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Pre- and postnatal *Lactobacillus reuteri* treatment alters DNA methylation of infant T helper cells

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Abstract

Background: Perinatal childhood exposures, including probiotic supplementation, may affect epigenetic modifications and impact on immune maturation and allergy development. The aim of this study was to assess the effects of pre- and postnatal *Lactobacillus reuteri* supplementation on DNA methylation in relation to immune maturation and allergy development.

Methods: DNA methylation patterns were investigated for allergy-related T helper subsets using a locus-specific method and at a genome-wide scale using the Illumina 450K array. From a randomised, double-blind, placebo-controlled allergy prevention trial with pre- and postnatal probiotic supplementation, CD4+ T helper cells were obtained at birth (from cord blood), and 12 and 24 months of age (total (placebo/probiotics); locus-specific method: CB = 32 (17/15), 12 months = 24 (9/15), 24 months = 35 (15/20); Illumina: CB = 19 (10/9), 12 months = 10 (6/4), 24 months = 19(11/8)).

Results: Comparing probiotics to placebo, the greatest genome-wide differential DNA methylation was observed at birth, where the majority of sites were hypomethylated, indicating transcriptional accessibility in the probiotic group. Bioinformatic analyses, including network analyses, revealed a module containing 91 genes, enriched for immune-related pathways such as chemotaxis, PI3K-Akt, MAPK and TGF- β signalling. A majority of the module genes were associated with atopic manifestations (OR = 1.43, $P = 2.4 \times 10^{-6}$), and a classifier built on this model could predict allergy development (AUC = 0.78, $P = 3.0 \times 10^{-3}$). Pathways such as IFN- γ signalling and T-cell activation were more hypermethylated at birth compared with later in life in both intervention groups over time, in line with DNA methylation patterns in the *IFNG* locus obtained by the locus-specific methodology.

Forsberg and Huoman shared first authorship.

Gustafsson and Jenmalm shared senior authorship.

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Conclusion: Maternal *L. reuteri* supplementation during pregnancy alters DNA methylation patterns in CD4+ T cells towards enhanced immune activation at birth, which may affect immune maturation and allergy development.

KEYWORDS

allergy, CD4+ T helper cells, DNA methylation, infants, *Lactobacillus reuteri*, postnatal, prenatal, probiotics

1 | INTRODUCTION

Individuals developing allergies already exhibit several differences in their immune system at birth,¹ indicating the importance of foetal life in immune development. Prenatal exposures to maternal dietary and microbial factors may along with genetic susceptibility affect the development of, for example, allergic disease.² Epigenetics constitutes a link between the genetic make-up of the foetus and environmental exposures provided by the mother, ultimately affecting the immune maturation of the new-born *post-partum*.¹⁻⁴ Epigenetic mechanisms modify the accessibility of DNA for transcription and comprise heritable changes in gene expression without altering the DNA sequence. Several processes modify epigenetic memory, for example, by post-translational histone modifications and methylation of DNA CpG dinucleotides.⁵⁻⁷ DNA methylation is generally considered a repressor of transcriptional activity and, in relation to other epigenetic modifications, more stable.^{6,7} In neonates, DNA methylation patterns appear to favour the Th2 skewing of immune responses, which may enable development of allergic disease.^{8,9} During pregnancy, the maternal microbial environment programmes immune development of the child¹ via epigenetic mechanisms, by regulating appropriate maturation of innate immunity as well as T helper (Th) and regulatory T (Treg) cell responses.^{1-2,6} Furthermore, the differentiation of CD4+ T cells such as Th1, Th2, Th17 and Treg cells is epigenetically regulated, also by DNA methylation.⁶

Several methods are available for the analysis of DNA methylation patterns both at a genome-wide level, and on a targeted scale. Utilising a T helper subset locus-specific method, DNA methylation patterns of single CpGs in the gene promoters of four distinct T helper cell subsets, that is Th1, Th2, Th17 and Treg cells, may be investigated. The DNA methylation patterns of these sites accurately predict commitment of naïve T helper cells into the different subsets, and as these sites are not incorporated into epigenome-wide approaches, they remain interesting to study in allergy development.¹⁰ Recently, investigations of epigenome-wide DNA methylation signatures related to allergy development in childhood have revealed associations with sensitisation, as well as food allergies and the development of asthma.^{4,11-13} However, comprehensive studies of the epigenome in CD4+ T cells early in life in allergy-prone children supplemented with probiotics are not available as yet.

