa, no differences were detected at the age of 1 month, but at the age of 6 months, the relative abundance of the class Clostridia was higher in children who developed AE compared to children who did not (p = 0.0044, false discovery rate (FDR) = 0.030), (Fig. 1). At this age, 14 out of 19 children had already developed clinical eczema.

The effect of maternal probiotic intervention on infant's gut microbiota composition

Second, we analyzed microbiota differences between the intervention groups, irrespective of possible development of AE. The gut microbiota alpha and beta diversities did not significantly differ among the children whose mothers received either of the probiotic interventions or placebo at the ages of 1 or 6 months (Fig. 2). At both time points, Actinomycetota and Bacillota were the most common phyla in children in both probiotic intervention groups and in the placebo group (Fig. 3). The relative abundance of Actinomycetota decreased and the relative abundance of Bacillota increased from the age of 1 month to the age of 6 months in all three groups. At the family level, *Bifidobacteriaceae* were the dominant bacteria at both time points, but their relative abundance decreased from 1 to 6 months of age. Children whose mothers received placebo appeared to exhibit a slightly lower relative abundance of *Bifidobacteriaceae* as compared to those whose mothers received either probiotic intervention. In the taxonomic profiling, no statistically significant differences were detected at the age of 1 month. At the age of 6 months, Bacillota tended to have higher relative abundance in children in the placebo group (p = 0.0038, FDR = 0.018), (Fig. 3).

The effect of maternal probiotic intervention on gut microbiota composition in children developing and not developing atopic eczema

Third, we analyzed the differences between children who developed AE and those who did not, while also taking the intervention into account. When the gut microbiota composition of the children in the

intervention groups was analyzed separately for those who developed or did not develop AE, no significant differences were found in alpha diversity (Fig. 4). When focusing only on children in two probiotic intervention groups, we observed that children who developed AE exhibited significantly lower gut microbiota richness compared to those who did not ($p = 0.040$) at 6 months of age (Fig. 4). There were no differences in beta diversity between children who developed AE and children who did not develop AE among children whose mothers received probiotic interventions. When focusing only on the children whose mothers received the probiotic combination of *Lacticaseibacillus rhamnosus* LPR and *Bifidobacterium longum* BL999, there were no significant differences in alpha diversity or in beta diversity between children who developed AE and children who did not at 6 months of age. The same was seen in diversity analyses of children whose mothers received the probiotic combination of *Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999. In the taxonomic profiling of children whose mothers received either probiotic, the relative abundance of the class Coriobacteriia was found to be significantly higher in children who did not develop AE compared to those who did at 6 months of age ($p = 0.013$, FDR = 0.045). This significance remained when the probiotic intervention groups were analyzed separately ($p = 0.0011$, FDR = 0.0075), (Fig. 5). When focusing only on children whose mothers received the probiotic combination of *Lacticaseibacillus rhamnosus* LPR and *Bifidobacterium longum* BL999, no significant differences were seen at the family or genus levels between children developing and not developing AE, but when focusing on children whose mothers received the probiotic combination of *Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999, the taxonomic profiling showed significantly lower relative abundance of the family *Oscillospiraceae* ($p < 0.0001$, FDR < 0.0001) and genera *Blautia* ($p < 0.0001$, FDR < 0.0001) and *Intestinibacter* ($p = 0.0002$, FDR = 0.0008) in children who develop AE compared to those who did not (Fig. 5).

Discussion

This study suggests that maternal probiotic administration, timed during the perinatal period and breastfeeding, conveys the risk reduction of AE in high-allergy-risk children primarily through mechanisms other than long-lasting microbiota modulation. Our original clinical trial [9] demonstrated that maternal probiotic intervention significantly reduces the risk of clinically diagnosed AE in children with high allergy risk. The present study shows that the maternal probiotic intervention is associated only with modest compositional changes in infant gut microbiota that do not consistently correlate with the development of AE.