In our placebo-controlled, randomised, multicentre allergy prevention trial, participants were supplemented with the probiotic

Key Message

Perinatal probiotic supplementation may affect epigenetic modifications, and epigenome-wide studies of CD4+ T cells in allergy-prone children supplemented with probiotics early in life could reveal mechanistic insights into allergy development. In this study, we assessed the effects of pre- and postnatal *Lactobacillus reuteri* supplementation on DNA methylation in relation to immune maturation and allergy development. The epigenome in CD4+ T cells from children receiving perinatal probiotic treatment was altered towards enhanced immune activation and maturation, particularly at the time of birth, suggesting that probiotics may have immune stimulating effects on the developing foetus.

strain *Lactobacillus reuteri*: mothers from gestational week (GW) 36 until delivery and the infants throughout their first year of life.¹⁴ In this cohort, probiotic supplementation resulted in significantly less IgE-associated eczema at 2 years of age¹⁴ and reduced allergen responsiveness during infancy,¹⁵ but no effect on respiratory allergies at 7 years of age.¹⁶ Other childhood probiotic allergy prevention trials have also shown promising results in preventing IgE-associated eczema, but not asthma or other allergic diseases.¹⁷ Combined pre- and postnatal supplementation seems to give the most beneficial allergy-preventive effects, indicating that epigenetic mechanisms can be important in infant immune maturation.¹⁸ Hence, studying epigenetic modifications during perinatal treatment with *L. reuteri* could give mechanistic insights into the potential allergy-preventive effects of probiotics and its possible regulation by DNA methylation.

The aim of this study was to examine epigenetic patterns in cord blood and peripheral blood CD4+ T helper cells from children in our probiotic intervention study, and to investigate whether these patterns differ depending on supplementation, age or allergic outcome. The DNA methylation patterning of allergy-related T helper cells was investigated utilising a T helper subset locus-specific DNA methylation analysis, and whole-epigenome Illumina 450K DNA methylation analyses were performed to gain insights into the global effects of the intervention. We hypothesised that probiotic supplementation

will lead to hypomethylation, and hence in general to increased transcriptional accessibility, of a subset of immune-related genes in CD4+ T helper cells, both at targeted immunoregulatory CpG sites and at the global scale.

2 | METHODS

For a brief overview of the study, see Figure 1 and Table 1. Detailed information on study design and utilised methods is provided in the Online supplementary material section.

3 | RESULTS

3.1 | T helper subset locus-specific DNA methylation analysis reveals age-dependent differences in immunoregulatory loci

DNA methylation patterns of the T helper cell-associated loci *IL13*, *IL17*, *IFNG* and *FOXP3* did not differ with regard to intervention group or the development of allergic disease. However, age-dependent differences were apparent. The *IFNG* and *IL17* promoters were highly methylated at birth, with significantly less methylation at all later time points (Figure 2A,D) in comparison with at birth. In contrast, the *FOXP3* (Figure 2B) and *IL13* (Figure 2C) loci were significantly more methylated at 12 and 24 months than at birth. In general, the *IL17* locus displayed the highest methylation levels (Figure 2D), and DNA methylation was significantly lower in adults compared with children in all examined loci.

3.2 | Whole-genome DNA methylation patterns show most differences between probiotic and placebo-supplemented infants at birth

Clear differences were observed between probiotic and placebo-supplemented infants in initial multidimensional scaling (MDS) analyses at birth, while fewer differences were seen at 12 and 24 months (Figure 3A-C). Differentially methylated probes (DMPs, defined as mean log2 methylation difference (MMD) $> \pm 5\%$ and FDR < 0.1) were extracted, revealing a total of 1629 significant DMPs when comparing the intervention groups at birth, while no or few DMPs were found at 12 and 24 months of age (Figure 3D-F, Tables S1-S4). Among the 1629 DMPs, more than twice as many were hypomethylated than hypermethylated (68% vs 32%). Genomic inflation analyses at birth revealed a slight over-significance in our data (inflation factor of 1.2, bias = -0.003, Figure S1). However, because of the weak inflation, any false positives are avoided through the subsequent *P*-value correction using FDR correction. Thus, no additional correction for inflation was deemed necessary. Additionally, permutation and bootstrap analyses (MATLABs *mattest*, 100 000

re-shufflings) confirmed the statistical robustness of the data, as all of the 1629 probes showed nominally significant *P*-values according to both tests. No clear differences were found when comparing children developing any type of atopic manifestations with those who did not, separately at different time points (results not shown). Separate within-group comparisons over time, from birth to 12 and 24 months of age, respectively, were performed (Figure S2, Tables S1, S5-S10) and further mapped to genes. Throughout the first year of life, in genes common to both intervention groups, DMPs were mainly hypermethylated (Figure S3A) and were enriched for pathways such as T-cell activation and IFN- γ signalling (Figure S2B). For the placebo-specific DMPs, hypermethylated DMPs were enriched for pathways of viral immune regulation, whereas genes containing hypomethylated DMPs showed enrichment for mainly non-immune pathways (Figure S2B). Similar results were obtained for the comparison with 2 years of age (Figures S3B and S4).

3.3 | Network approach reveals an immune-associated gene module

As the comparison between the probiotic and placebo groups at birth revealed the most DMPs, these were further mapped to genes, resulting in 1015 genes containing one or more DMPs, of which a majority (65.5%) contained only hypomethylated sites (Figure S5). Enrichment analyses on these genes revealed GO terms mainly related to cell signalling, cell migration and TGF- β signalling (Table S11). In order to further elucidate the potential functional role of the methylation changes in these DMP-containing genes, these were analysed from a network perspective. For this purpose, we computed an intervention module by running four established module identification methods, based on DNA methylation data and publicly available data from the STRING database, and selected a consensus of genes that were predicted by at least two methods and interconnected (see Online supplementary material). The resulting module consisted of 91 genes with 306 direct protein-protein interactions (Figure 4A). Of these module genes, more than half (53 genes) originated from the set of DMP-containing genes in the probiotics vs. placebo comparison at birth, while the remaining 38 constituted novel genes from the interaction network (Figure 4A, Table S12). The module showed overall enrichment for immune-related pathways, such as leucocyte migration and PI3 kinase activity (Figure 4B). Examinations of the network structure of the module identified four large clusters, enriched for chemokine signalling, PI3K-Akt and MAPK signalling, and TGF- β signalling as well as transcription and translation (Figure 4A). Inspecting these 91 genes more thoroughly, 26 genes were highly connected (>10 connections in network, Table S12). Of these highly connected genes, five of the genes that showed more than 5% difference in methylation had previously been associated with the development of allergic outcomes (Table S13). Two of these genes were hypermethylated (*PRKCA*

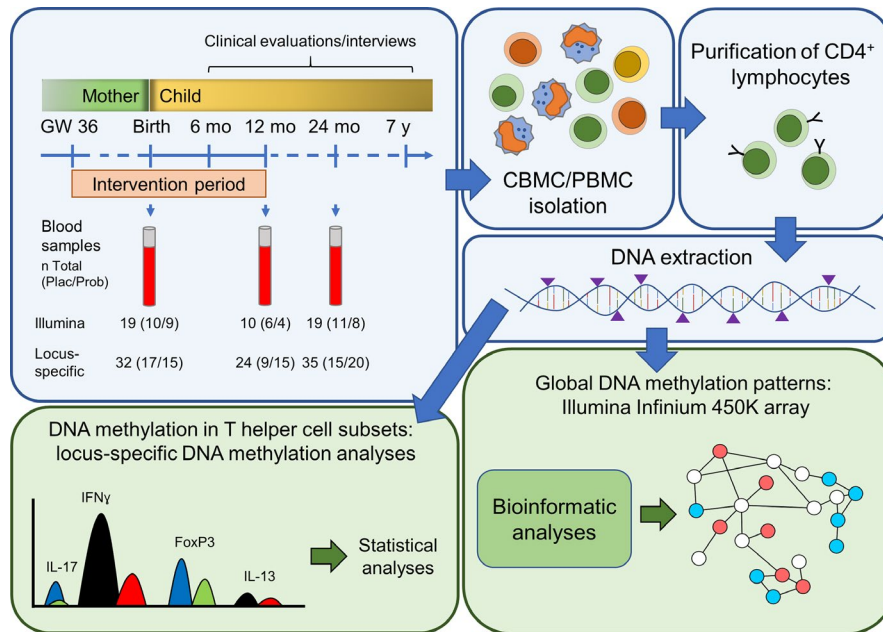


FIGURE 1 Flow scheme for the study. From children in a probiotic intervention study where the mother was treated from GW 36 until birth and the child from birth throughout the first year of life, blood samples were retrieved at birth (cord blood), 12 and 24 mo of age. Thereafter, CBMCs/PBMCs were isolated, and CD4⁺ T cells were purified from these populations. Subsequently, DNA extraction was performed, and utilised for either targeted immune-related (T helper subset locus-specific DNA methylation analysis) or global (Illumina 450K) investigation of DNA methylation patterns, followed by statistical and bioinformatic analyses. The number of samples per method is stated as total (placebo/probiotics) at the different time points. CBMC—cord blood mononuclear cell, GW—gestational week, PBMC—peripheral blood mononuclear cell

and *ITGA4*), and three were hypomethylated (*CCR10*, *OXGR1* and *GNAO1*).

3.4 | Classifier built on module associates with the development of allergic manifestations

In order to further evaluate the module computed on the comparison between probiotics and placebo at birth, the relationship of the module to the development of atopic manifestations was studied. The power of the module (containing 91 genes) to predict which children were going to remain healthy ($n = 17$, $n\text{Prob} = 5$, $n\text{Plac} = 12$, Table S14) or develop atopic manifestations (sensitisation and/or symptoms cumulatively up to 7 years of age, $n = 24$, $n\text{Prob} = 12$, $n\text{Plac} = 12$) was investigated by testing the DMPs of the module ($n = 2734$). These DMPs were extracted from all 91 genes in the network, not only from the genes found to be of interest in the differential DNA methylation analyses. Significantly more genes were differentially methylated between children who later developed atopic manifestations and those who remained healthy ($n = 61$ genes, $\text{OR} = 1.43$, $P = 2.4 \times 10^{-6}$), with most DMPs being hypomethylated (Figure 5A, Table S12). This further supported the possibility of using the module to construct a classifier of the response (see Online supplementary material), which could predict allergy development (as validated by means of leave-one-out cross-validation, average cross-validation $\text{AUC} = 0.78$, $P = 3.0 \times 10^{-3}$ from 100 permutations, Figure 5B).