In the present study, the development of AE was associated with higher relative abundance of Clostridia at the age of 6 months, and in children whose mothers received either of the probiotic intervention, also with lower abundance of the class Coriobacteriia and lower richness. In the infants whose mothers received the probiotic *Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999, the development of AE was associated with lower abundance of *Oscillospiraceae* family, and *Blautia* and *Intestinibacter* genera. In previous studies, several different bacterial taxa have been associated with the development of AE and some evidence of lower microbial diversity exists [17]. Whilst some studies have suggested a causal link between microbial changes and the development of AE [18,19], in our study, the compositional changes were only present at the age of 6 months, and thus, we cannot ensure that these changes occurred prior to the development of AE.

The effect of maternal probiotic intervention on infant gut microbiota at 1 and 6 months of age was minor. No effect was observed in biodiversity measures. In taxonomic profiling, only Bacillota were observed to have a tendency for lower abundance in children in probiotic intervention groups at 6 months of age, but no other taxa emerged to explain it.

Most likely, specific probiotics reduce the risk of AE through the interaction of several mechanisms. Previous experiments have demonstrated that probiotics can enhance gut barrier functions [6]. This may be pivotal as AE has been recognized as an epithelial barrier disease, albeit the focus has been more on the skin. Interestingly, an increased permeability with subsequent activation of humoral immune responses is observed in both milieus in atopic patients [6,20,21]. Specific probiotics reportedly control both anti-inflammatory and proinflammatory mediators [6,7,22] and may thus depress atopic inflammation and induce tolerance. During early breastfeeding, the reduced ratio of bifidobacteria to clostridia has previously been suggested to associate with the susceptibility of high-allergy-risk children to develop atopy [23]. Moreover, bifidobacteria have been observed to control inflammation in the host [24]. Thus, the early gut microbiota and immunological effects of specific probiotics seem to intertwine. Birth and breastfeeding are

the main events that initiate the formation of the early gut microbiota and most of the bacteria in the infant gut can be traced to the mother [25]. As the maternal probiotic intervention during pregnancy and breastfeeding achieved significant risk reduction of AE with no consistent microbial changes, the effect of these specific probiotics likely stems from the very early interaction between the child and the probiotic bacteria that activate immunological mechanisms, or mechanisms yet to discover, rather than microbiota modulation. In some children, the probiotic intervention was not sufficient for protection against development of AE and likely, an unknown external trigger might be required.

The main strengths of this study are the uniform study population and the prospective design. All children shared similar hereditary risk as the mothers exhibited atopic sensitization and had clinical manifestations of atopic disease. Additionally, all children were vaginally delivered, breastfed at least for a month, and were not exposed to neonatal antibiotics, minimizing confounding. The number of participants was relatively small, and therefore it is possible that there may have existed differences in the gut microbiota that this study was unable to detect. This is particularly true in microbiome research, in view of the fact that an important part of the human microbiome remains unidentified. Although significant differences did arise, limitations related to the small sample size should be acknowledged." It would have been interesting to investigate maternal gut microbiotas, but these analyses were not available. AE was diagnosed by licensed physicians and no parental assessment or recall was applied. Some children may have developed AE after 2 years of follow-up, but longer follow-up period would have increased the likelihood of confounding factors.

The development of AE is associated with specific changes in the infant gut microbiota. This might originate from the depletion of microbial contacts during pregnancy and infancy in accordance with the disappearing microbiota hypothesis. Maternal probiotic intervention reduces the risk of atopic eczema in high-allergy-risk children. While microbiota modulation might contribute to this process, control of inflammation is probably of greater importance to convey the strong clinical effect. More studies are needed to uncover the intricate origin of atopic eczema and the mechanism of action of probiotics.

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Statement of Ethics

The present study was conducted ethically in accordance with the Declaration of Helsinki. Oral and written informed consents were obtained from the caregivers before entering the trial. The fecal samples were collected during the original clinical trial, which was approved by the Ethics Committee of the Hospital District of Southwest Finland (10/2004, §290).

Conflict of Interest Statement

The authors have no conflict of interest to declare.