4 | DISCUSSION

We have demonstrated that perinatal probiotic supplementation affects global DNA methylation patterns of CD4⁺ cells in supplemented infants and that this effect is most pronounced at birth. The identified DMPs were mainly hypomethylated, indicating that the probiotic intervention may mainly modulate the epigenome towards transcriptional accessibility. However, this pattern was not observed at 12 and 24 months of age, highlighting the significance of the prenatal period for immune maturation later in life.^{17,18} As differences in methylation between probiotics and placebo were most pronounced at birth, we proceeded with network analyses to investigate the biological relevance of these findings. The obtained module was enriched for immune-related signalling pathways involving *i.a.* PI3K/MAPK, TGF- β and chemokine signalling. The majority of genes in the module were hypomethylated, suggesting that probiotic intervention may mainly lead to transcriptional activation of these genes, and thereby possibly activate pathways concerning, for example, chemotaxis, T-cell receptor signalling and immunoregulatory functions. The ubiquitous PI3K/MAPK signalling pathways are also involved in signal transduction downstream of the T-cell receptor.¹⁹ In support of our findings, studies on DNA methylation revealed genes such as *MAPK1* and *PI3KD* being differentially methylated in egg-allergic children.¹² The Treg-associated cytokine TGF- β plays a pivotal role in immunoregulatory processes such as induction of allergen

TABLE 1 Descriptive data for the children in the T helper subset locus-specific DNA methylation analyses and Illumina 450K analyses

	T helper subset locus-specific DNA methylation analysis (n/N)			Illumina 450K (n/N)		
	CB	12 mo	24 mo	CB	12 mo	24 mo
<i>Lactobacillus reuteri</i>	44 (14/32)	61 (14/23)	57 (20/35)	47 (9/19)	40 (4/10)	42 (8/19)
Boys	31 (10/32) ^a	30 (7/23) ^a	40 (14/35)	42 (8/19)	20 (2/10)	26 (5/19)
First-born	53 (17/32)	52 (12/23)	49 (17/35)	47 (9/19)	50 (5/10)	47 (9/19)
Parental smoking birth	6 (2/32)	4 (1/23)	9 (3/35)	0 (0/19)	0 (0/10)	5 (1/19)
Furred pets	6 (2/32)	22 (5/23)	14 (5/35)	5 (1/19)	40 (4/10) ^a	21 (4/19)
Maternal atopy	78 (25/32)	87(20/23)	91 (32/35) ^a	84 (16/19)	100 (10/10)	90 (17/19)
Breastfeeding 3 m	74 (17/23)	57 (13/23)	69 (24/35)	75 (9/12)	70 (7/10)	79 (15/19)
Breastfeeding 6 m	83 (19/23)	83 (19/23)	91 (32/35)	83 (10/12)	80 (8/10)	95 (18/19)
Antibiotics 0-12 m	17 (4/23)	26 (6/23)	23 (8/35)	17 (2/12)	30 (3/10)	26 (5/19)
Antibiotics 12-24 m	57 (13/23)	52 (12/23)	34 (12/35)	58 (7/12)	50 (5/10)	26 (5/19)
Day care 0-12 m	0 (0/23)	0 (0/23)	3 (1/35)	0 (0/12)	0 (0/10)	0 (0/19)
Day care 12-24 m	91 (21/23)	91 (21/23)	89 (31/35)	92 (11/12)	90 (9/10)	84 (16/19)

Note: ^aSignificantly different from the original study (n = 188), as investigated by chi-square test or Fisher's exact test (for sample sizes with five observations or less).

In comparison with the original study, in the T helper subset locus-specific DNA methylation analyses, significantly fewer boys were included at birth and 12 mo, and significantly more children had atopic mothers at the 24-mo time point. In the Illumina analyses, significantly more children had pets in their homes at the 12-mo time point in comparison with the original study. $P < .05$.

Nine and seven infants were lost to follow-up at 24 mo from birth for T helper subset locus-specific DNA methylation analyses and Illumina analyses, respectively, but were included in the study to investigate effects of maternal probiotic supplementation at birth.

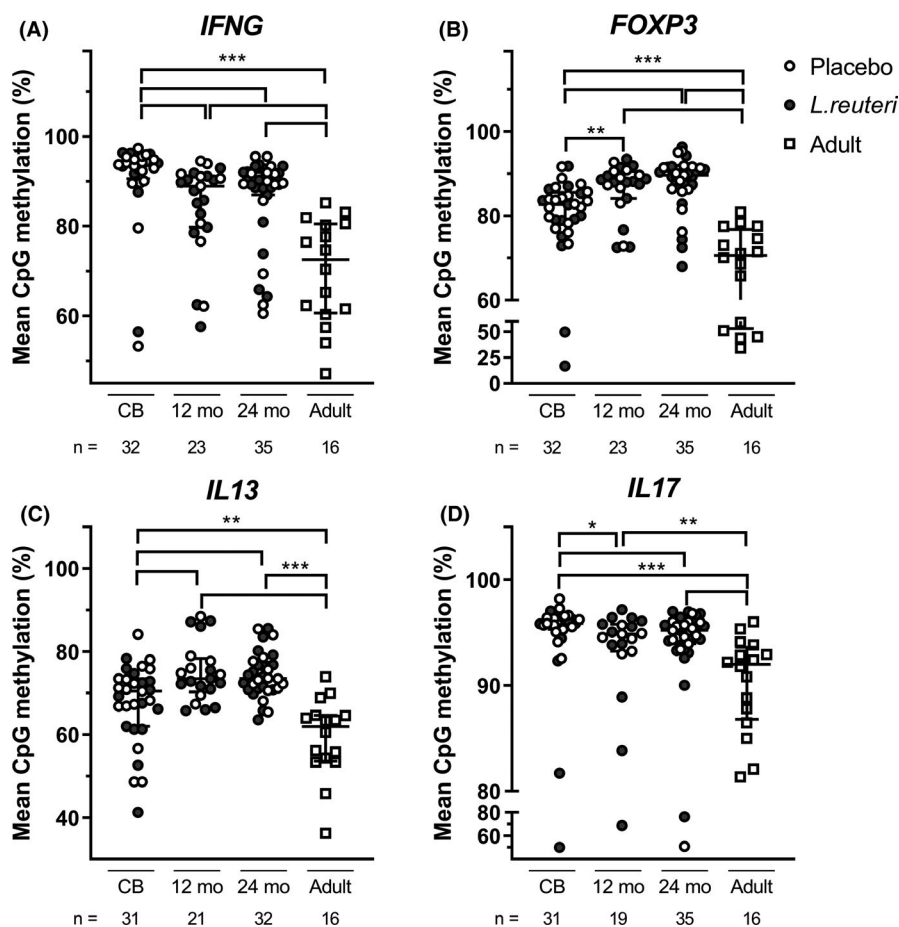


FIGURE 2 Targeted immune-related DNA methylation patterns in T helper cell-associated loci. Age-related changes in specific T helper cell lineage-associated CpG methylation patterns located in proximity to the transcription start site of the respective lineages were analysed by means of T helper subset locus-specific DNA methylation analysis. Mean DNA methylation levels in A *IFNG*, B *FOXP3*, C *IL13* and D *IL17* are displayed at birth (CB), 12 and 24 mo in infants and in a group of adults. Groups were compared using the Kruskal-Wallis test, followed by Dunn's multiple comparison test. FDR-corrected P -values (q -values) are reported as follows, *** $q < 0.001$, ** $q < 0.01$, * $q < 0.05$. CB, cord blood; FDR, false discovery rate