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Author Contributions

Reetta Puisto: data curation, formal analysis, writing - original draft

Carlos Gómez-Gallego: formal analysis, writing – original draft, writing – review and editing

María Carmen Collado: formal analysis, writing – review and editing

Olli Turta: data curation, writing – review and editing

Samuli Rautava: data curation, supervising, conceptualization, writing – review and editing

Erika Isolauri: supervising, conceptualization, writing - review and editing

Data Availability Statement

The data that support the findings of this study are not publicly available due to their containing information that could compromise the privacy of research participants but are available from corresponding author (R.P.) upon reasonable request.

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Figure legends

Fig. 1. Gut microbiota alpha diversity, beta diversity and taxonomic profiling at 1 and 6 months of age in children developing and not developing atopic eczema.
The gut microbiota diversity analyses in children developing and not developing atopic eczema by 2 years of age by Shannon index, observed richness and permutational analysis of variance (PERMANOVA) at 1 month of age (a – c), respectively, and at 6 months of age (d – f). The difference in the relative abundance of the class Clostridia at 6 months of age by DESeq2 in children who developed and did not develop atopic eczema ($p = 0.0044$, FDR = 0.030) (g).

Fig. 2. Gut microbiota alpha diversity and beta diversity at 1 and 6 months of age in children in the probiotic intervention groups and the placebo group.
The gut microbiota diversity analyses in children whose mothers received placebo, probiotic combination 1 (*Lacticaseibacillus rhamnosus* LPR and *Bifidobacterium longum* BL999) or probiotic combination 2 (*Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999) by Shannon index, observed richness and PERMANOVA at 1 month of age (a – c), respectively, and at 6 months of age (d – f).

Fig. 3. Relative abundance of the most common microbial taxa and taxonomic profiling at 1 and 6 months of age in children in the probiotic intervention groups and children in the placebo group.
The relative abundance of the most common phyla at 1 (a) and 6 (b) months of age and the relative abundance of the most common families at 1 (c) and 6 (d) months of age in children whose mothers received placebo, probiotic combination 1 (*Lacticaseibacillus rhamnosus* LPR and *Bifidobacterium longum* BL999) or probiotic combination 2 (*Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999). The difference in the relative abundance of the phylum Bacillota at 6 months of age by DESeq2 in children whose mothers received the placebo, the probiotic combination 1 or the probiotic combination 2 ($p = 0.0038$ FDR = 0.018) (e).

Fig. 4. Gut microbiota alpha diversity and beta diversity at 6 months of age in the probiotic intervention groups and the placebo groups with and without developing atopic eczema.
The gut microbiota diversity analyses by Shannon index (a) and observed richness (b) at 6 months of age in children whose mothers received placebo, probiotic combination 1 (*Lacticaseibacillus rhamnosus* LPR and *Bifidobacterium longum* BL999) or probiotic combination 2 (*Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999) and did or did not develop atopic eczema by 2 years of age. The gut microbiota diversity analyses by Shannon index (c), observed richness (d) and PERMANOVA (e) at 6 months

of age in children whose mothers received either of the probiotic combinations and did or did not develop atopic eczema by 2 years of age.

Fig. 5. Taxonomic profiling in children in the probiotic intervention groups at 6 months of age.
The relative abundance of the class Coriobacteriia by DESeq2 at 6 months of age in children whose mothers received either probiotic combination ($p = 0.013$, FDR = 0.045) (a) or either the probiotic combination 1 (*Lacticaseibacillus rhamnosus* LPR and *Bifidobacterium longum* BL999) or probiotic combination 2 (*Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999) ($p = 0.0011$, FDR = 0.0075) (b) and did or did not develop atopic eczema. The relative abundance of *Oscillospiraceae* ($p < 0.0001$, FDR < 0.0001) (c), *Blautia* ($p < 0.0001$, FDR < 0.0001) (d) and *Intestinibacter* ($p = 0.0002$, FDR = 0.0008) (e) by DESeq2 at 6 months of age in children whose mothers received the probiotic combination 2 (*Lacticaseibacillus paracasei* ST11 and *Bifidobacterium longum* BL999) and who developed or did not develop atopic eczema.