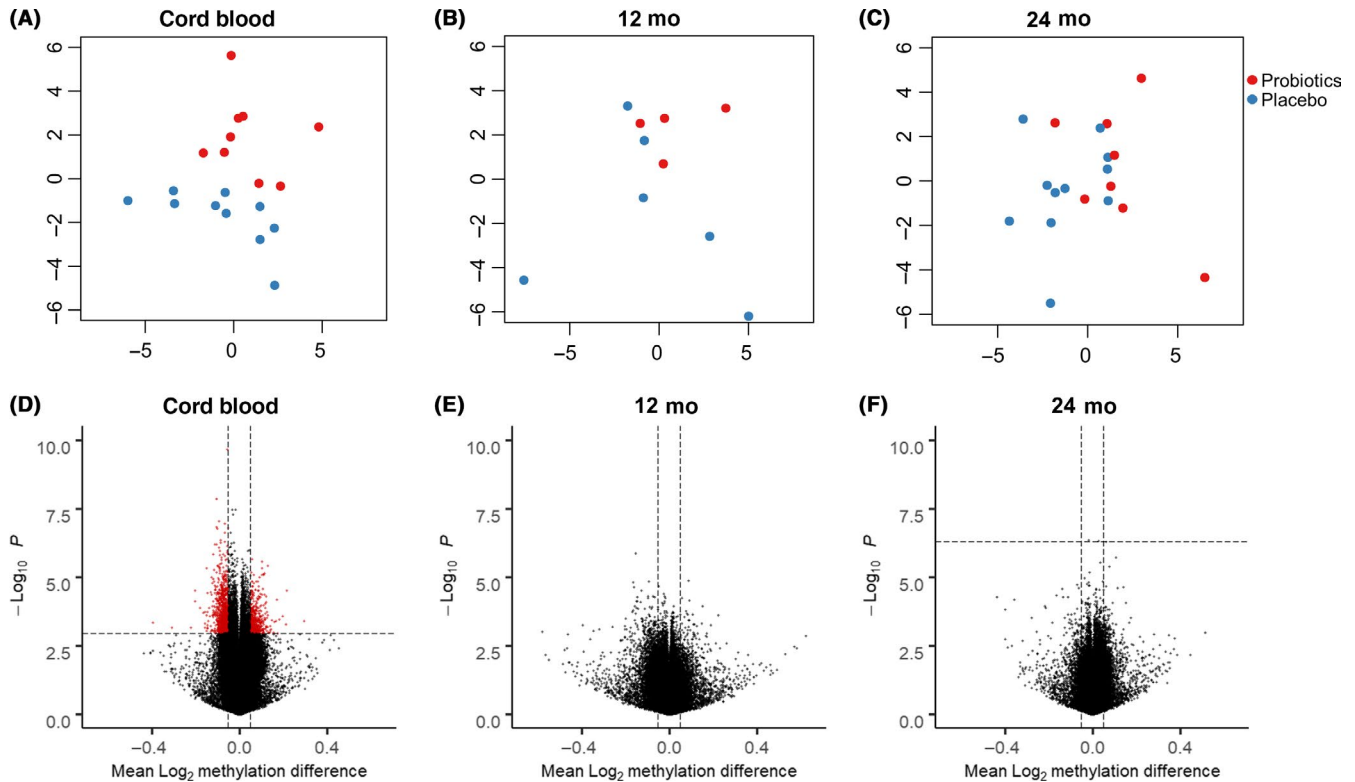


FIGURE 3 Multidimensional scaling (MDS) analysis and volcano plots of differential DNA methylation. MDS was performed on data after filtering, BMIQ normalization and cell-type correction in samples from birth (CB, panel A), 12 months (B) and 24 months (C), respectively. Red dots indicate probiotics, and blue dots denote placebo. Based on the comparison between probiotics and placebo, differential methylation for each probe ($-\log_{10} P$ -values plotted against mean log2 methylation difference (MMD)) is illustrated as volcano plots in CB at birth (D), and at 12 months (E) and 24 months (F). The differential methylation analysis included fitting a linear model to the obtained data and FDR correction for multiple testing. The horizontal dashed line represents a cut-off of $FDR < 0.1$, and the vertical dashed lines correspond to $MMD < -0.05$ and > 0.05 , respectively. Red dots indicated differentially methylated probes fulfilling both criteria for FDR and MMD described above. BMIQ, beta mixture quantile dilation; CB, cord blood; FDR, false discovery rate; MDS, multidimensional scaling; MMD, mean log2 methylation difference

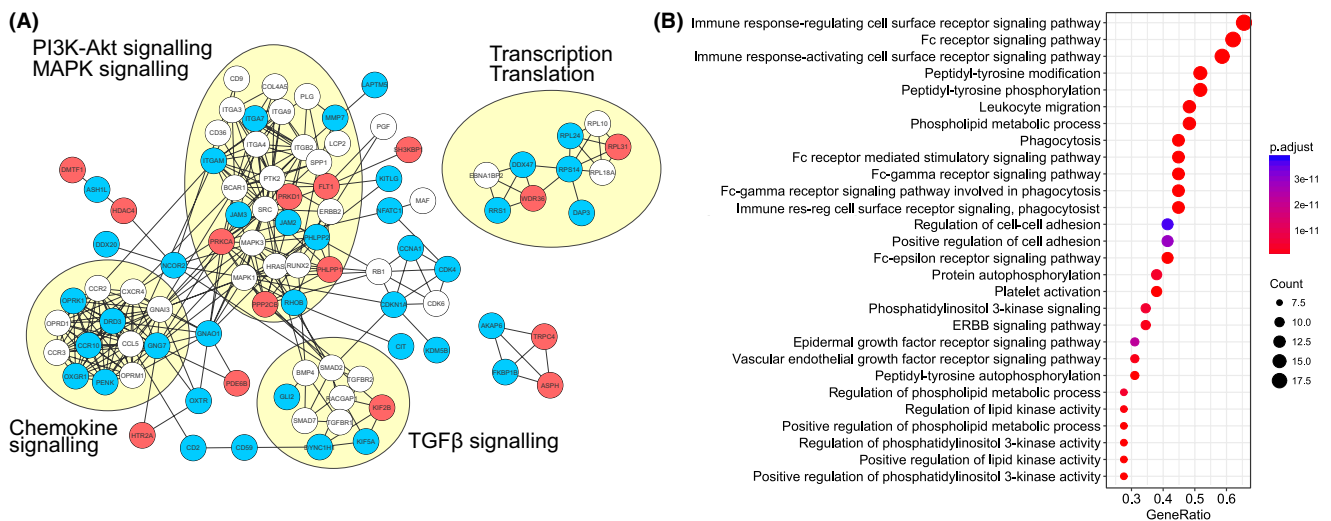
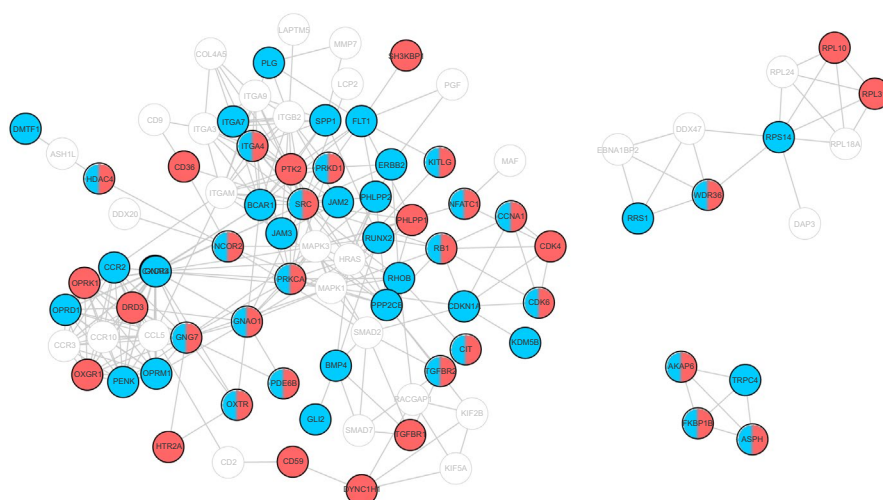


FIGURE 4 Network illustration of module and gene enrichment of module genes. A. Network visualisation of the module identified based on genes containing significant DMPs ($FDR < 0.1$). Nodes represent genes and connecting lines represent protein-protein interactions (STRING combined score > 0.7). Blue nodes correspond to genes containing only hypomethylated DMPs, and red nodes correspond to genes containing only hypermethylated DMPs. Yellow areas display intra-connected subunits, each analysed for biological pathway enrichment using the online tool DAVID. B. Enriched pathways based on all module genes illustrated as a dot plot. The x-axis represents the gene ratio, dot size corresponds to gene count, and dot colour represents adjusted enrichment P-values. DMP, differentially methylated probe; FDR, false discovery rate

(A)



(B)

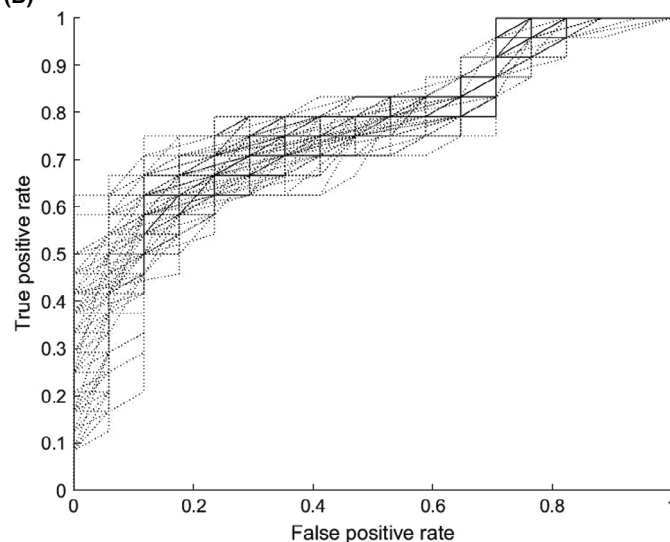


FIGURE 5 Visualisation of the module network in relation to the development of atopic manifestations and the allergy predictive power of the module. The left-hand panel A visualises the module network based on relation to the development of atopic manifestations. Module genes that contain probes showing differential methylation when comparing samples from allergic and healthy individuals ($P < .05$) are highlighted in colour. Blue denotes the presence of only hypomethylated probes, red indicates the presence of only hypermethylated probes, and colouring with both blue and red indicates the presence of both hypo- and hypermethylated probes. The right-hand panel B illustrates receiver operating characteristics (ROCs) for the proposed ensemble learner from 100 different repetitions (to account for stochasticity between the runs) when trained only using the probes in the consensus module genes, which were derived from network analysis of probiotics vs. placebo at birth. In each repetition, cross-validated predictions of the learner were calculated to test whether the learner predicted individuals who later developed atopic manifestations or remained healthy up to 7 y of age. The mean area under the curve (AUC) for the 100 learners was 0.78

tolerance, by transducing intracellular signals through SMAD proteins.²⁰ Martino and co-workers found that global DNA methylation patterns in CD4+ T cells from healthy children were enriched in immune-related gene GO terms involved in, for example, TGF- β signalling at 12 months compared with at birth,²¹ suggesting that microbial and environmental exposure throughout the first year of life induced immunoregulatory responses. Intriguingly, network analyses of DNA methylation data in cord blood from children born to asthmatic mothers revealed *SMAD3* as the most connected node in the network,⁴ and Lund et al recently showed that, in children with atopic asthma and a previous history of rhinovirus-induced wheezing, the greatest differential methylation was for the *SMAD3* gene.¹¹ Collectively, these studies corroborate the importance of the TGF- β , PI3K and MAPK pathways for immune maturation throughout childhood. Examinations of the most highly connected genes in the network revealed five differentially methylated ($>\pm 5\%$ MMD) genes that previously had been related to allergy development. The most differentially hypermethylated *PRKCA*, coding for protein kinase C (PKC) alpha, has been

associated with asthma development in genetic studies,^{22,23} and neonatal PKC alpha expression correlates with allergen-stimulated PBMC IL-5 levels at 1 year of age.²⁴ The likewise hypermethylated gene *ITGA4*, coding for integrin subunit alpha 4, is differentially expressed in children developing asthma.²⁵ When coupled with the integrin beta 7 subunit, the heterodimeric protein drives preferential homing of T cells to the gut mucosa.²⁶ The expression of integrin alpha4beta7 in allergen-stimulated CD4+ T cells is also elevated in allergic compared with healthy subjects.²⁷ This is particularly interesting, as the probiotic treatment interacts with the gut mucosa. Furthermore, the CC chemokine receptor 10 (CCR10) was hypomethylated in this study and drives recruitment of T cells to the skin via the epidermally expressed CC chemokine CCL27.²⁸ Significantly increased expression of CCR10 in circulating CD4+ cells was demonstrated in individuals with atopic dermatitis and asthma,²⁹ and in experimental allergic rhinitis, CCR10+ CD4+ memory T cells were present in the nasal mucosa.³⁰ As *L. reuteri* supplementation in this cohort leads to decreased development of IgE-associated eczema,¹⁴ these findings are of notable interest.

The most differentially hypomethylated gene was the G-protein GNAO1, with lower expression in PBMCs from allergic rhinitis patients than in healthy subjects.³¹ The likewise hypomethylated GPCR-related gene *OXR1* binds leukotrienes such as LTE_4 in murine models.³² Other leukotriene receptors, such as *CysLT1*, are upregulated in CD4+ T cells of allergic individuals when stimulated with Der p1 allergen³³ and upon treatment with the Th2 cytokine IL-33 in healthy individuals.³⁴ Whether these methylation differences translate to differential protein expression or function remains to be elucidated. It is tempting to speculate that probiotic supplementation leads to improved immune activation at birth compared with non-treated individuals, due to the observed association with epigenome-wide hypomethylation in this study. Furthermore, as food-allergic children have decreased lymphoproliferative responses in infancy, this could possibly be beneficial in relation to allergy development.¹² Recently, a pilot study combining pre- and postnatal *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* supplementation revealed general hypomethylation of immune-related genes such as *IL-6R*, *IL-5*, *CD38* and *STAT-3*, in treated compared with non-treated children,³⁵ corroborating our findings, suggesting that probiotic supplementation promotes transcriptional accessibility of immune response genes.

To determine whether the module genes were related to the development of atopic manifestations, we employed a linear model of module-related DNA methylation probes with sample time points as co-variables. This approach revealed a number of genes (61 of 91) being differentially methylated in relation to the development of atopic manifestations, encouraging us to build a classifier to evaluate the development of allergic disease. The resulting classifier was able to distinguish healthy individuals from children with atopic manifestations. Although the limited sample size may have affected the accuracy of the classifier, our findings suggest that the created module is not only related to the probiotic intervention, but possibly also related to allergy-preventive effects in our population.

To examine local DNA methylation sites in the promoters of four immunoregulatory T helper cell subsets, that is Th1 (*IFNG*), Th2 (*IL13*), Th17 (*IL17*) and Treg (*FOXP3*) cells, we employed a T helper subset locus-specific DNA methylation analysis method. These four loci are not available in the Illumina 450K panel, and their importance in T helper cell differentiation is established.¹⁰ While neither intervention nor atopic manifestations development had any significant effect on the locus-specific sites, an age-dependent change in methylation status was revealed. In agreement with studies showing lower methylation levels in the Th2-associated *IL13* promoter region in CD4+ cells from cord blood,⁸ we observed significantly less methylation of *IL13* at birth, compared with 12 and 24 months of age. Similarly, the *FOXP3* DNA methylation levels increased from birth throughout infancy, whereas the Th1-associated *IFNG* locus methylation decreased from birth onwards, in line with the higher degree of DNA methylation demonstrated in the *IFNG* promoter region in CD4+ cells from neonates compared with both children^{9,36} and adults.^{9,36,37} Interestingly, a recent study on cow's milk-allergic children reported increased

DNA methylation in *IL4* and *IL5*, increased demethylation in *FOXP3* and less DNA methylation in *IFNG* and *IL-10* after 12-month treatment with a milk formula containing *Lactobacillus rhamnosus* GG.³⁸ As DNA methylation generally corresponds to decreased transcriptional accessibility,⁶ the decrease in *IFNG* and *FOXP3* methylation may reflect normal immune maturation of Th1 and Treg responses during the first years of life, continuing into adulthood. This is further corroborated by the findings of changes in DNA methylation over time within the treatment groups, revealing a large number of common DMPs, which represent biological processes involved in Th1 immunity and the regulation of viral infections, such as IFN- γ -mediated responses as well as I κ B/NF κ B signalling.

There are some limitations to the present study. The limited sample size may have affected our outcomes, and longitudinal analyses were unfortunately not possible. However, we do reveal differences in line with what others have shown^{4,11-12,21} in our cross-sectional analyses, and the means by which we attempted to assure robustness and statistical soundness of the data should at least partly compensate for this. Bootstrap and permutation analyses on the 1629 DMPs at birth revealed significant nominal *P*-values for both methods, indicating that the differential methylation changes are robust. In the downstream network analyses, as a means to further improve the stringency of possible findings, we required that any differences found should be present for a specific probe in at least two of the four module packages tested in order to be considered significant.

To our knowledge, this is the first study to assess the epigenetic developmental trajectory in infant CD4+ T cells after pre- and postnatal probiotic supplementation. In conclusion, the epigenome in CD4+ T cells from children receiving probiotic treatment pre- and postnatally is altered towards enhanced immune activation and maturation, particularly at the time of birth, suggesting that probiotics have immune-stimulating effects on the developing foetus.

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CONFLICT OF INTEREST

T. Abrahamsson and M. Jenmalm have received funding for clinical trials and honoraria from Biogaia AB for lectures. M. Jenmalm has also received consultant fees and travel support from Nutricia/Danone.

AUTHOR CONTRIBUTIONS

MCJ and TA designed the study. TA and LJN were responsible for the clinical evaluation of the children. JH and AF performed the

experimental work. SS, JH and AF performed statistical analyses and presented the data. MG provided expert knowledge on the performance of network statistical analyses. RBM and JE provided expert knowledge on setting up the experimental method. All authors interpreted and discussed the results. JH, SS and AF drafted the manuscript. All authors contributed to and approved the final draft for publication.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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